Handbook of Experimental Pharmacology 240

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Pharmacology of Mitochondria

Springer

Handbook of Experimental Pharmacology

Volume 240

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ISSN 0171-2004 ISSN 1865-0325 (electronic)
Handbook of Experimental Pharmacology
ISBN 978-3-319-57311-3 ISBN 978-3-319-57313-7 (eBook)
DOI 10.1007/978-3-319-57313-7

Library of Congress Control Number: 2017940040

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer International Publishing AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

In 1870, Eduard Pfluger demonstrated that respiration takes place in cells and around a full century ago, in 1910, Kingsbury postulated that respiration takes place in "mitochondria." However, Carl Benda coined the term "mitochondria" (thread granules) in 1898 to describe these ubiquitous cellular structures. After the initial discovery of mitochondria, the respiratory chain was conceptualized by Keilin, Warburg, Hartree, and others in between the 1910s and 1930s. From the 1920s to 1950s, key aspects of aerobic metabolism were elucidated. The chemiosmosis hypothesis for ATP generation was then proposed by Peter Mitchell in 1961. This era was followed by mitochondrial isolation and the publication of first high-resolution images of mitochondria by George Palade and Fritiof Sjostrand. In the 1960s and 1970s, much of the focus remained on ATP synthesis, electron transport chain (ETC), and anion translocators. With the sequence of the human mitochondrial genome in 1981, a new era of mitochondrial biology was initiated resulting in the recognition of mitochondrial inheritance. In 1998, the first mitochondrial proteome was published, and eventually by 2008, the complete catalog of mitochondrial proteins, "MitoCarta," was unveiled. The ever growing interest in mitochondria and pharmacology, as well as the physiology associated with them, is widely discussed in several scientific meetings and literature.

Classically, mitochondria were defined as the key regulators of cellular energy. However, now it is universally accepted that they are dynamic, interconnected, and integrated with other cellular organelles. In addition to their role in the generation of ATP by oxidative phosphorylation, mitochondria are involved in controlling cell metabolism and are key players in cellular calcium signaling, free radical homeostasis, lipid transport and biosynthesis, apoptosis, cell cycle and differentiation, and cellular aging. They also participate in retrograde signaling with nucleus and cross talk with endoplasmic reticulum either directly or via mitochondria-associated membranes. *Pharmacology of Mitochondria*, the focus of this handbook, is a unique effort by leading experts and researchers to assimilate information on pharmacology and physiology of mitochondria. Topics covered in the handbook expand beyond canonical roles assigned to mitochondria. The information presented will be highly useful for mitochondrial biologists and researchers working in physiology, medicine, and plant sciences.

In the *Handbook of Pharmacology*, information is delineated from mitochondrial genetics to mitochondrial diseases and physiology. Even though mitochondria possess their own genes, the majority of proteins imported into mitochondria are encoded by nuclear DNA and a chapter in our book titled "Nuclear Transcription Factors in the Mitochondria: A New Paradigm in Fine-Tuning Mitochondrial Metabolism" provides an overview of nuclear-encoded factors. Ionic homeostasis plays a major role in maintaining the structural and functional integrity of mitochondria. The majority of the proteins involved in maintaining the mitochondrial ionic homeostasis are encoded by the nuclear DNA; however, information on these proteins responsible for transporting ions is still in its infancy. Several ion channels and transporters involved in mitochondrial ionic homeostasis have been shown to present by either pharmacological or genetic approaches. In this handbook, chapters titled "The Mitochondrial Permeability Transition Pore and ATP Synthase"; "The Roles of Mitochondrial Cation Channels Under Physiological Conditions and in Cancer"; "Anion Channels of Mitochondria"; "Guide to the Pharmacology of Mitochondrial Potassium Channels"; and "The Mitochondrial Ca^{2+} Uniporter: Structure, Function, and Pharmacology" provide an overview of different classes of ion channels and transporters present in mitochondria, pharmacology associated with them, and their roles in diseases.

Mitochondrial structural and functional dynamics are associated with human physiological and pathological conditions. To maintain the structural and functional integrity of mitochondria, they continuously undergo fission, fusion, and trafficking, regulate lipid transportations, modulate reactive oxygen species production, and regulate cellular metabolism. Any deviation or abnormality from mitochondrial structural and functional integrity can result in human pathological conditions and diseases. Several chapters titled "Mitochondrial Fission in Human Diseases"; "Mitochondrial Cholesterol and the Paradox in Cell Death"; "Mitochondrial Changes in Cancer"; "The Emerging Role of Mitochondrial Targeting in Kidney Disease"; "Mitochondrial Dynamics as a Therapeutic Target for Treating Cardiac Diseases"; "Mitochondria in Alzheimer's Disease and Diabetes-Associated Neurodegeneration: License to Heal!"; "Leber Hereditary Optic Neuropathy: A Mitochondrial Disease Unique in Many Ways"; and "Leber Hereditary Optic Neuropathy: Exemplar of an mtDNA Disease" summarize the association of mitochondrial structural and functional integrity with human diseases.

Pharmacologically, mitochondria have undergone a renaissance in the last two decades. Several novel approaches at pharmacological and genetic levels have been incorporated to the mitochondrial medicine. The recent first live birth of three-parent baby carrying mitochondria from donor mother is the spectacular breakthrough in treating mitochondrial diseases. However, several new approaches and treatments are required to treat mitochondrial diseases and disorders. Chapters discussing new as well as existing therapeutic approaches in this handbook "Mitochondria-Targeted Agents: Mitochondriotropics, Mitochondriotoxics, and Mitocans"; "Mitochondrial Flashes: Elemental Signaling Events in Eukaryotic Cells"; "Role of Mitochondrial Reactive Oxygen Species in the Activation of Cellular Signals, Molecules, and Function"; "MITO-Porter for Mitochondrial

Delivery and Mitochondrial Functional Analysis"; and "Toxicity of Antiepileptic Drugs to Mitochondria" provide highly useful overview on mitochondria in pharmacology. In the current era the amount of data generated by multidisciplinary approaches on a daily basis, there is an urgent need of integrating the big data to derive useful information and a chapter focused on understanding the complexity of mitochondrial phenome titled "Equipping Physiologists with an Informatics Tool Chest: Toward an Integrated Mitochondrial Phenome" provides an excellent platform for the same.

The authors and editors of the *Pharmacology of Mitochondria* hope that the chapters presented herein will provide extremely beneficial and inspiration information to mitochondrial enthusiasts, researchers, students, and clinicians. The chapters contributed by leading mitochondrial researchers in the *Handbook of Pharmacology* will take us through the novel pharmacological strategies via mitochondria to understand their physiological and pathological role as well as present them as therapeutic targets. We hope that the handbook will motivate the current and new generation of researchers to pursue the unanswered questions and understand the pharmacological and physiological implications of this fascinating and complicated organelle, "mitochondrion."

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Contents

Nuclear Transcription Factors in the Mitochondria: A New Paradigm in Fine-Tuning Mitochondrial Metabolism

Naresh Babu V. Sepuri, Prasad Tammineni, Fareed Mohammed, and Arunkumar Paripati

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Abstract

Noncanonical functions of several nuclear transcription factors in the mitochondria have been gaining exceptional traction over the years. These transcription factors include nuclear hormone receptors like estrogen, glucocorticoid, and thyroid hormone receptors: p53, IRF3, STAT3, STAT5, CREB, NF-kB, and MEF-2D. Mitochondria-localized nuclear transcription factors regulate mitochondrial processes like apoptosis, respiration and mitochondrial transcription albeit being nuclear in origin and having nuclear functions.

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H. Singh, S.-S. Sheu (eds.), *Pharmacology of Mitochondria*,
Handbook of Experimental Pharmacology 240, DOI 10.1007/164_2016_3

Hence, the cell permits these multi-stationed transcription factors to orchestrate and fine-tune cellular metabolism at various levels of operation. Despite their ubiquitous distribution in different subcompartments of mitochondria, their targeting mechanism is poorly understood. Here, we review the current status of mitochondria-localized transcription factors and discuss the possible targeting mechanism besides the functional interplay between these factors.

Keywords
Metabolism • Mitochondria • Nuclear receptors and transcription factors • Protein import • Protein targeting and signaling

1 Introduction

Mitochondria are crucial organelles involved in various cellular processes. Besides the production of ATP for cellular needs, they are also participating in the metabolism of fatty acids and amino acids. Also, mitochondria play a significant role in apoptosis by integrating the extracellular cues with intracellular signaling pathways. Further, the involvement of mitochondria in cell proliferation, motility, and ion homeostasis is well documented (McBride et al. 2006). Thus, it's not surprising that mitochondrial dysfunction is often associated with various disease conditions like cancer and neurodegenerative disorders. Paradoxically, mitochondrial DNA encodes only for a handful of proteins. A majority of mitochondrial proteins are nuclear encoded. Mitochondria, though, have a handful of proteins encoded by mitochondrial DNA to execute all these functions; it depends on a majority of nuclear-encoded proteins for its optimal function. These nuclear-encoded mitochondrial proteins are synthesized on cytosolic ribosomes and transported to the mitochondria through mitochondrial protein import machinery (Mokranjac and Neupert 2009). Therefore, the mitochondria and nucleus act in concert with each other to mount an appropriate response toward extracellular stimuli. For example, nuclear transcription factors and coactivators like NRF-1, NRF-2, and PGC-1 regulate the expression of mitochondrial OXPHOS subunits to achieve fine-tuning of mitochondrial function toward altered metabolic demands of the cell (Leigh-Brown et al. 2010; Scarpulla et al. 2012). For this reason, considerable interest has been gained toward understating the role of nuclear receptors, transcription factors, and other signaling proteins in the establishment of harmonic equilibrium between these two organelles.

Nuclear receptors and transcription factors are activated in response to growth factors and cytokines to regulate the gene expression in the nucleus. Some of these transcription factors control mitochondrial function indirectly by regulating the expression of mitochondria-associated protein factors. On the other hand, increasing evidence suggests that some of the nuclear receptors and transcription factors are also present in mitochondria to potentially influence various mitochondrial functions (Szczepanek et al. 2012). For example, glucocorticoids and other transcription factors regulate mitochondrial transcription, while p53 and IRF3 regulate apoptosis and respiration. Also, p53 and STAT3 are known to influence the opening

of mitochondrial permeability transition and also affect ROS generation from the mitochondrial OXPHOS system (Szczepanek et al. 2012). Sufficiently reasonable attempts have been made to understand the role of various mitochondrial transcription factors. However, information on their mitochondrial targeting is lagging behind as the nuclear transcription factors entering the mitochondria lack the canonical mitochondrial targeting sequence.

In this review, we will provide an outline of various transcription factors associated with mitochondria and discuss how their submitochondrial localization contributes to their overall biological function. Also, we will summarize the potential mechanism of their transport to mitochondria and involvement of these mitochondrial transcription factors in retrograde signaling and functional interplay between these factors.

2 Nuclear Hormone Receptors Present in the Mitochondria

2.1 Estrogen Receptor

Estrogen receptors (ESRs), ESR1 and ESR2, belong to the nuclear receptor superfamily of transcription factors responsible for estrogen-mediated transcriptional induction of genes in reproductive and nonreproductive tissues. ESR1 and ESR2 are highly homologous proteins except in the DNA-binding domains that render ESR2 to be a poor transcription factor when compared to ESR1. Noncanonical functions of estrogen receptors in the cytosol, endoplasmic reticulum, and plasma membrane have been well documented. ESR1, ESR2, and its isoform ERβ2 have been found to be present in various tissues and cell lines (Cammarata et al. 2004; Chen et al. 2004; Milner et al. 2005; Solakidi et al. 2005). Mitochondria-localized ESR plays a significant role in apoptosis and reduces ROS by activating manganese superoxide dismutase (MnSOD) enzyme. When cancer cells are irradiated with UV, it induces mROS species generation and activation of c-Jun N-terminal kinase (JNK), and protein kinase C (PKC) δ. This triggers apoptotic cell death. Estradiol (E2) inhibits all these events, by directly activating manganese superoxide dismutase (Pedram et al. 2006). Mitochondria-localized ESR2 has also been shown to suppress apoptosis induced by cisplatin in non-small cell lung cancer cells. ESR interacts with Bad and inhibits translocation of Bax to mitochondria (Xie et al. 2015). It also has been shown that downregulation of ESR2 enhances Bax activation and translocation to mitochondria in a ligand-independent manner (Liang et al. 2015). Interestingly, in ovarian cancer, it was observed that cytoplasmic ERβ2 is associated with lower apoptotic rate, whereas mitochondria-localized ERβ2 inhibits apoptosis by interacting with Bad protein (Ciucci et al. 2015). Recently, it has been shown that ESR1 affects beta-oxidation in mitochondria by directly interacting with beta subunit of HADHB (hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase) (Zhou et al. 2012). In addition to these transcription factors, recent reports also suggest the possible role of G-protein-coupled estrogen receptor 1 (GPER1) in modulating the opening of mitochondrial permeability transition pore via phosphorylation of ERK1/ERK2/

GSK-3β (Bopassa et al. 2010; Kabir et al. 2015). However, further studies are needed to understand the mechanistic details of GPER1 action on mitochondria.

2.2 Glucocorticoid Receptor

GRs are expressed in all tissues and cell types and regulate cell metabolism, immune response, and development in response to glucocorticoids such as cortisol. GRs are the first nuclear receptors reported to be present in mitochondria. Mobilization of GR from the cytoplasm to mitochondria has been observed when rats were administered with dexamethasone. However, the precise mechanism of this translocation is not clear (Demonacos et al. 1993). GR has been detected in mitochondria of HeLa cells, cytoplasm, and mitochondria of rat brain synaptosomes (Moutsatsou et al. 2001; Scheller et al. 2000). GR binds to glucocorticoid response element (GRE) consensus sequence present on cytochrome c oxidase I and III genes and the D-loop region of mitochondrial DNA (Demonacos et al. 1995). These studies indicate that GR present in the mitochondria may be regulating mitochondrial transcription independent of its nuclear role. Overexpression of mitochondrial GR increases RNA synthesis, ATP production, and cytochrome c oxidase I protein (Psarra and Sekeris 2011). Further, it has been shown that GR forms a complex with Bcl-2 before its translocation into mitochondria in corticosterone-treated brain cells. This translocation inhibits the release of cytochrome c and calcium ions from mitochondria, thereby protecting the neuron (Du et al. 2009). In contrast, mitochondria-localized GR has been shown to induce apoptosis in dexamethasone-treated thymocytes where the mitochondrial import of GR correlates with the release of cytochrome c and activation of caspase 3 (Talaber et al. 2009).

2.3 Thyroid Hormone Receptor

Thyroid hormone receptor is a nuclear receptor that regulates cellular metabolism and heart rate by activating transcription of genes in response to thyroid hormone. It has been 3 decades since Sterling and his colleagues discovered the presence of thyroid hormones in mitochondria (Sterling et al. 1984). Thyroid receptors TRα and TRβ are encoded by two genes c-erbA-1 and c-erbA-2, respectively. p43 and p28 are alternate translational initiation products of TRα, and these are exclusively localized to mitochondria. Mitochondria-localized TRα induces the expression of mitochondrial 12S rRNA by binding to the thyroid hormone response element of mitochondrial DNA (Morrish et al. 2006). p43 depleted mice exhibits decreased mitochondrial DNA replication and respiratory chain activities. p43 has also been implicated in maintaining glucose homeostasis and insulin secretion. It would be interesting to see the effect of p43 depletion in pancreatic cells and on the mitochondrial and nuclear cross talk. Depletion of p43 may cause decreased levels of Glut2 and Kir62 that may ultimately lead to pancreatic cell dysfunction and

lowered insulin secretion (Blanchet et al. 2012). Further, overexpression of p43 induces a shift in skeletal muscle fiber types and causes muscle atrophy during aging through two muscle-specific E3 ubiquitin ligases, atrogin-1/MAFbx and MuRF1 (Casas et al. 2009; Casas et al. 2008). This data demonstrates the physiological importance of mitochondrial thyroid hormone receptor. Interestingly, human dermal fibroblasts overexpressing p43 undergo cellular transformation by upregulating c-Jun and c-Fos and concomitantly decreasing the expression of tumor suppressor genes p53, p21WAF1, and Rb (Grandemange et al. 2005) (Fig. 1).

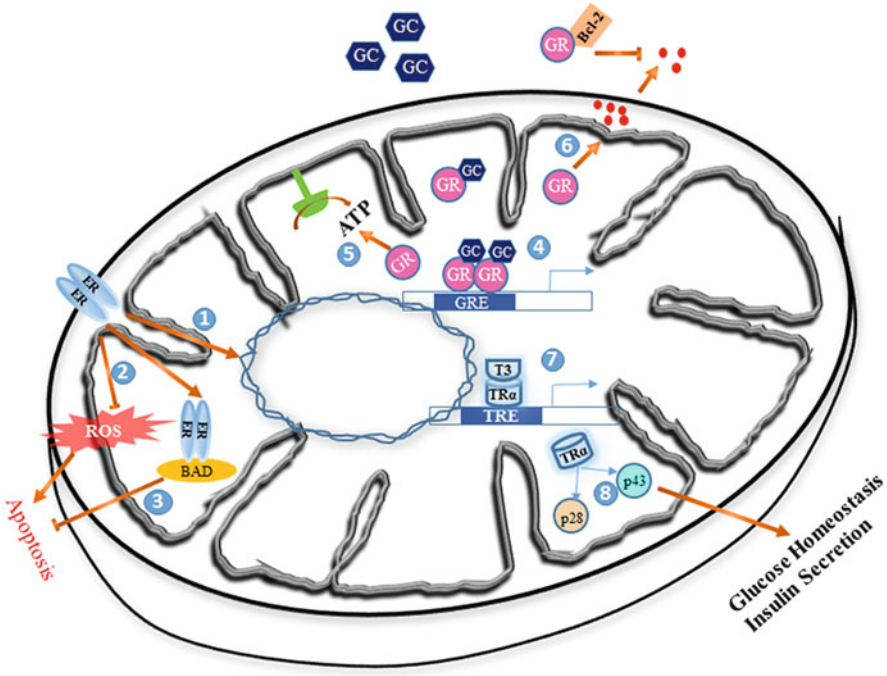

Fig. 1 Mitochondria-localized nuclear hormone receptors: nuclear hormone receptors that are present in mitochondria are estrogen receptor (ESR), glucocorticoid receptor (GR), and thyroid receptor (TR). Numbers in the figure represent the action of receptors, which are (*1*) transcriptional regulation of mitochondrial genes by ESR upon binding to its ligand. (*2*) ESR decreases the amount of ROS generated in mitochondria by activating MnSOD. (*3*) ESR interacts with Bad, thereby reducing the translocation of BAX to mitochondria. (*4*) GR interacts with GRE present in mitochondria and regulates the transcription independent of nucleus. (*5*) Overexpression of mitochondrial GR increases the production of ATP. (*6*) GR complexes with Bcl-2 and inhibits the translocation of cytochrome c. (*7*) TR interacts with TRE in mitochondria and regulates transcription. (*8*) p43, alternate translational product of TRα, has shown to play a role in insulin secretion and glucose homeostasis

3 Nuclear Transcription Factors Associated with Mitochondria

3.1 Interferon Regulatory Factor 3

IRF3 belongs to an interferon regulatory transcription factor family and is known to play a major role in innate immune response. Though IRF3 may not be localized to the mitochondria, it is known to regulate mitochondrial functions in a transcription-independent manner. In support of this theory, IRF3 mutant's deficiency in its transcription activity is still capable of activating apoptosis by releasing cytochrome c from mitochondria (Chattopadhyay et al. 2010). It is well documented that mitochondria-associated proteins and MAVS are required to orchestrate the IRF3 nuclear-mediated antiviral signaling and apoptosis. For carrying out its nuclear and mitochondrial roles, cytosolic IRF3 has to be activated by mitochondria-associated MAVS through synthetic or viral generated dsRNA-like vesicular stomatitis viruses. For IRF3 to influence mitochondrial functions, a pool of cytosolic-activated IRF3 interacts initially with Bax, a pro-apoptotic Bcl2 family protein through its BH3 domain. This interaction triggers Bax translocation to mitochondria, and its subsequent oligomerization on the mitochondrial membrane results in the release of cytochrome c and initiation of apoptosis cascade. Intriguingly, Bax seems to be also required for phosphorylation and nuclear translocation of IRF3 (Sharif-Askari et al. 2007). It is not clear whether Bax is directly involved in the phosphorylation of IRF3. More studies are required to understand if additional factors are involved in the interaction between phosphorylated IRF3 and Bax and also the mechanism that prevents the entry of cytosolic IRF3 into nucleus. A more definitive evidence is required to completely rule out the possibility of IRF3 entry into mitochondria.

3.2 p53

p53 is a tumor suppressor protein that responds to a myriad of stresses that include oxidative stress, DNA damage, nutrient stress, and ischemia (Zilfou and Lowe 2009). It is believed that p53-induced apoptotic changes were mediated by its ability to activate transcription of a particular section of genes within the nucleus; however, recent reports suggest that this ability to induce apoptosis and necrosis is attributed to its noncanonical role in the mitochondria. Most of the apoptotic signals, like gamma radiation, oncogenic deregulation, hypoxia, and genotoxic and oxidative stress, stimulate the translocation of p53 from cytosol to the outer mitochondrial membranes and to interact with multi-domain Bcl-2 family of proteins so that it can promote membrane permeabilization and apoptosis (Marchenko et al. 2007). p53 interacts with Bcl-2 family of proteins such as pro-apoptotic BAX and BAK and anti-apoptotic proteins Bcl-xL and Bcl-2. During stress, p53 competes with anti-apoptotic Mcl1 protein for its interaction with BAK in order to induce apoptosis (Leu et al. 2004). Similarly, p53 interaction with BAX disrupts the

sequestration of BAX by Bcl-xL. This disruption allows for the oligomerization of BAX on the outer mitochondrial membranes and opening of the permeability transition pore (PTP) (Chipuk et al. 2004). Unlike other transcription factors that reside in the mitochondria, the DNA-binding domain of p53 is critical for its mitochondrial function. In addition to its influence on outer mitochondrial Bcl2 proteins, p53 is also present in the subcompartments of mitochondria. A fraction of p53 resides in the mitochondrial matrix and interacts with cyclophilin D (CypD). This interaction facilitates the ROS-mediated opening of PTP and induction of necrosis. Thus, mitochondria-localized p53 is required for induction of mitochondria-mediated apoptosis and necrosis. In addition, another fraction of p53 that is also present in the mitochondrial matrix interacts with mtHSP70, mtHSP60, mitochondrial DNA polymerase γ, and mtTFA. However, the precise function of matrix-localized p53 in mitochondrial transcription, DNA binding, and protein folding is yet to be understood. Matrix-localized p53 has also been shown to sequester MnSOD to initiate apoptosis (Zhao et al. 2005). It appears that necrosis is operated when apoptotic process is stalled and necrosis seems to be operated only under oxidative stress conditions. However, it is not clear whether there is any cross talk between these two pathways to initiate the death signaling. Elucidation of the upstream signaling mechanism that determines the translocation of p53 to different subcompartments of mitochondria will provide valuable insights into p53 function vis-a-vis its mitochondrial positioning.

Though the mitochondrial translocation mechanism of p53 is not clear, mitochondrial p53 is known to regulate the OPA1 processing, mitochondrial dynamics in cisplatin (cis-diamminedichloroplatinum (II)), and CDDP-induced mitochondrial fragmentation (Kong et al. 2014). In response to oxidative stress, p53 interacts with Drp1, a mitochondrial fission protein before translocating to mitochondria. Although the mechanism of p53 translocation is yet to be unraveled, there are some indications of the possible path that p53 might be taking. It has been shown that Mdm2-dependent mono-ubiquitination of cytosolic p53 triggers p53 translocation to mitochondria (Marchenko et al. 2007). Drp1 may act as a facilitator for the Mdm2-dependent mono-ubiquitination of p53 (Guo et al. 2014). The translocated p53 probably undergoes deubiquitination in a HAUSP-dependent manner to revert back to a fully functional form on mitochondrial membrane. Curiously, serine phosphorylated p53 appears to be accumulated in the mitochondrial matrix of mouse cortical neurons (Pei et al. 2014). This suggests that posttranslational modifications of p53 may be employed as a signature for the accurate targeting of p53 into the mitochondrial subcompartments. This possibility is all the more appealing as p53 is known to undergo different kinds of posttranslational modifications including methylation and acetylation besides phosphorylation. Pharmacological drugs and stress conditions can directly modulate mitochondrial translocation of p53 without affecting its nuclear translocation (Strom et al. 2006). Hence, understanding the mechanistic details of p53 translocation to mitochondria might be useful for novel therapeutic interventions as p53 regulates tumor development and ischemic reperfusion injury.

3.3 cAMP Response Element-Binding Protein

The cAMP response element-binding (CREB) protein is a transcriptional factor known to be involved in synaptic transmission and neuron survival. CREB is activated by a set of kinases such as cyclic AMP-dependent protein kinase A (PKA), extracellular regulated kinases (ERKs), and calcium-activated calmodulin kinases (CaMKs) (Altarejos and Montminy 2011). CREB activates transcription upon binding to cAMP-responsive elements present in the upstream region of target genes. Although CREB does not contain any mitochondrial localization signals, it has been shown that CREB gets imported into mitochondria through the TOM complex in a membrane potential-dependent manner with the aid of mitochondrial matrix-residing heat shock protein, mtHSP70. mtHSP70 is known to be involved in the unfolding and translocation of proteins across the mitochondrial inner membrane (Lee et al. 2005). In support of CREB's movement to mitochondria, CREB-binding sites have been detected on the D-loop of mitochondrial genome by chromatin immunoprecipitation (ChIP) assay (Marinov et al. 2014). Mitochondrial depletion of CREB has shown to decrease the expression of several mitochondria-encoded RNAs of complex I with a concomitant reduction in the complex I activity (Lee et al. 2005).

Intriguingly, recent reports have suggested the presence of phosphorylated CREB in mitochondria similar to phosphorylated p53. It is possible that mitochondria choose a similar mechanism for import of both CREB and p53. CREB has also been implicated in the regulation of neuronal survival by regulating mitochondrial gene expression in response to various stimuli in a cell (De Rasmo et al. 2009). Besides the aforementioned functions of CREB in the mitochondria, it also appears to be responsible for pathophysiology of Huntington disease (HD) (Bogdanov et al. 1998; Lee et al. 2005). In addition, CREB is also known to be involved in the regulation of cell death. CREB binds to the CRE element present in the promoter of Bcl-2 gene to induce Bcl-2 overexpression that causes inhibition of apoptosis (Wilson et al. 1996). Hence, CREB multitasks to contribute not only to the activation of a subset of nuclear genes but also in the regulation of apoptosis and neuronal survival.

3.4 NF-kB

NF-kB family of transcription factors responds toward diverse stimuli which result in the expression of genes involved in inflammation, metabolism, cancer, and development. It is well documented that NF-kB transcription factors regulate mitochondrial metabolism through a canonical transcriptional activation pathway. However, there are reports suggesting that they might be playing additional roles in a transcription-independent manner. Along with its inhibitor, Ik-B, several members of the NF-kB family are present in mitochondria. NF-kB was shown to interact with ATP-ADP translocator-1 (Bottero et al. 2001). This interaction promotes the mitochondrial recruitment of NF-kB with a concomitant decrease in

its nuclear activity. This result is further corroborated with decreased expression of known nuclear anti-apoptotic NF-kB target genes, Bcl-xL and c-IAP-2 (Zamora et al. 2004).

Coincidentally, RelA, a NF-kB family member, is also present in the mitochondria, binds to mitochondrial DNA, and inhibits the expression of cytochrome c oxidase III (CoxIII) (Cogswell et al. 2003). It is striking to note that mitochondrial p53 levels negatively correlate with mitochondrial RelA levels. This potential interplay between mitochondrial p53 and RelA levels is further supported by the fact that overexpression of p53 mitigates the inhibitory effect of RelA on mitochondrial gene expression (Johnson et al. 2011). It has been proposed that the actions of p53 and RelA on mitochondrial respiration influence the metabolic switch from OXPHOS to glycolysis (Johnson et al. 2011).

3.5 Signal Transducer and Activator of Transcription

In response to a variety of cytokine stimuli, signal transducer and activator of transcription (STAT) family proteins translocate to the nucleus from cytoplasm to regulate target gene expression (Bromberg et al. 1999). These transcription factors often work together or in opposite manner to regulate various cellular processes. In fact, they regulate energy metabolism and mitochondrial function by modulating the expression of nuclear-encoded mitochondrial genes (Avalle et al. 2012; Walker et al. 2009). Despite their well-established nuclear functions, it has been reported that a pool of STATs are also present in mitochondria and regulate diverse mitochondrial functions (Bourke et al. 2013; Chueh et al. 2010; Wegrzyn et al. 2009). To date, only three of the STAT family members have been reported to be present in mitochondria. These are STAT3, STAT5, and STAT1.

STAT3 was the first STAT family member to be found in the mitochondria. It was shown to regulate the activities of complexes I and II (Wegrzyn et al. 2009). Mitochondrial STAT3 was also shown to mediate the Ras-induced cellular transformation (Gough et al. 2009). Though Tyr705 phosphorylation is required for nuclear functions of STAT3, mitochondrial functions of STAT3 also require phosphorylation, however, at Ser727 (Gough et al. 2009; Wegrzyn et al. 2009). Mitochondrial STAT3 was also shown to protect against ischemic injury by preventing the leakage of electrons from complex I of ETC (Szczepanek et al. 2011). Studies indicate that mitochondrial STAT3 interacts with CypD, thereby suggesting a possible role of this transcription factor in permeability transition (Boengler et al. 2010). A recent study also suggested the involvement of STAT3 in gene expression. SIRT1 is a major NAD-dependent deacetylase and an important marker for cardiovascular, neurological, and aging disorders. SIRT1 is known to be involved in mitochondrial metabolism through deacetylation of PGC-1α and LKB1 (an upstream kinase of AMPK) besides regulating STAT3-mediated mitochondrial respiration (Nemoto et al. 2005). SIRT1 knockdown in MEFs enhances the mitochondrial respiration rate and enzyme activities due to the accumulation of phosphorylated STAT3 in the mitochondria (Bernier et al. 2011).

Taken together, the above studies underscore the importance of the intricate interplay between STAT3 and SIRT1 for executing their mitochondrial functions.

STAT5 is a transcription factor majorly involved in the growth and development of blood cells. However, recently it was also shown to be present in mitochondria. The IL-2 treatment increases mitochondrial recruitment of STAT5 to mitochondria. It also binds to D-loop of mitochondrial DNA and interacts with an E2 subunit of mitochondrial pyruvate dehydrogenase complex (Chueh et al. 2010).

STAT1, a key regulator of antiviral immune response, is also localized to mitochondria. Though there is no function ascribed to this transcription factor, it may be repressing the mitochondrial gene expression as IFN-β inhibition activates STAT1 (Bourke et al. 2013), mitochondrial RNAs, as well as nuclear-encoded mRNAs of ETC.

3.6 Myocyte-Specific Enhancer Factor-2D

The myocyte-specific enhancer factor-2 (MEF-2) family of transcription factors play a major role in immune response, muscle differentiation, and carbohydrate metabolism. Though the involvement of MEF-2 family protein in mitochondrial biogenesis has been known for a long time, only recently the localization of MEF-2D in mitochondria was demonstrated (She et al. 2011). MEF-2D binds to the consensus sequence present in the light strand of the mitochondrial DNA that encodes a complex I subunit called ND6. Disruption of MEF-2D resulted in decreased complex I activity, increased ROS production, and decreased ATP levels. Rotenone treatment decreases the binding of MEF-2D to the ND6 promoter (She et al. 2011). In addition, MMP+ treatment resulted in declined levels of MEF-2D and ND6, which is associated with decreased neuronal viability in brains from MMP+-treated mice. Intriguingly, reduced levels of ND6 and mitochondrial MEF-2D have been documented in postmortem brains of PD patients. These results suggest the crucial role of MEF-2D in regulating the mitochondrial metabolism through modulation of ND6 (Fig. 2).

4 Mechanistic Insights into the Mitochondrial Transport of Nuclear Receptors and Transcription Factors

Mitochondria being a semiautonomous organelle, a majority of its proteins are nuclear DNA encoded. These proteins are synthesized on the cytosolic ribosomes and imported into mitochondria posttranslationally. Nuclear-encoded mitochondrial matrix-targeted proteins, in general, possess cleavable, N-terminal pre-sequence which forms an amphipathic alpha helical structure on the mitochondrial surface. Import receptors that are present on the outer and inner mitochondrial membranes known as TOM and TIM complex, respectively, drive the precursor protein across the inner membrane into the matrix with the help of matrix-localized Hsp70 molecular motor (Schulke et al. 1999; Schulke et al. 1997). Most of the inner

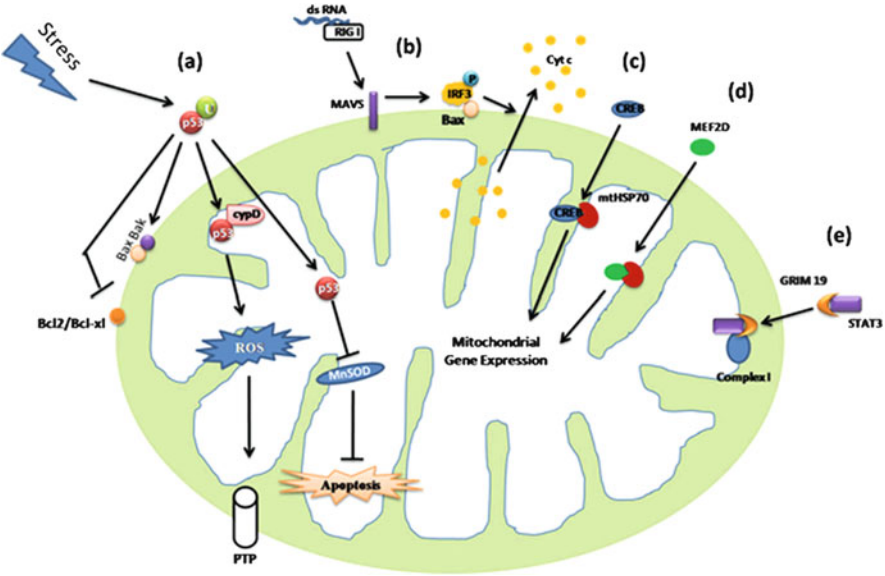

Fig. 2 Mitochondria-localized nuclear transcription factors and their role. (*a*) During stress conditions, mono-ubiquitylated p53 translocates into mitochondria. Matrix-localized p53 sequesters MnSOD and initiates apoptosis. In matrix p53 interacts with CypD and mediates ROS-mediated permeability transition pore opening. p53 interacts with Bcl2 family proteins and anti-apoptotic proteins Bcl-xL and Bcl2. (*b*) During viral infections, RIGI binds to viral dsRNA and interacts with mitochondrial MAVS protein, which activates IRF3. This activated IRF3 interacts with Bax and induces the release of cytochrome c for apoptosis initiation. (*c*, *d*) CREB and MEF-2D translocate to mitochondria by mtHSP70 and induce mitochondrial gene expression. (*e*) GRIM-19 acts as a chaperon to import STAT3 into mitochondria

membrane, outer membrane, and intermembrane space mitochondrial proteins do not contain any cleavable N-terminal targeting sequence; instead, the targeting sequences are embedded within the mature protein sequence (Chacinska et al. 2009). Precursor proteins that contain internal targeting sequence are recognized and made to traverse through the outer membrane receptors Tom70 followed by Tom20 and Tom40/Tom22 of the TOM complex (Anandatheerthavarada et al. 2008; Sepuri et al. 2007). However, pre-sequence-containing proteins are first recognized by Tom20 followed by Tom40/Tom22 of TOM complex. Besides, the mitochondrial recruitment of these proteins requires a cytosolic chaperone system. Both Hsp90 and Hsp70 chaperones guide proteins that harbor an internal targeting sequence, while Hsp70 alone is sufficient to accompany proteins with pre-sequence to the outer membrane receptors.

In contrast nuclear transcription factors despite lacking either of the canonical or non-canonical mitochondrial targeting sequences can translocate to the mitochondria. These proteins make a significant contribution to the myriad functions of mitochondria (Szczepanek et al. 2012). The investigation into their mitochondrial

recruitment has become an active area of research in cell biology. Attempts were made to study the role of Hsp70, Hsp90, TOM complex, and alternative translation initiation in the mitochondrial recruitment of few transcription factors like CREB, RelA, and p53. The exact mechanism adopted by mitochondria to import these nuclear transcription factors is yet to be unraveled despite studies that tried to understand the role of chaperones and mitochondrial import receptors in their import. Studies on alternative translation initiation have also been futile.

One of our studies had focused on the mitochondrial pool of STAT3 and its role in cellular respiration. Our studies showed that mitochondrial STAT3 plays a very critical role in a vast array of cellular processes. Using in vitro and in vivo studies, we have shown that the gene associated with retinoid interferon-induced cell mortality 19 (GRIM-19), a complex I subunit, involved in the recruitment of STAT3 into mitochondria. GRIM-19 acts like a chaperone to enhance the import and integration of STAT3 into mitochondrial complex I. GRIM-19 mediated import of STAT3 requires a Ser727 phosphorylation as a phospho-mutant fails to integrate into the membrane (Tammineni et al. 2013; Zhang et al. 2013).

5 Conclusions and Future Perspectives

In general, transcription factors translocate to the nucleus in response to extracellular cues and regulate gene expression. Recently, novel functions for these transcription factors were also being reported in mitochondria. These mitochondria-localized transcription factors regulate myriad cellular functions like apoptosis, cell survival, and mitochondrial gene expression. In fact, the list of transcription factors associated with mitochondria and their functions is increasing exponentially. These findings suggest a new paradigm that mitochondrial function of transcription factors serves as the key determinant of cell fate over their nuclear function (Szczepanek et al. 2012). Despite the significant role of nuclear transcription factors in mitochondria, our understanding of their mitochondrial functions and targeting is limited at this moment.

Mitochondrial proteins utilize either cleavable canonical mitochondrial targeting sequence or internal bipartite sequence for their transport to mitochondria (Chacinska et al. 2009). Since most of the transcription factors do not have either of the mitochondrial targeting sequences, understanding their mechanism of mitochondrial transport would be an active area of research. Initial studies in this direction identify the importance of some of the chaperones and mitochondria-associated proteins in this process. However, it would be of more interest to see whether these transcription factors compete or cooperate for their mitochondrial targeting or utilize unique or shared pathways for their transport to mitochondria. Since most of the transcription factors identified so far are associated with various disease conditions like cancer and cardiac injury, understanding the mechanism of mitochondrial recruitment may also provide novel insights into precise therapeutic interventions sparing their nuclear functions intact.

Moreover, each subcompartment of mitochondria is discrete in structure and function. Precise submitochondrial localization of transcription factors rather than mitochondrial localization per se would provide novel insights into our current understanding of various cellular functions.

Another important aspect would be to investigate the cross talk between various transcription factors in regulating the mitochondrial functions. The role of transcription factors in controlling each other's function in the nucleus is well established. However, it remains largely unknown whether such regulation also exists in the mitochondria to regulate either their targeting or mitochondrial function. For instance, STAT3 promotes cell proliferation and motility to promote tumorigenesis. On the other hand, STAT1, in general, triggers antiproliferative and pro-apoptotic responses in tumor cells (Avalle et al. 2012). As these two transcription factors are present in mitochondria and mitochondria play a significant role in tumorigenesis, it would be interesting to see how these transcription factors regulate mitochondrial function and whether loss of this balance sufficiently triggers tumorigenesis. Likewise, IRF3 and p53, though, respond to similar kinds of stresses and translocate to mitochondria. Do they have opposing effects on mitochondrial RNA expression? Hence, investigating the cross talk among transcription factors would probably provide novel insights into our current understanding of these processes.

Steady-state levels of most of the nuclear transcription factors, under normal physiological conditions, in the mitochondria are very minimal. These transcription factors are subject to change in response to various stimuli. Very little progress is being made in understanding the role of the posttranslational modification in directing the transcription factors to mitochondria. For instance, phosphorylation of STAT3 at Ser727 is shown to be essential for its mitochondrial functions (Gough et al. 2009; Wegrzyn et al. 2009), whereas upstream kinase or signal that is responsible for STAT3 phosphorylation in the context of mitochondria remains to be understood. Similarly, it remains to be elucidated the significance of mono-ubiquitination in mitochondrial targeting of p53 (Marchenko et al. 2007). Hence, further studies are needed to understand whether posttranslational modifications are sufficed to target them to mitochondria or increase their interaction with chaperons or other mitochondria-associated proteins to share their ride to mitochondria. It is also possible that phosphorylation may expose cryptic mitochondrial targeting sequence present (Robin et al. 2002) or induce conformational changes such that noncontiguous sequences brought together to generate the mitochondria-targeting sequence. However, further studies may shed light on the mechanistic details employed by mitochondria to import these nuclear transcription factors. Nevertheless, mounting evidence suggests that nuclear transcription factors in their novel milieu help in fine-tuning mitochondrial metabolism to evoke response toward environmental cues.

Acknowledgments We would like to thank all the members of Dr. Sepuri lab for critical comments on the manuscript. We are grateful to Dr. Thanuja Krishnamoorthy for critically evaluating the manuscript. We thank the funding agency SERB to Dr. Sepuri lab and DST-FIST

and UGC-SAP to the department. Mr. Arun Kumar and Fareed Mohammed are supported by CSIR and UGC Junior Research Fellowship, respectively.

References

Altarejos JY, Montminy M (2011) CREB and the CRTC co-activators: sensors for hormonal and metabolic signals. Nat Rev Mol Cell Biol 12:141–151. doi:10.1038/nrm3072

Anandatheerthavarada HK, Sepuri NB, Biswas G, Avadhani NG (2008) An unusual TOM20/TOM22 bypass mechanism for the mitochondrial targeting of cytochrome P450 proteins containing N-terminal chimeric signals. J Biol Chem 283:36060

Avalle L, Pensa S, Regis G, Novelli F, Poli V (2012) STAT1 and STAT3 in tumorigenesis: a matter of balance. JAKSTAT 1:65–72. doi:10.4161/jkst.20045

Bernier M, Paul RK, Martin-Montalvo A, Scheibye-Knudsen M, Song S, He HJ, Armour SM, Hubbard BP, Bohr VA, Wang L, Zong Y, Sinclair DA, de Cabo R (2011) Negative regulation of STAT3 protein-mediated cellular respiration by SIRT1 protein. J Biol Chem 286:19270–19279. doi:10.1074/jbc.M110.200311

Blanchet E, Bertrand C, Annicotte JS, Schlernitzauer A, Pessemesse L, Levin J, Fouret G, Feillet-Coudray C, Bonafos B, Fajas L, Cabello G, Wrutniak-Cabello C, Casas F (2012) Mitochondrial T3 receptor p43 regulates insulin secretion and glucose homeostasis. FASEB J 26:40–50. doi:10.1096/fj.11-186841

Boengler K, Hilfiker-Kleiner D, Heusch G, Schulz R (2010) Inhibition of permeability transition pore opening by mitochondrial STAT3 and its role in myocardial ischemia/reperfusion. Basic Res Cardiol 105:771–785. doi:10.1007/s00395-010-0124-1

Bogdanov MB, Ferrante RJ, Kuemmerle S, Klivenyi P, Beal MF (1998) Increased vulnerability to 3-nitropropionic acid in an animal model of Huntington's disease. J Neurochem 71:2642–2644

Bopassa JC, Eghbali M, Toro L, Stefani E (2010) A novel estrogen receptor GPER inhibits mitochondria permeability transition pore opening and protects the heart against ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 298:H16–H23. doi:10.1152/ajpheart.00588.2009

Bottero V, Rossi F, Samson M, Mari M, Hofman P, Peyron JF (2001) Ikappa b-alpha, the NF-kappa B inhibitory subunit, interacts with ANT, the mitochondrial ATP/ADP translocator. J Biol Chem 276:21317–21324. doi:10.1074/jbc.M005850200M005850200

Bourke LT, Knight RA, Latchman DS, Stephanou A, McCormick J (2013) Signal transducer and activator of transcription-1 localizes to the mitochondria and modulates mitophagy. JAKSTAT 2, e25666. doi:10.4161/jkst.256662013JAKS0144R1

Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, Darnell JE Jr (1999) Stat3 as an oncogene. Cell 98:295–303, doi: S0092-8674(00)81959-5 [pii]

Cammarata PR, Chu S, Moor A, Wang Z, Yang SH, Simpkins JW (2004) Subcellular distribution of native estrogen receptor alpha and beta subtypes in cultured human lens epithelial cells. Exp Eye Res 78:861–871. doi:10.1016/j.exer.2003.09.027S0014483503003907

Casas F, Pessemesse L, Grandemange S, Seyer P, Gueguen N, Baris O, Lepourry L, Cabello G, Wrutniak-Cabello C (2008) Overexpression of the mitochondrial T3 receptor p43 induces a shift in skeletal muscle fiber types. PLoS One 3, e2501. doi:10.1371/journal.pone.0002501

Casas F, Pessemesse L, Grandemange S, Seyer P, Baris O, Gueguen N, Ramonatxo C, Perrin F, Fouret G, Lepourry L, Cabello G, Wrutniak-Cabello C (2009) Overexpression of the mitochondrial T3 receptor induces skeletal muscle atrophy during aging. PLoS One 4, e5631. doi:10.1371/journal.pone.0005631

Chacinska A, Koehler CM, Milenkovic D, Lithgow T, Pfanner N (2009) Importing mitochondrial proteins: machineries and mechanisms. Cell 138:628–644. doi:10.1016/j.cell.2009.08.005

Chattopadhyay S, Marques JT, Yamashita M, Peters KL, Smith K, Desai A, Williams BR, Sen GC (2010) Viral apoptosis is induced by IRF-3-mediated activation of Bax. EMBO J 29:1762–1773. doi:10.1038/emboj.2010.50

Chen JQ, Delannoy M, Cooke C, Yager JD (2004) Mitochondrial localization of ERalpha and ERbeta in human MCF7 cells. Am J Physiol Endocrinol Metab 286:E1011–E1022. doi:10.1152/ajpendo.00508.200300508.2003

Chipuk JE, Kuwana T, Bouchier-Hayes L, Droin NM, Newmeyer DD, Schuler M, Green DR (2004) Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. Science 303:1010–1014. doi:10.1126/science.1092734303/5660/1010

Chueh FY, Leong KF, Yu CL (2010) Mitochondrial translocation of signal transducer and activator of transcription 5 (STAT5) in leukemic T cells and cytokine-stimulated cells. Biochem Biophys Res Commun 402:778–783. doi:10.1016/j.bbrc.2010.10.112

Ciucci A, Zannoni GF, Travaglia D, Scambia G, Gallo D (2015) Mitochondrial estrogen receptor beta2 drives antiapoptotic pathways in advanced serous ovarian cancer. Hum Pathol 46:1138–1146. doi:10.1016/j.humpath.2015.03.016

Cogswell PC, Kashatus DF, Keifer JA, Guttridge DC, Reuther JY, Bristow C, Roy S, Nicholson DW, Baldwin AS Jr (2003) NF-kappa B and I kappa B alpha are found in the mitochondria. Evidence for regulation of mitochondrial gene expression by NF-kappa B. J Biol Chem 278:2963–2968. doi:10.1074/jbc.M209995200M209995200

De Rasmo D, Signorile A, Roca E, Papa S (2009) cAMP response element-binding protein (CREB) is imported into mitochondria and promotes protein synthesis. FEBS J 276:4325–4333. doi:10.1111/j.1742-4658.2009.07133.x

Demonacos C, Tsawdaroglou NC, Djordjevic-Markovic R, Papalopoulou M, Galanopoulos V, Papadogeorgaki S, Sekeris CE (1993) Import of the glucocorticoid receptor into rat liver mitochondria in vivo and in vitro. J Steroid Biochem Mol Biol 46:401–413

Demonacos C, Djordjevic-Markovic R, Tsawdaroglou N, Sekeris CE (1995) The mitochondrion as a primary site of action of glucocorticoids: the interaction of the glucocorticoid receptor with mitochondrial DNA sequences showing partial similarity to the nuclear glucocorticoid responsive elements. J Steroid Biochem Mol Biol 55:43–55, doi: 096007609500159W [pii]

Du J, McEwen B, Manji HK (2009) Glucocorticoid receptors modulate mitochondrial function: a novel mechanism for neuroprotection. Commun Integr Biol 2:350–352

Gough DJ, Corlett A, Schlessinger K, Wegrzyn J, Larner AC, Levy DE (2009) Mitochondrial STAT3 supports Ras-dependent oncogenic transformation. Science 324:1713–1716. doi:10.1126/science.1171721

Grandemange S, Seyer P, Carazo A, Becuwe P, Pessemesse L, Busson M, Marsac C, Roger P, Casas F, Cabello G, Wrutniak-Cabello C (2005) Stimulation of mitochondrial activity by p43 overexpression induces human dermal fibroblast transformation. Cancer Res 65:4282–4291. doi:10.1158/0008-5472.CAN-04-3652

Guo X, Sesaki H, Qi X (2014) Drp1 stabilizes p53 on the mitochondria to trigger necrosis under oxidative stress conditions in vitro and in vivo. Biochem J 461:137–146. doi:10.1042/BJ20131438

Johnson RF, Witzel II, Perkins ND (2011) p53-dependent regulation of mitochondrial energy production by the RelA subunit of NF-kappaB. Cancer Res 71:5588–5597. doi:10.1158/0008-5472.CAN-10-4252

Kabir ME, Singh H, Lu R, Olde B, Leeb-Lundberg LM, Bopassa JC (2015) G protein-coupled estrogen receptor 1 mediates acute estrogen-induced cardioprotection via MEK/ERK/GSK-3beta pathway after ischemia/reperfusion. PLoS One 10, e0135988. doi:10.1371/journal.pone.0135988

Kong B, Wang Q, Fung E, Xue K, Tsang BK (2014) p53 is required for cisplatin-induced processing of the mitochondrial fusion protein L-Opa1 that is mediated by the mitochondrial metallopeptidase Oma1 in gynecologic cancers. J Biol Chem 289:27134–27145. doi:10.1074/jbc.M114.594812

Lee J, Kim CH, Simon DK, Aminova LR, Andreyev AY, Kushnareva YE, Murphy AN, Lonze BE, Kim KS, Ginty DD, Ferrante RJ, Ryu H, Ratan RR (2005) Mitochondrial cyclic AMP response element-binding protein (CREB) mediates mitochondrial gene expression and neuronal survival. J Biol Chem 280:40398–40401. doi:10.1074/jbc.C500140200

Leigh-Brown S, Enriquez JA, Odom DT (2010) Nuclear transcription factors in mammalian mitochondria. Genome Biol 11:215. doi:10.1186/gb-2010-11-7-215

Leu JI, Dumont P, Hafey M, Murphy ME, George DL (2004) Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex. Nat Cell Biol 6:443–450. doi:10.1038/ncb1123ncb1123

Liang J, Xie Q, Li P, Zhong X, Chen Y (2015) Mitochondrial estrogen receptor beta inhibits cell apoptosis via interaction with Bad in a ligand-independent manner. Mol Cell Biochem 401:71–86. doi:10.1007/s11010-014-2293-y

Marchenko ND, Wolff S, Erster S, Becker K, Moll UM (2007) Monoubiquitylation promotes mitochondrial p53 translocation. EMBO J 26:923–934. doi:10.1038/sj.emboj.7601560

Marinov GK, Wang YE, Chan D, Wold BJ (2014) Evidence for site-specific occupancy of the mitochondrial genome by nuclear transcription factors. PLoS One 9, e84713. doi:10.1371/journal.pone.0084713PONE-D-13-34454

McBride HM, Neuspiel M, Wasiak S (2006) Mitochondria: more than just a powerhouse. Curr Biol 16:R551–R560. doi:10.1016/j.cub.2006.06.054

Milner TA, Ayoola K, Drake CT, Herrick SP, Tabori NE, McEwen BS, Warrier S, Alves SE (2005) Ultrastructural localization of estrogen receptor beta immunoreactivity in the rat hippocampal formation. J Comp Neurol 491:81–95. doi:10.1002/cne.20724

Mokranjac D, Neupert W (2009) Thirty years of protein translocation into mitochondria: unexpectedly complex and still puzzling. Biochim Biophys Acta 1793:33–41. doi:10.1016/j.bbamcr.2008.06.021

Morrish F, Buroker NE, Ge M, Ning XH, Lopez-Guisa J, Hockenbery D, Portman MA (2006) Thyroid hormone receptor isoforms localize to cardiac mitochondrial matrix with potential for binding to receptor elements on mtDNA. Mitochondrion 6:143–148. doi:10.1016/j.mito.2006.04.002

Moutsatsou P, Psarra AM, Tsiapara A, Paraskevakou H, Davaris P, Sekeris CE (2001) Localization of the glucocorticoid receptor in rat brain mitochondria. Arch Biochem Biophys 386:69–78. doi:10.1006/abbi.2000.2162

Nemoto S, Fergusson MM, Finkel T (2005) SIRT1 functionally interacts with the metabolic regulator and transcriptional coactivator PGC-1{alpha}. J Biol Chem 280:16456–16460. doi:10.1074/jbc.M501485200

Pedram A, Razandi M, Wallace DC, Levin ER (2006) Functional estrogen receptors in the mitochondria of breast cancer cells. Mol Biol Cell 17:2125–2137. doi:10.1091/mbc.E05-11-1013

Pei L, Shang Y, Jin H, Wang S, Wei N, Yan H, Wu Y, Yao C, Wang X, Zhu LQ, Lu Y (2014) DAPK1-p53 interaction converges necrotic and apoptotic pathways of ischemic neuronal death. J Neurosci 34:6546–6556. doi:10.1523/JNEUROSCI.5119-13.2014

Psarra AM, Sekeris CE (2011) Glucocorticoids induce mitochondrial gene transcription in HepG2 cells: role of the mitochondrial glucocorticoid receptor. Biochim Biophys Acta 1813:1814–1821. doi:10.1016/j.bbamcr.2011.05.014

Robin MA, Anandatheerthavarada HK, Biswas G, Sepuri NB, Gordon DM, Pain D, Avadhani NG (2002) Bimodal targeting of microsomal CYP2E1 to mitochondria through activation of an N-terminal chimeric signal by cAMP-mediated phosphorylation. J Biol Chem 277:40583–40593. doi:10.1074/jbc.M203292200M203292200

Scarpulla RC, Vega RB, Kelly DP (2012) Transcriptional integration of mitochondrial biogenesis. Trends Endocrinol Metab 23:459–466. doi:10.1016/j.tem.2012.06.006

Scheller K, Sekeris CE, Krohne G, Hock R, Hansen IA, Scheer U (2000) Localization of glucocorticoid hormone receptors in mitochondria of human cells. Eur J Cell Biol 79:299–307. doi:10.1078/S0171-9335(04)70033-3

Schulke N, Sepuri NB, Pain D (1997) In vivo zippering of inner and outer mitochondrial membranes by a stable translocation intermediate. Proc Natl Acad Sci U S A 94:7314–7319

Schulke N, Sepuri NB, Gordon DM, Saxena S, Dancis A, Pain D (1999) A multisubunit complex of outer and inner mitochondrial membrane protein translocases stabilized in vivo by translocation intermediates. J Biol Chem 274:22847–22854

Sepuri NB, Yadav S, Anandatheerthavarada HK, Avadhani NG (2007) Mitochondrial targeting of intact CYP2B1 and CYP2E1 and N-terminal truncated CYP1A1 proteins in Saccharomyces cerevisiae--role of protein kinase A in the mitochondrial targeting of CYP2E1. FEBS J 274:4615–4630. doi:10.1111/j.1742-4658.2007.05990.x

Sharif-Askari E, Nakhaei P, Oliere S, Tumilasci V, Hernandez E, Wilkinson P, Lin R, Bell J, Hiscott J (2007) Bax-dependent mitochondrial membrane permeabilization enhances IRF3-mediated innate immune response during VSV infection. Virology 365:20–33. doi:10.1016/j.virol.2007.03.011

She H, Yang Q, Shepherd K, Smith Y, Miller G, Testa C, Mao Z (2011) Direct regulation of complex I by mitochondrial MEF2D is disrupted in a mouse model of Parkinson disease and in human patients. J Clin Invest 121:930–940. doi:10.1172/JCI43871

Solakidi S, Psarra AM, Sekeris CE (2005) Differential subcellular distribution of estrogen receptor isoforms: localization of ERalpha in the nucleoli and ERbeta in the mitochondria of human osteosarcoma SaOS-2 and hepatocarcinoma HepG2 cell lines. Biochim Biophys Acta 1745:382–392. doi:10.1016/j.bbamcr.2005.05.010

Sterling K, Campbell GA, Brenner MA (1984) Purification of the mitochondrial triiodothyronine (T3) receptor from rat liver. Acta Endocrinol (Copenh) 105:391–397

Strom E, Sathe S, Komarov PG, Chernova OB, Pavlovska I, Shyshynova I, Bosykh DA, Burdelya LG, Macklis RM, Skaliter R, Komarova EA, Gudkov AV (2006) Small-molecule inhibitor of p53 binding to mitochondria protects mice from gamma radiation. Nat Chem Biol 2:474–479. doi:10.1038/nchembio809

Szczepanek K, Chen Q, Derecka M, Salloum FN, Zhang Q, Szelag M, Cichy J, Kukreja RC, Dulak J, Lesnefsky EJ, Larner AC (2011) Mitochondrial-targeted Signal transducer and activator of transcription 3 (STAT3) protects against ischemia-induced changes in the electron transport chain and the generation of reactive oxygen species. J Biol Chem 286:29610–29620. doi:10.1074/jbc.M111.226209

Szczepanek K, Lesnefsky EJ, Larner AC (2012) Multi-tasking: nuclear transcription factors with novel roles in the mitochondria. Trends Cell Biol 22:429–437. doi:10.1016/j.tcb.2012.05.001

Talaber G, Boldizsar F, Bartis D, Palinkas L, Szabo M, Berta G, Setalo G Jr, Nemeth P, Berki T (2009) Mitochondrial translocation of the glucocorticoid receptor in double-positive thymocytes correlates with their sensitivity to glucocorticoid-induced apoptosis. Int Immunol 21:1269–1276. doi:10.1093/intimm/dxp093

Tammineni P, Anugula C, Mohammed F, Anjaneyulu M, Larner AC, Sepuri NB (2013) The import of the transcription factor STAT3 into mitochondria depends on GRIM-19, a component of the electron transport chain. J Biol Chem 288:4723–4732. doi:10.1074/jbc.M112.378984

Walker SR, Nelson EA, Zou L, Chaudhury M, Signoretti S, Richardson A, Frank DA (2009) Reciprocal effects of STAT5 and STAT3 in breast cancer. Mol Cancer Res 7:966–976. doi:10.1158/1541-7786.MCR-08-0238

Wegrzyn J, Potla R, Chwae YJ, Sepuri NB, Zhang Q, Koeck T, Derecka M, Szczepanek K, Szelag M, Gornicka A, Moh A, Moghaddas S, Chen Q, Bobbili S, Cichy J, Dulak J, Baker DP, Wolfman A, Stuehr D, Hassan MO, Fu XY, Avadhani N, Drake JI, Fawcett P, Lesnefsky EJ, Larner AC (2009) Function of mitochondrial Stat3 in cellular respiration. Science 323:793–797. doi:10.1126/science.1164551

Wilson BE, Mochon E, Boxer LM (1996) Induction of bcl-2 expression by phosphorylated CREB proteins during B-cell activation and rescue from apoptosis. Mol Cell Biol 16:5546–5556

Xie Q, Huang Z, Liu Y, Liu X, Huang L (2015) Mitochondrial estrogen receptor beta inhibits non-small cell lung cancer cell apoptosis via interaction with Bad. Nan Fang Yi Ke Da Xue Xue Bao 35:98–102

Zamora M, Merono C, Vinas O, Mampel T (2004) Recruitment of NF-kappaB into mitochondria is involved in adenine nucleotide translocase 1 (ANT1)-induced apoptosis. J Biol Chem 279:38415–38423. doi:10.1074/jbc.M404928200M404928200

Zhang Q, Raje V, Yakovlev VA, Yacoub A, Szczepanek K, Meier J, Derecka M, Chen Q, Hu Y, Sisler J, Hamed H, Lesnefsky EJ, Valerie K, Dent P, Larner AC (2013) Mitochondrial localized Stat3 promotes breast cancer growth via phosphorylation of serine 727. J Biol Chem 288:31280–31288. doi:10.1074/jbc.M113.505057

Zhao Y, Chaiswing L, Velez JM, Batinic-Haberle I, Colburn NH, Oberley TD, St Clair DK (2005) p53 translocation to mitochondria precedes its nuclear translocation and targets mitochondrial oxidative defense protein-manganese superoxide dismutase. Cancer Res 65:3745–3750. doi:10.1158/0008-5472.CAN-04-3835

Zhou Z, Zhou J, Du Y (2012) Estrogen receptor alpha interacts with mitochondrial protein HADHB and affects beta-oxidation activity. Mol Cell Proteomics 11(M111):011056. doi:10.1074/mcp.M111.011056

Zilfou JT, Lowe SW (2009) Tumor suppressive functions of p53. Cold Spring Harb Perspect Biol 1:a001883. doi:10.1101/cshperspect.a001883

The Mitochondrial Permeability Transition Pore and ATP Synthase

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Contents

The original version of this chapter was revised. An erratum to this chapter can be found at DOI 10.1007/164_2016_87

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H. Singh, S.-S. Sheu (eds.), *Pharmacology of Mitochondria*,
Handbook of Experimental Pharmacology 240, DOI 10.1007/164_2016_5

Abstract

Mitochondrial ATP generation by oxidative phosphorylation combines the stepwise oxidation by the electron transport chain (ETC) of the reducing equivalents NADH and $FADH_2$ with the generation of ATP by the ATP synthase. Recent studies show that the ATP synthase is not only essential for the generation of ATP but may also contribute to the formation of the mitochondrial permeability transition pore (PTP). We present a model, in which the PTP is located within the c-subunit ring in the F_o subunit of the ATP synthase. Opening of the PTP was long associated with uncoupling of the ETC and the initiation of programmed cell death. More recently, it was shown that PTP opening may serve a physiologic role: it can transiently open to regulate mitochondrial signaling in mature cells, and it is open in the embryonic mouse heart. This review will discuss how the ATP synthase paradoxically lies at the center of both ATP generation and cell death.

Keywords

ATP synthase • Bioenergetics • Electron transport chain • Embryonic heart • Mitochondria • Permeability transition pore

1 Mitochondrial Energy Production

1.1 Metabolic Substrates and Energy Supply

Oxidative phosphorylation (OXPHOS) can be defined as the oxidation of metabolic substrates by cytosolic and then mitochondrial enzymes to release energy that is transferred to ATP, which is the basic energetic currency of the cell (Fig. 1). The main metabolic substrates for all cellular processes are carbohydrates, fatty acids, and proteins. Carbohydrates are transformed into glucose and, via the glycolytic pathway, pyruvate, which enters the mitochondrion where it is decarboxylated and acetylated by pyruvate dehydrogenase to produce acetyl coenzyme A (CoA). A second source of acetyl-CoA is the β-oxidation of fatty acids in the mitochondrial matrix. Proteins can be hydrolyzed into peptides and amino acids, which are then deaminated and converted into pyruvate or acetyl-CoA. In the mitochondrial matrix, acetyl-CoA enters the tricarboxylic acid (TCA, aka. citric acid, Krebs) cycle, which generates NADH and $FADH_2$, the substrates for the mitochondrial ETC, which is located in the mitochondrial cristae membrane.

1.2 The Electron Transport Chain

The mitochondrial ETC consists of five large, multi-protein complexes (Fig. 1). The proximal complexes (I, II, III, and IV) use the energy supplied by electron transport

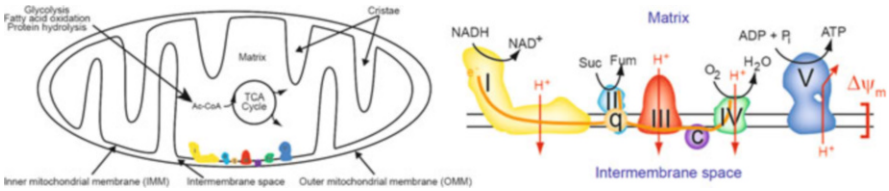

Fig. 1 Model of oxidative phosphorylation and the electron transport chain. (*Left*) Macroscopic diagram of a mitochondrion and metabolic pathways. Mitochondria utilize carbons from the metabolism of glucose, fatty acids, proteins, and other metabolic pathways to feed the TCA cycle, which produces NADH. Mitochondrial structures that are indicated are as follows: the cristae, the inner mitochondrial membrane (IMM), the intermembrane space, the matrix, and the outer mitochondrial membrane (OMM). (*Right*) Microscopic diagram of the electron transport chain. The oxidation of **NADH** to $\mathbf{NAD^+}$ donates electrons ($\mathbf{e^-}$) to complex I (**I**, NADH-ubiquinone oxidoreductase/dehydrogenase), where they are eventually passed to ubiquinone (**q**, coenzyme Q). In the TCA cycle, succinate (**Suc**) is oxidized to fumarate (**Fum**) at complex II (**II**, succinate dehydrogenase, the electron carrier is $FADH_2$), and electrons are passed to q. Electrons flow from q to complex III (**III**, ubiquinol/cytochrome *c* oxidoreductase/dehydrogenase) to cytochrome *c* (**C**) to complex IV (**IV**, cytochrome *c* oxidase), where they reduce O_2 to water. Complexes I, III, and IV pump protons ($\mathbf{H^+}$) across the inner mitochondrial membrane to generate the membrane potential ($\Delta\psi_m$) that complex V (**V**, ATP synthase, F_1F_o ATPase) taps to synthesize ATP. The matrix and intermembrane space are indicated

to generate an electrochemical gradient across the mitochondrial inner membrane (IMM), which complex V (also known as ATP synthase or F_1F_o-ATP synthase) is used to produce ATP. The subunits of complexes I, III, IV, and V are encoded in both the nuclear and mitochondrial genome, while the four subunits of complex II are nuclear encoded (Wallace 1999).

Electrons enter the ETC at three sites: complex I, complex II, and ubiquinone (Fig. 1). Oxidation of NADH in the hydrophilic, matrix-protruding arm of complex I donates electrons that flow through a series of iron-sulfur-containing subunits to eventually reduce ubiquinone. Complex II of the ETC is also the succinate dehydrogenase enzyme of the TCA cycle, and the oxidation of succinate to fumarate reduces complex II, which then reduces ubiquinone. At least three other enzymes can also directly reduce the ubiquinone pool (Nicholls and Ferguson 2013). Ubiquinol (reduced ubiquinone) is then oxidized by complex III, which then uses these electrons to reduce cytochrome c. Cytochrome c in the intermembrane space then reduces complex IV, leading to the final redox reaction where complex IV reduces oxygen to water.

Energy released from the flow of electrons is used by complexes I, III, and IV to pump protons against their electrochemical gradient into the intermembrane space (Fig. 1). This gradient is called the electrochemical proton gradient ($\Delta\mu_H$, proportional to the proton motive force, Δp) that is the product of the electrical ($\Delta\psi_m$) and proton (ΔpH) gradients across the IMM. Maintenance of this gradient is essential to OXPHOS (see below) and requires high capacitance of the inner mitochondrial membrane so that neither $\Delta\psi_m$ nor ΔpH are dissipated. Mitochondria prevent such leaks by tightly regulating any leak currents as well as the many ion channels and transporters that reside in the IMM (reviewed in Szabo and Zoratti 2014).

The major source of mitochondrial reactive oxygen species (ROS) is the ETC (Fig. 1), although other enzymes may play lesser roles. Slipping of electron flowing through complexes I and III can form superoxide anions in the matrix, while complex III can also release superoxide into the intermembrane space (Nickel et al. 2014). In addition, it was recently suggested that complex II can also produce ROS (Quinlan et al. 2012).

1.3 Electron Transport Chain Assembly and Respirasomes

The four proximal members of the ETC are large, multi-subunit protein complexes that must be assembled to function efficiently. Each requires additional assembly factors, and their assembly may be controlled by the assembly of the other complexes, but the exact mechanisms that regulate the assembly of each remain unclear. Assembly proceeds through sub-complexes, monomers, dimers, and higher-order homo- and hetero-oligomers (Fig. 2).

An example of this assembly is complex I. In mammals, it is the largest and most complicated ETC complex and contains at least 44 subunits that combine to form a membrane-embedded hydrophobic domain and a hydrophilic domain containing a chain of the redox centers that protrudes into the matrix (Hirst 2013). It remains unclear how the assembly of these subunits into a functional complex I monomer is regulated, but additional assembly factors/proteins are required (Mimaki et al. 2012). In addition, its assembly can be stabilized if it is co-assembled into respirasome supercomplexes (Fig. 2) (Calvaruso et al. 2012).

Assembly of respirasomes adds an additional layer of complexity to ETC activity. Technical advances and the development of blue and clear-native electrophoresis show that complexes I, III, and IV (along with ubiquinone and cytochrome *c*) have the tendency to form respiratory active supercomplexes (Dudkina et al. 2008; Wittig et al. 2007). The formation of respirasomes appears to confer a bioenergetic advantage, because the close proximity of the complexes increases the efficiency of electron transfer (Cogliati et al. 2013; Genova and Lenaz 2013). Respirasomes are functionally active even after purifying them by blue native electrophoresis (Acin-Perez et al. 2008). It appears that complexes III and IV begin the process of the respirasome formation followed by the addition of partially assembled and still de-active complex I (Moreno-Lastres et al. 2012). Not only does respirasome

Fig. 2 Electron transport chain assembly into respirasomes. When the individual complexes of the ETC transfer electrons by random collision, electron flux is less efficient (*left*). However, the assembly of complexes I, III, and IV with ubiquinone and cytochrome *c* into supercomplexes called respirasomes greatly increases the efficiency of electron transfer and oxygen consumption

formation increase ETC efficiency, but it also decreases ROS production, perhaps due to the more efficient flow of electrons through the chain (Maranzana et al. 2013). However, it is not clear whether respirasomes are permanent structures (solid model) or form depending on the bioenergetic requirements of the cell (plastic model) (Cogliati et al. 2013; Genova and Lenaz 2013; Lapuente-Brun et al. 2013).

1.4 ATP Synthase

The proximal four complexes of the ETC accept and transfer electrons from NADH and the TCA cycle, and subsequent proton translocation by complexes I, III, and IV from the mitochondrial matrix into the intermembrane space creates the proton motive force (Δp) to drive ATP synthesis by ATP synthase (complex V or F_1F_o ATP synthase; Jonckheere et al. 2012). The monomer of the mammalian ATP synthase is a ~600 kDa protein complex of 15 subunits and consists of 2 functional units (Fig. 3): the membrane-embedded F_o subunit and the matrix facing F_1 subunit.

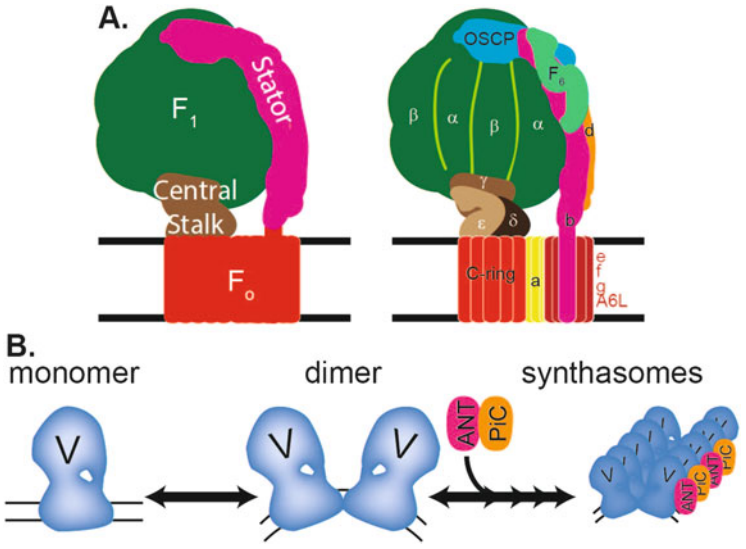

Fig. 3 ATP synthase structure and assembly into synthasomes. (**a**) Diagrams of structure of an ATP synthase monomer. (*Left*) The structural components of the complex are F_1 (*green*), F_o (*orange*), central stalk (*brown*), and stator (*pink*). (*Right*) The position of the 15 subunits in the complex is labeled (adapted from Dickson et al. 2006). The F_1 component contains the α- and β-subunits. The F_o component contains the a-, b-, c-, e-, f-, g-, and A6L-subunits. The F_1 and F_o components are connected by the central stalk (δ-, ε-, and γ-subunits) and the stator (b-, d-, F_6-, and OSCP-subunits). (**b**) ATP synthase monomers combine to form dimers, which then assemble with adenine nucleotide translocase (ANT) and the phosphate carrier (PiC) into supercomplexes called synthasomes that increase the efficiency of energy (ATP) production and energy transfer into the sarcoplasm. Ribbons of synthasomes likely increase this efficiency and mold the cristae into tubular structures

In bovine heart, F_o contains a ring of 8 very hydrophobic c-subunits and the subunits a, b, e, f, g, and A6L. A central stalk composed of the subunits δ, ε, and γ connects the c-subunit ring to the catalytic F_1 unit, which consists of a hexamer of alternating α and β subunits, where ATP synthesis and hydrolysis occur. Finally, a lateral stalk or stator containing the subunits b, d, and F6 and the oligomycin-sensitivity conferring protein (OSCP) connects the lateral portion of F_o to the top of F_1. Movement of protons between the c- and a-subunit causes rotation of the c-subunit ring and provides the energy necessary for F_1 to synthesize ATP (Walker 2013).

ATP synthase monomers are enzymatically active, but recent studies show that in vivo the ATP synthase forms dimers and ribbons of even-numbered oligomers (Fig. 3) (Bornhovd et al. 2006; Davies et al. 2011; Wittig and Schagger 2008). Oligomerization of ATP synthase has been shown to shape the cristae membranes, and this may confer a physiologic advantage (Cogliati et al. 2013; Davies et al. 2011). For example, ATP synthase oligomerization is essential to build and to maintain the mitochondrial membrane potential and local proton charge to increase ATP synthase activity (Bornhovd et al. 2006). Since the mitochondrial ATP synthase harbors the PTP (see below), elucidating the functional regulation of monomers, dimers, and oligomers will provide important knowledge of how the ATP synthase transforms into the PTP (see below for detailed discussion).

The ATP synthase also forms supercomplexes with the mitochondrial creatine kinase (mtCK), adenine nucleotide translocase (ANT), and phosphate carrier (PiC) (Fig. 3). These "synthasomes" connect the machinery required for ATP generation (ATP synthase) to that of ADP/ATP and phosphate exchange (ANT, PiC) and energy transfer to the cytoplasm (ANT, mtCK), therefore creating a regulatory unit where energy production and exchange pathways meet (Chen et al. 2004; Saks et al. 2012).

2 The Permeability Transition Pore

The permeability transition was first described in the 1950s as an acute swelling and uncoupling of mitochondria when exposed to high calcium concentrations and phosphate, oxidative stress, or other conditions (Fig. 4; for more details of the history of the PT, see Bernardi 2013). The term permeability transition was introduced by Haworth and Hunter, who first described the pharmacological properties of the permeability transition pore (PTP) (Haworth and Hunter 1979; Hunter and Haworth 1979a, b). Their work and that of many others indicated that during PT, a large pore/ion channel opens to allow molecules of up to 1.5 kDa across the IMM.

2.1 Physiologic Consequences of the Permeability Transition

For a long time, the opening of the PTP was considered to be a traumatic event leading to cell death (Fig. 4). Increased permeability of the IMM causes

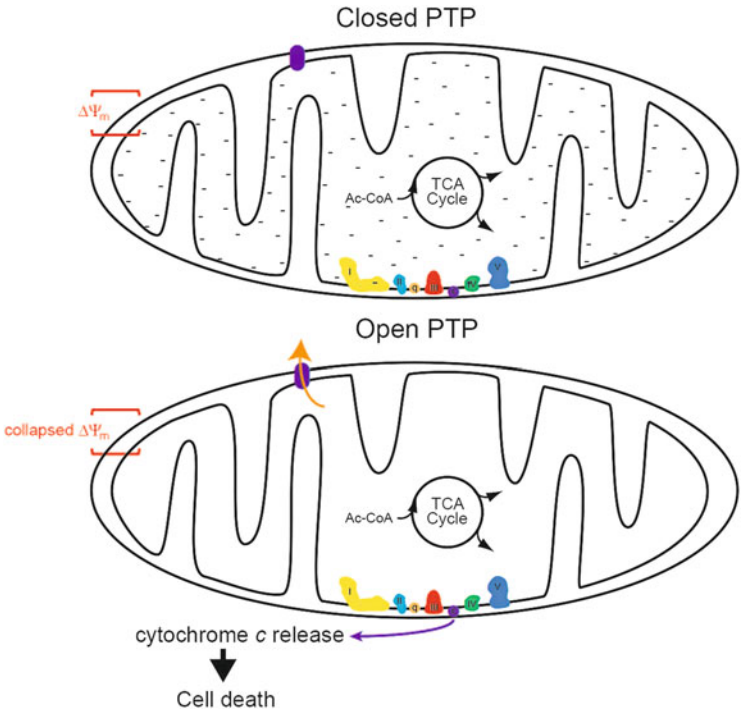

Fig. 4 The mitochondrial permeability transition pore. Under most conditions, the PTP is closed (*Upper*), maintaining the high capacitance of the IMM, the relative negative charge of the matrix, and the mitochondrial membrane potential ($\Delta\psi_m$). When the PTP is open (*Lower*), the capacitance of the IMM falls, the negative charge of the matrix dissipates, and $\Delta\psi_m$ collapses. This can also lead to release of cytochrome *c*, which, along with other factors, initiates cell death pathways. Note also that transient PTP opening can occur, and this does not lead to cell death and may serve a physiologic function

mitochondrial swelling due to the high osmotic pressure of the matrix, and this swelling leads to rupture of the outer mitochondrial membrane (OMM) and the release of proteins (cytochrome *c*, apoptosis inducing factor) from the mitochondria that push cell death pathways past the point of no return (Petit et al. 1997). In addition, the energetic failure due to uncoupling of the IMM and subsequent reversal of the ATP synthase reaction to consume ATP is also thought to play a role in cell death. Over the years, the PTP has been extensively studied for its role in ischemic injury in brain, heart, and other organs as well as in neurodegenerative conditions (Bonora et al. 2015). In the heart, data suggest that opening of the PTP during early reperfusion after ischemia is a harmful event that precipitates further damage to the myocardium (Griffiths and Halestrap 1993). Additional data suggest that cell death pathways may regulate or be regulated by the PTP, and the general consensus now is that uncontrolled opening of the PTP leads to cellular necrosis and, perhaps, apoptosis (Baines 2011; Bernardi 2013).

However, more recent data suggest that transient PTP opening can serve a physiologic purpose. Transient opening was described in the late 1990s (Huser and Blatter 1999; Ichas and Mazat 1998; Jouaville et al. 1998; Petronilli et al. 1999). In the heart, transient opening of the PTP during preconditioning can be protective, thus serving a physiological role even during injury (Hausenloy et al. 2004). This transient PTP opening in striated muscle mitochondria may be associated with transient increases in ROS (so-called superoxide flashes) which are proposed to serve as a signaling mechanism (Wang et al. 2008, 2012) Furthermore, it has been hypothesized that transient opening of the PTP releases mitochondrial matrix Ca^{2+} to maintain mitochondrial homeostasis (Elrod et al. 2010), although this function of the PTP has recently been questioned (De Marchi et al. 2014).

In the embryonic heart, the PTP appears to be open at early stages of myocyte differentiation (Beutner et al. 2014; Hom et al. 2011). The early heart derives most of its energy from anaerobic glycolysis (Porter et al. 2011), and the activity of the ETC is low (Beutner et al. 2014). At an early stage of development (mouse embryonic day 9.5), myocytes have an open PTP, low $\Delta\psi_m$, and high levels of ROS, and closure of the PTP using CsA increases $\Delta\psi_m$ and decreases ROS, leading to further myocyte differentiation (Hom et al. 2011). These changes in PTP activity are associated with increased assembly and activation of the ETC at or after embryonic day 11.5 (Beutner et al. 2014).

2.2 Defining the Permeability Transition Pore

In the late 1980s, patch-clamping experiments defined the biophysical, electrophysiological, and pharmacological properties of the PTP and began to establish criteria for defining PTP activity (Table 1) (Kinnally et al. 1989; Petronilli et al. 1989; Sorgato et al. 1987). In 1987, the first patch-clamping recordings of liver mitochondrial inner membrane isolated from cuprizone-fed mice demonstrated an ~100 pS channel, although its relationship to the PTP remains unclear (Sorgato et al. 1987). Then, in 1989, a putative PTP was recorded by patch-clamping mitoplasts (Petronilli et al. 1989). Channel activity occurred at positive potentials of the patch pipette and was found either in whole organelle mode or in single channel recordings in the organelle-attached configuration. Gating was less common at negative potentials and prolonged openings, and fewer subconductance states at negative patch potentials were observed. The activity was slightly anion selective and had multiple conductance states ranging from 30 pS to a peak conductance of 1.3 nS, with the lower conductances attributed to substates of the larger channel openings. The mitochondrial multi-conductance channel (MCC) in mouse liver mitoplasts was also described in 1989 to have similar properties (Kinnally et al. 1989). During recordings, this channel initially had low activity that progressively increased with time. Rectification was observed at both positive and negative potentials, and channel activity was weakly cation selective with multiple conductances ranging from 10 to 1,000 pS.

Table 1 Electrophysiological properties of the PTP and ATP synthase

Preparation	Conductance	Selectivity	Rectification	Reference
PTP				
IMM, patch clamping, induced by cuprizone	~100 pS	Slight anion selectivity	Not determined	Sorgato et al. (1987)
Mitoplast, patch clamping	Multiple conductances (30 pS to 1.3 nS)	Slight anion selectivity	Positive rectification at positive potentials and negative rectification at negative potentials	Petronilli et al. (1989)
Mitoplast, patch clamping (mitochondrial multi-conductance channel)	Multiple conductances (10 pS to 1 nS)	Slight cation selectivity	Variable rectification at positive and negative potentials	Kinnally et al. (1989)
ATP synthase				
ATP synthase dimers in lipid bilayers	Peak conductance of 1–1.3 nS with multiple subconductance states	Not determined	Not determined	Giorgio et al. (2013)
ATP synthase monomers in liposomes, patch clamping	Multiple conductances (~800 pS)	Not determined	Not determined	Alavian et al. (2014)
Purified ATP synthase c-subunits in lipid bilayers	Multiple conductances (15 pS to 2 nS)	Relative cation selectivity	Not tested	Azarashvili et al. (2014)
Purified ATP synthase c-subunits in liposomes, patch clamping	Multiple conductances (100 pS to 2 nS)	Slight cation selectivity	Slightly negative rectification	Alavian et al. (2014)

These studies also delineated the activators and inhibitors that are used today to investigate the PTP (Table 2). The PTP can be induced by elevated mitochondrial matrix Ca^{2+}, ROS, inorganic phosphate (P_i), fatty acids, and intracellular acidification. In contrast, it is inhibited by adenine nucleotides, divalent cations ($Mg^{2+} > Mn^{2+} > Ba^{2+} > Sr^{2+}$), and acidification of the matrix. Early in these investigations, it was found that cyclosporine A (CsA), an immunosuppressant that was known to inhibit cyclophilin proteins, inhibits these large conductance channels in the IMM by binding to a site in the mitochondrial matrix (Szabo and Zoratti 1991). Sub-micromolar concentrations of CsA inhibited a Ca^{2+}-activated, large conductance (1.3 nS) channel, but not the lower (~100 pS) substate conductances,

Table 2 Activators and inhibitors of the PTP

Activators	Reference	Inhibitors	Reference
Acidification, intracellular	Gendron et al. (2001)	Acidification, matrix	Haworth and Hunter (1979) and Nicolli et al. (1993)
Atractyloside	Hunter and Haworth (1979a)	ATP/ADP/AMP	Haworth and Hunter (1979), Hunter and Haworth (1979a), and Hunter and Haworth (1979b)
Calcium	Hunter et al. (1976)	Bongkrekic acid	Hunter and Haworth (1979a)
Fatty acids	Hunter et al. (1976)	Cyclosporine A	Crompton et al. (1988) and Fournier et al. (1987)
ROS	Brookes et al. (2004)	Mg^{2+} and other divalent cations	Hunter and Haworth (1979b)
Inorganic phosphate	Hunter et al. (1976)		
Phenylarsine oxide	Lenartowicz et al. (1991)		
Polyhydroxybutyrate	Elustondo et al. (2013)		
Polyphosphate	Seidlmayer et al. (2012)		

suggesting that this activity might be due to a separate ion channel (Szabo and Zoratti 1991). In addition, PTP activity could be induced by atractyloside (an inhibitor of ANT; see below) and phenylarsine oxide (an oxidizing agent) and inhibited by bongkrekic acid (an inhibitor of ANT; see below) (Lenartowicz et al. 1991). It should be emphasized that many of these agents, such as CsA, alter the amount of matrix Ca^{2+} required to open the PTP; thus, they are said to sensitize or desensitize the PTP to Ca^{2+}, but the mechanisms of this sensitization remain unknown.

2.3 The Search for the Identity of the Permeability Transition Pore

Since the electrophysiological studies of the late 1980s, much effort has been made to define the molecular identity of the PTP. A long list of candidates has been proposed, but subsequent studies demonstrated that many of these factors were either not related to the PTP or merely regulated its function (summarized in Table 3). Potential PTP candidates included the targets of common inducers and inhibitors of the PTP, such as ANT and cyclophilin D (CypD). Additional proteins that were proposed to be the PTP because their function seemed to control its activity include hexokinase (HK), mtCK, PiC, TSPO (translocator protein of 18 kDa, previously called the peripheral benzodiazepine receptor), and VDAC (voltage-dependent anion channel). For the most part, these proteins were

Table 3 Proposed PTP and PTP regulatory molecules

Protein/complex (Abbreviation, *gene*)	Evidence for role in PTP		Probable role in the PTP
	For	Against	
Adenine nucleotide translocase (ANT, *Slc25a4-6*)	PTP-like activity of ANT in bilayers (Ruck et al. 1998), effect of ADP, ATP, ATR and bongkrekic acid on PTP activity (Haworth and Hunter 1979; Hunter and Haworth 1979a, b)	Deletion of ANT1 and ANT2 (*Slc25a4* and *Slc25a5*) does not eliminate PTP activity, but decreases its sensitivity to Ca^{2+} (Kokoszka et al. 2004)	Regulates PTP activity, perhaps through interactions with ATP synthase as part of the synthasome
ATP synthase	Binds to or associates with PTP regulatory molecules (ANT, CypD, mtCK, PiC; see text). PTP activity found in ATP synthase dimers and the C-subunit ring (Alavian et al. 2011; Giorgio et al. 2013)	The exact mechanisms of PTP regulation within ATP synthase remain unknown	Although ATP synthase dimers have been proposed to create the pore of the PTP, more evidence exists suggesting that the F_O C-ring does this, but how this is regulated remains unresolved
Creatine kinase, mitochondrial (mtCK, *Ckmt1A*, *1B*, *2*)	Knockout of Ckmt1 increases susceptibility to PTP (Datler et al. 2014)		Regulatory, connects energy-producing and energy-consuming mechanism (Saks et al. 2012)
Cyclophilin D (CypD, *Ppif*)	Inhibition of CypD with CsA closes the PTP	Deletion of CypD does not eliminate PTP (Baines et al. 2005; Basso et al. 2005; Nakagawa et al. 2005; Schinzel et al. 2005)	Regulatory, binds to ATP synthase at OSCP but exact mechanism unknown
Hexokinase (*Hk1-4*)	Phosphorylation of glucose by mitochondrial bound HK I and II inhibits PTP (Azoulay-Zohar et al. 2004)		Regulatory, binds to VDAC, creates micro-compartments with high capacitive coupling to favor ADP/ATP exchange (Vyssokikh and Brdiczka 2003)
Phosphate carrier (PiC, *Slc25a3*)	Binds to ANT and CypD (Leung et al. 2008)	Deletion of PiC does not eliminate PTP activity, but decreases its sensitivity to Ca^{2+}	Regulates PTP activity, perhaps through interactions with ATP synthase as

(continued)

Table 3 (continued)

Protein/complex (Abbreviation, *gene*)	Evidence for role in PTP		Probable role in the PTP
	For	Against	
		(Gutierrez-Aguilar et al. 2014; Kwong et al. 2014)	part of the synthasome
Polyphosphate and Polyhydroxybutyrate	The naturally occurring molecules increase PTP (Elustondo et al. 2013; Seidlmayer et al. 2012; Stotz et al. 2014)		Regulates PTP activity, perhaps through interactions with ATP synthase and the C-ring
Spastic paraplegia 7 (*Spg7*)	Binds to CypD and VDAC, and its knockdown inhibits the PT (Shanmughapriya et al. 2015)		Regulates the PTP through currently unknown mechanisms. Its role in pore formation is unknown
Translocator protein of 18 kDa (TSPO, *Tspo*)	TSPO was isolated with ANT and VDAC (McEnery et al. 1992)	Deletion of TSPO does not eliminate PTP activity (Sileikyte et al. 2014)	Probably plays no role in creating the PTP
Unfolded proteins	He and Lemasters (2002)		Unknown
Voltage-dependent anion carrier (VDAC, *Vdac1-3*)	Pathway for cytochrome c release (Shimizu et al. 2000)	Deletion of VDAC1, VDAC2, and VDAC3 does not eliminate PTP activity (Baines et al. 2007)	Interaction with HK or mtCK keeps VDAC in anion-selective state (Vyssokikh and Brdiczka 2003)

eliminated as candidates to form the pore of the PTP by experiments where their expression was deleted (summarized in Table 3). Finally, it has also been hypothesized that unfolded proteins in the IMM might form a nonspecific, high-conductance pore (He and Lemasters 2002), and other recent reports suggest that polyphosphate chains and polyhydroxybutyrate may, when in the presence of elevated Ca^{2+}, regulate or participate in the formation of the PTP (Elustondo et al. 2013; Seidlmayer et al. 2012).

Two particular IMM translocators (ANT and PiC) were purported to form the PTP. In the 1990s, ANT was the prime candidate (Beutner et al. 1998; Crompton et al. 1998; Halestrap and Davidson 1990), because the PTP activator atractyloside/carboxyatractyloside and the PTP inhibitor bongkrekic acid both inhibit the ADP/ATP translocase activity of ANT. Interestingly, these agents have different effects on the conformation of ANT (Brustovetsky and Klingenberg 1996; Haworth

and Hunter 2000). In addition, several papers showed that the ANT can form channels with properties of the PTP, although issues with the purity of these preparations may have led to this conclusion (Brustovetsky and Klingenberg 1996; Ruck et al. 1998). Later, the PiC was proposed as an integral component of the PTP due to its binding to two proteins that regulate the PTP, ANT, and CypD (Leung et al. 2008). However, more recent studies have demonstrated by genetic deletion of ANT1 and 2 and of the PiC that these proteins are not essential to PTP formation, although these studies still support regulatory roles for these translocators (Gutierrez-Aguilar et al. 2014; Kokoszka et al. 2004; Kwong et al. 2014) (Table 3).

CypD, a peptidyl-prolyl *cis*/*trans* isomerase, is the most important regulator of the PTP and was a candidate to create the pore. However, deletion of CypD in mice did not eliminate PTP activity, although this did decrease the sensitivity of the PTP to Ca^{2+} and oxidative stress (Baines et al. 2005; Basso et al. 2005; Elrod and Molkentin 2013; Nakagawa et al. 2005; Schinzel et al. 2005). Although it is well known that CypD is the target of CsA, which is known to prevent its binding to target proteins, how this regulates the PTP and to what protein CypD binds to confer this activity remains controversial (see below). Interestingly, it was recently found that CypD binds to SPG7 (spastic paraplegia 7), an IMM protein that may link CypD to VDAC in the OMM (Shanmughapriya et al. 2015). SPG7 appears to be an important regulator of the PTP, but its role in creating the pore of the PTP remains untested.

3 ATP Synthase and the Permeability Transition Pore

3.1 ATP Synthase and the Permeability Transition Pore Interact

Metabolic pathways are known to regulate the PTP. For example, glycolysis in the cytoplasm can regulate PTP activity via the binding of hexokinase II to the OMM (Pasdois et al. 2013). In addition, since the ETC proteins make up the majority of the proteins in the IMM, it is logical to assume that the PTP is either physically associated with the ETC or derived from its components. In fact, electron transport chain activity regulates the PTP, as an increase in mitochondrial energization ($\Delta\psi_m$) inhibits the PT, while de-energization/depolarization (a fall in $\Delta\psi_m$) enhances it (Di Lisa et al. 2011; Haworth and Hunter 1979; Hunter and Haworth 1979a, b). Furthermore, inhibition of complex I using rotenone or metformin can inhibit the PTP when CypD is present at low levels, and it was proposed that CypD may bind to complex I (Li et al. 2012).

However, recent evidence has suggested an interaction between ATP synthase and the PTP. Many of the proteins that are known to regulate the activity of the PTP can also bind directly or indirectly to ATP synthase and/or CypD. First, CypD can bind directly to ATP synthase at the OSCP (oligomycin-sensitivity conferring protein) subunit (Giorgio et al. 2009, 2010). Second, CypD can bind to Bcl-2 (Eliseev et al. 2009), while Bcl-X_L, an anti-apoptotic member of the Bcl-2 family,

interacts with the β-subunit of ATP synthase and increases the efficiency of ATP production by decreasing a leak current in the IMM (Alavian et al. 2011; Chen et al. 2011).

Third, CypD binds to ANT and the PiC and disruption of this association affects PTP activity (Leung et al. 2008; Woodfield et al. 1998). This interaction is very interesting. As discussed above, ANT and the PiC interact with ATP synthase in synthasomes that also contain mtCK, which binds to ANT (Beutner et al. 1998; Leung et al. 2008; Saks et al. 2012; Vyssokikh et al. 2001; Woodfield et al. 1998). Furthermore, the interaction between ANT and VDAC may confer indirect associations between synthasomes and both HK and TSPO in the OMM (McEnery et al. 1992).

3.2 The Permeability Transition Pore Lies Within or Around ATP Synthase

In the last 2 years, more direct evidence supports the idea that the pore of the PTP is associated with ATP synthase (Alavian et al. 2014; Azarashvili et al. 2014; Bonora et al. 2013; Giorgio et al. 2013). In particular, work from the Bernardi group suggests that the PTP forms in the membrane surrounding dimers of ATP synthase (Giorgio et al. 2013). To extend their work showing that CypD binds to the stator of ATP synthase (Giorgio et al. 2009), they showed that this binding is specifically at the OSCP subunit and that decreasing the expression of OSCP decreases the association of CypD with ATP synthase (Giorgio et al. 2013). Furthermore, they demonstrated that a novel inhibitor of ATP synthase that binds to OSCP, benzodiazipine-423, inhibits the interaction of OSCP and CypD and, like CypD, opens the PTP in response to Ca^{2+}. These data suggested that ATP synthase and in particular OSCP are somehow associated with and control the PTP. They also showed that conditions that promote ATP hydrolysis by ATP synthase decreased the sensitivity of the PTP to Ca^{2+} compared to ATP synthesis and that OSCP may control this Ca^{2+} sensitivity.

The Bernardi group purified ATP synthase monomers and dimers from native gels, incorporated these into lipid bilayers, and tested for PTP ion channel activity. It is important to note that monomer and dimer preparations contained no associated CypD, ANT, or VDAC, but that purified dimers did contain complexes I and III. No conductance was reportedly observed in monomer preparations, but dimers exhibited conductances of 1–1.3 nS with multiple subconductance states (Table 1). Conductance required the presence of Ca^{2+} and P_i, was induced by benzodiazepine-423, was inhibited by Mg^{2+} and adenine nucleotides, and was not affected by typical PTP sensitizing (atractyloside, phenylarsine oxide) and inhibiting (CsA, bongkrekic acid) agents. Additional data from this group suggested that dimerization was important for PTP formation, because deletion of the e- and d-subunits of yeast ATP synthase decreased PTP opening (Carraro et al. 2014). Based on these data, the authors proposed that the PTP forms at the membrane interface of the dimers and is perhaps related to changes in the lipid

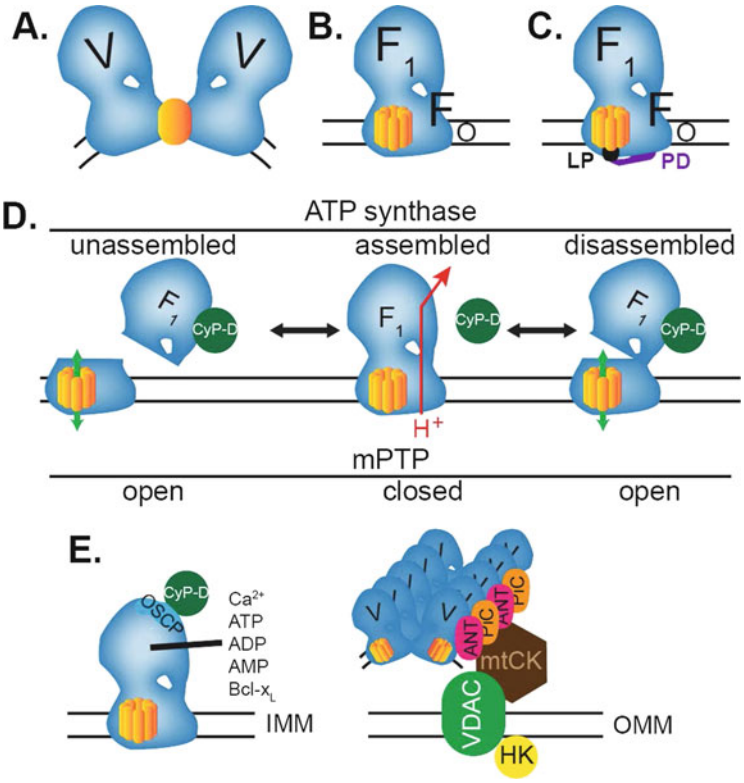

Fig. 5 Models of the permeability transition pore derived from ATP synthase. (**A**–**C**) Three current models of the PTP as part of ATP synthase. In (**A**), the PTP lies within the membrane between a dimer of ATP synthase (**V**, modified from (Bernardi 2013). In (**B**), the PTP is the C-ring of the F_o component of ATP synthase. In (**C**), the lipid plug (**LP**) in the central pore of the C-ring is controlled by the p-side density (**PD**) to regulate C-ring conductance. (**D**) For ATP synthase assembly (*left*), F_1 is added to the C-ring and other components of F_o. Assembly (*center*) allows the flow of protons ($\mathbf{H^+}$) between the C-ring and the stator, causing rotation of the C-ring, which provides energy for F_1 to synthesize ATP. In this model, binding of CypD to ATP synthase during assembly promotes the presence of unassembled C-rings that create the open mPTP in the embryonic heart (*left*). Assembly and pore formation are reversible (*double arrows*), and CypD also causes disassembly of ATP synthase to create the pore in mature cells (*right*). (**E**) Control of PTP opening by CypD occurs due to its binding to OSCP, while Ca^{2+}, adenine nucleotides (ATP, ADP, and AMP), and Bcl-x_L may control PTP by binding to ATP synthase at other sites. Other proteins may regulate the PTP via the assembly of ATP synthase, ANT, and PiC into synthasomes that associate with mtCK in the intermembrane space, VDAC in the OMM, and HK attached to the OMM

environment in that region upon PTP induction (Fig. 5a) (Bernardi 2013), but no further information has been published that clarifies this hypothesis.

3.3 The C-Ring of ATP Synthase Creates the Permeability Transition Pore

The data presented in the last two sections suggest that ATP synthase is related to the PTP in some way, but the exact molecular identity of the pore is not defined in these studies. However, additional reports in recent years suggest that the pore of the PTP lies within the c-subunit, a major membranous component of ATP synthase. Membranous pores are usually formed by integral membrane proteins, but, as outlined above, the only membrane-delimited IMM proteins (ANT, PiC) that have been proposed to form the PTP were not required for PTP in genetic deletion models. The ATP synthase subunits a, b, and c are highly hydrophobic membrane proteins and evolutionally conserved, making them candidates to form the PTP. However, the a-subunit is not required for the PTP, as it is absent in ρ0 cells that lack mitochondrial DNA, but do undergo PT (Masgras et al. 2012).

Attention has focused on the c-subunit because the c-subunits form a ring in the membrane and have ion-conducting properties. The known C-ring structures consist of 8 (bovine heart) and 10–15 (bacteria) c-subunits, and bacterial C-rings have a torus shape with a central hole (Pogoryelov et al. 2007). In the late 1990s, reconstitution experiments by the McGeoch laboratory showed that purified mammalian c-subunits have ion channel activity (McGeoch and Guidotti 1997; McGeoch et al. 2000; McGeoch and Palmer 1999). Exposure of these purified subunits to water induces a conformational change whereby a channel with a diameter of 3 nm (McGeoch and McGeoch 2008), close to the estimated size (2.3 nm) of the PTP pore, is formed (Crompton and Costi 1990). Finally, c-subunit homologues in the V_O component of vacuolar H^+-ATPases line a water-accessible pore structure and are involved in creating gap junctions between cells (Harrison et al. 2003; Jones et al. 1995; Peters et al. 2001).

A recent study published evidence in 2013 that the c-subunit was important for the PT (Bonora et al. 2013). When all three mammalian isoforms of the c-subunit (*ATP5G1*, *ATP5G2*, *ATP5G3*) were deleted in HeLa cells using siRNA, there was no effect on ATP production, as these cells are glycolytic. However, depletion of c-subunits prevented ionomycin-induced opening of the PTP, mitochondrial fragmentation, oxidative stress-induced mitochondrial depolarization, and release of cytochrome *c*. Overexpression of ATP5G1 had the opposite effect on PTP. Under conditions of Ca^{2+} overload (ionomycin) or oxidative stress, c-subunit depletion prevented cell death in HeLa cells and attenuated glutamate-induced cell death in primary cultures of cortical neurons.

PTP-like activity has been observed using mitochondrial proteins purified by chloroform/methanol extraction. In 2005, the Pavlov lab attributed such activity to polyhydroxybutyrate, although they later concluded that this preparation probably contained the c-subunit (Elustondo et al. 2013). In addition, work from the Saris lab over the last 15 years suggests pore-forming activities of the c-subunit. In the early 2000s, they isolated a phosphorylated form of the c-subunit from liver mitochondria and found that it induced, while a c-subunit antibody inhibited, the PT in isolated mitochondria and that dephosphorylation of this protein was associated with

opening of the PTP (Azarashvili et al. 2002). Furthermore, this isolated fraction increased conductance of lipid membranes, an effect that was blocked by c-subunit antibodies (Azarashvili et al. 2002). These results were further explored in 2014, when they demonstrated that dephosphorylated c-subunit was more effective than phosphorylated c-subunit at inducing PT and that the c-subunit could bind Ca^{2+} (Azarashvili et al. 2014). Furthermore, the conductance of the c-subunit in lipid bilayers was found to be 15 pS to 2 nS, consistent with previous recordings of the PTP (Table 1).

Starting in 2004, the Jonas laboratory began to study the molecular identity of the PTP when they found that overexpression of the anti-apoptotic protein, Bcl-x_L, in neurons increased cytoplasmic ATP levels yet decreased oxygen consumption, consistent with the idea that Bcl-x_L overexpression increases mitochondrial bioenergetic efficiency (Alavian et al. 2011; Chen et al. 2011). Furthermore, Bcl-x_L was found to bind to the β-subunit of ATP synthase to increase ATP synthesis, and its depletion or inhibition increased an IMM leak current in patch-clamping experiments (Alavian et al. 2011).

These data suggested that a regulated leak current lay within the ATP synthase and the Jonas and Porter laboratories began to study this further (Alavian et al. 2014). Patch clamping of purified ATP synthase subunits reconstituted in liposomes revealed that extremely pure c-subunit preparations with no other associated proteins formed channels in proteoliposomes. These channels had multi-conductance states that ranged from 100 pS to 2 nS, were slightly cation selective (Table 1), and were inhibited by c-subunit antibodies and high concentrations of ATP but not CsA. Additional patch clamp studies were performed on purified ATP synthase monomers that contained no CypD. In contrast to results reported by Giorgio et al. (Giorgio et al. 2013), these studies revealed that calcium and purified CypD induced an ~1 nS conductance (with multiple subconductance states, Table 1). Similar experiments, using mitoplasts, submitochondrial vesicles (purified IMMs), and urea-stripped submitochondrial vesiicles stripped of non-membrane-embedded proteins with urea, revealed multiple levels of regulation of this C-ring channel. The conductance in mitoplasts and submitochondrial vesicles had the expected properties of the PTP – activation by Ca^{2+} and inhibition by CsA. However, when the c-subunit is stripped of non-membrane-embedded proteins, submitochondrial vesicles were neither responsive to Ca^{2+} nor inhibited by CsA, suggesting that regulatory components were peripheral to the c-subunit and were removed by urea. Interestingly, the conductance associated with the C-ring could be inhibited with specific purified ATP synthase F_1 components.

Additional experiments revealed that the C-ring expands upon exposure to calcium to open the PTP and that mutation of the c-subunit to make the C-ring larger increased its conductance and enhanced PTP opening and associated cell death in neurons. In contrast, depletion of the c-subunit attenuated PTP opening and cell death.

Combined, these data suggest a model whereby in the intact synthase complex the central stalk and F_1 of the ATP synthase inhibit opening of the c-subunit channel; in contrast the C-ring of ATP synthase is exposed upon induction of the

PTP (Fig. 5b, d). Release of the C-ring from ATP synthase was demonstrated by exposing mitochondria to calcium and immunocapturing the ATP synthase using an antibody directed at the F_1 (Alavian et al. 2014). These studies showed that calcium exposure indeed destabilizes, in a CypD-dependent manner, the connection between the stalk and the c-subunit, disrupting protein/protein interaction between the c-subunit and F_1. Furthermore, this dissociation was prevented when CsA or ADP was added to prevent PTP opening (Alavian et al. 2014).

4 A Current Model of the Permeability Transition Pore

Overall, the data presented above suggest a model, in which the PTP is contained within the C-ring of ATP synthase (Fig. 5b). In this model, induction of the PT disrupts ATP synthase to unmask the face of the C-ring to the matrix and expose its central pore. As molecules flow through this pore, the IMM depolarizes and the ETC uncouples (Figs. 4 and 5d). The PTP can be regulated by CypD due to its binding to OSCP on ATP synthase and by Ca^{2+}, adenine nucleotides, and Bcl-x_L at the level of ATP synthase (Fig. 5e). Furthermore, control of ATP synthase assembly and PTP opening by other proteins known to regulate this process (ANT, PiC, SPG7, mtCK, VDAC, and HK) occurs at the level of the synthasome where the ATP synthase forms connections to the latter three proteins in the intermembrane space or at the OMM.

However, this model does not explain all of the current data, and many other issues must be resolved to refine this model. The first issue is whether it is logical and possible for ATP synthase to disassemble and expose a pore within its central structure when the mitochondrion faces stressful situations. Experimental conditions in which this occurs are consistent with known matrix calcium concentrations; the 60 μM Ca^{2+} required to open the PTP is well within the range of physiological Ca^{2+} concentrations found within the mitochondrial matrix or adjacent to the mitochondria in Ca^{2+} microdomains (Csordas et al. 2001; Rizzuto et al. 2000, 2009; Rizzuto and Pozzan 2006; Schneggenburger and Neher 2005). In addition, the opening of the PTP is reversible (see above), so under physiologic conditions, PTP opening might only occur as a transient exposure of the C-ring pore with subsequent reconstitution, as has been observed for ATP synthase (Pedersen et al. 1978).

The inhibition of the C-ring pore by F_1 components of ATP synthase (Alavian et al. 2014) may play a role in transient pore opening. Alternatively blockade of the PTP by F_1 components may prevent PTP opening when ATP synthase becomes disassembled or has not yet assembled as it is forming (Havlickova et al. 2010), but further work will need to be done to address this issue. However, under certain conditions, this disassembly may become irreversible and safety mechanisms may fail, thus causing pathophysiologic PT and rupture of the OMM. Finally, it is also important to note that opening of only a few C-rings/PTPs would be required to depolarize the IMM, as this pore has such a large conductance, so even a low

probability event of ATP synthase disruption could lead to devastating consequences for the mitochondrion.

Structural studies suggest that the center of the C-ring contains lipids that would prevent the observed conductance. However, a recent cryo-electron microscopic study suggests that this lipid plug lies close to the matrix side of the ring center and this may be attached to a portion of ATP synthase composed of the e-subunit (Zhou et al. 2015). Furthermore, a recent review of this work suggested that this p-side density (or death finger) could pull the lipid plug toward the matrix, opening the center of the C-ring to allow conductance (Fig. 5c) (Gerle 2016). Additional experiments must be done to explore this potential mechanism of C-ring activity.

Many questions are unanswered regarding the regulation of the PTP. Probably the most interesting question is: what is the mechanism, by which Ca^{2+} and ROS regulate the PTP? These molecules play the most central and powerful roles in this process, but where they bind or what they modify to regulate PTP opening is still completely unknown. Most likely, they affect the proteins described in this chapter, but other targets may be involved. For example, lipids such as cardiolipin likely play a major role in the assembly and control of both of these structures and provide a site for Ca^{2+}-mediated regulation.

Despite the long-standing and justified idea that CypD plays a central role in PTP regulation, the mechanism, by which it does this, is unclear. It has recently been assumed that the binding of CypD to OSCP is important for this function; as suggested by Giorgio et al., CypD can induce PTP opening in purified ATP synthase preparations (Alavian et al. 2014), but it is not isolated with ATP synthase monomers or dimers (Alavian et al. 2014; Giorgio et al. 2013). Thus, its binding to OSCP/ATP synthase may be weak. It is possible that other, more important targets, such as ANT or SPG7, are as important as OSCP, but this remains to be determined. It is also unclear how the protein folding (peptidyl-prolyl, *cis-trans* isomerase) activity of CypD is important for its regulation of PTP function, as suggested by mutation studies (Lin and Lechleiter 2002).

The exact role of the other proteins that control the PTP (Table 3) remains unclear, and the mechanisms by which they exert their effects can only be surmised from incomplete data. Figure 5e suggests one possible model, but more work must be done to establish the importance of the association of the synthasome with mtCK, VDAC, and HK in the regulation of PTP. Furthermore, how the ANT inhibitors, atractyloside and bongkrekic acid, have opposite effects on the PTP is a mystery, but the answer probably lies in their ability to lock ANT into two totally different conformations, as discussed above. It is possible that defining the effects of these agents on ANT may also shed light on the role of CypD's enzymatic activity, as it was reported that Ca^{2+} stabilization of ANT's "c" conformation is related to its proline isomerization (Pestana et al. 2010).

5 Summary

OXPHOS and the mitochondrial ETC are essential for eukaryotic life as they produce the majority of ATP in most cells. However, disruption of mitochondrial function can lead to cell death, and the PTP plays a major role in both the energetic failure and the activation of programmed cell death pathways that lead to cellular demise. The molecular identity of the PTP has long been sought, and recent evidence suggests a model where ATP synthase, the major producer of ATP in the cell, also contains within its core the PTP. Further work must be done to delineate the exact nature of the pore of the PTP, how it is derived from ATP synthase, and what controls this process.

Acknowledgments American Heart Association Grant 12GRNT12060233 to GAP. NIH NS045876; NS081746 to EAJ.

References

Acin-Perez R, Fernandez-Silva P, Peleato ML, Perez-Martos A, Enriquez JA (2008) Respiratory active mitochondrial supercomplexes. Mol Cell 32:529–539

Alavian KN, Li H, Collis L, Bonanni L, Zeng L, Sacchetti S, Lazrove E, Nabili P, Flaherty B, Graham M, Chen Y, Messerli SM, Mariggio MA, Rahner C, Mcnay E, Shore GC, Smith PJ, Hardwick JM, Jonas EA (2011) Bcl-xL regulates metabolic efficiency of neurons through interaction with the mitochondrial F1FO ATP synthase. Nat Cell Biol 13:1224–1233

Alavian KN, Beutner G, Lazrove E, Sacchetti S, Park HA, Licznerski P, Li H, Nabili P, Hockensmith K, Graham M, Porter GA Jr, Jonas EA (2014) An uncoupling channel within the c-subunit ring of the F1FO ATP synthase is the mitochondrial permeability transition pore. Proc Natl Acad Sci U S A 111:10580–10585

Azarashvili TS, Tyynela J, Odinokova IV, Grigorjev PA, Baumann M, Evtodienko YV, Saris NE (2002) Phosphorylation of a peptide related to subunit c of the F0F1-ATPase/ATP synthase and relationship to permeability transition pore opening in mitochondria. J Bioenerg Biomembr 34:279–284

Azarashvili T, Odinokova I, Bakunts A, Ternovsky V, Krestinina O, Tyynela J, Saris NE (2014) Potential role of subunit c of F0F1-ATPase and subunit c of storage body in the mitochondrial permeability transition. Effect of the phosphorylation status of subunit c on pore opening. Cell Calcium 55:69–77

Azoulay-Zohar H, Israelson A, Abu-Hamad S, Shoshan-Barmatz V (2004) In self-defence: hexokinase promotes voltage-dependent anion channel closure and prevents mitochondria-mediated apoptotic cell death. Biochem J 377:347–355

Baines CP (2011) The mitochondrial permeability transition pore and the cardiac necrotic program. Pediatr Cardiol 32:258–262

Baines CP, Kaiser RA, Purcell NH, Blair NS, Osinska H, Hambleton MA, Brunskill EW, Sayen MR, Gottlieb RA, Dorn GW, Robbins J, Molkentin JD (2005) Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. Nature 434:658–662

Baines CP, Kaiser RA, Sheiko T, Craigen WJ, Molkentin JD (2007) Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death. Nat Cell Biol 9:550–555

Basso E, Fante L, Fowlkes J, Petronilli V, Forte MA, Bernardi P (2005) Properties of the permeability transition pore in mitochondria devoid of Cyclophilin D. J Biol Chem 280:18558–18561

Bernardi P (2013) The mitochondrial permeability transition pore: a mystery solved? Front Physiol 4:95

Beutner G, Ruck A, Riede B, Brdiczka D (1998) Complexes between porin, hexokinase, mitochondrial creatine kinase and adenylate translocator display properties of the permeability transition pore. Implication for regulation of permeability transition by the kinases. Biochim Biophys Acta 1368:7–18

Beutner G, Eliseev RA, Porter GA Jr (2014) Initiation of electron transport chain activity in the embryonic heart coincides with the activation of mitochondrial complex 1 and the formation of supercomplexes. PLoS One 9, e113330

Bonora M, Bononi A, DE Marchi E, Giorgi C, Lebiedzinska M, Marchi S, Patergnani S, Rimessi A, Suski JM, Wojtala A, Wieckowski MR, Kroemer G, Galluzzi L, Pinton P (2013) Role of the c subunit of the FO ATP synthase in mitochondrial permeability transition. Cell Cycle 12:674–683

Bonora M, Wieckowski MR, Chinopoulos C, Kepp O, Kroemer G, Galluzzi L, Pinton P (2015) Molecular mechanisms of cell death: central implication of ATP synthase in mitochondrial permeability transition. Oncogene 34:1475–1486

Bornhovd C, Vogel F, Neupert W, Reichert AS (2006) Mitochondrial membrane potential is dependent on the oligomeric state of F1F0-ATP synthase supracomplexes. J Biol Chem 281:13990–13998

Brookes PS, Yoon Y, Robotham JL, Anders MW, Sheu SS (2004) Calcium, ATP, and ROS: a mitochondrial love-hate triangle. Am J Physiol Cell Physiol 287:C817–C833

Brustovetsky N, Klingenberg M (1996) Mitochondrial ADP/ATP carrier can be reversibly converted into a large channel by Ca2+. Biochemistry 35:8483–8488

Calvaruso MA, Willems P, VAN DEN Brand M, Valsecchi F, Kruse S, Palmiter R, Smeitink J, Nijtmans L (2012) Mitochondrial complex III stabilizes complex I in the absence of NDUFS4 to provide partial activity. Hum Mol Genet 21:115–120

Carraro M, Giorgio V, Sileikyte J, Sartori G, Forte M, Lippe G, Zoratti M, Szabo I, Bernardi P (2014) Channel formation by yeast F-ATP synthase and the role of dimerization in the mitochondrial permeability transition. J Biol Chem 289:15980–15985

Chen C, Ko Y, Delannoy M, Ludtke SJ, Chiu W, Pedersen PL (2004) Mitochondrial ATP synthasome: three-dimensional structure by electron microscopy of the ATP synthase in complex formation with carriers for Pi and ADP/ATP. J Biol Chem 279:31761–31768

Chen YB, Aon MA, Hsu YT, Soane L, Teng X, Mccaffery JM, Cheng WC, Qi B, Li H, Alavian KN, Dayhoff-Brannigan M, Zou S, Pineda FJ, O'Rourke B, Ko YH, Pedersen PL, Kaczmarek LK, Jonas EA, Hardwick JM (2011) Bcl-xL regulates mitochondrial energetics by stabilizing the inner membrane potential. J Cell Biol 195:263–276

Cogliati S, Frezza C, Soriano ME, Varanita T, Quintana-Cabrera R, Corrado M, Cipolat S, Costa V, Casarin A, Gomes LC, Perales-Clemente E, Salviati L, Fernandez-Silva P, Enriquez JA, Scorrano L (2013) Mitochondrial cristae shape determines respiratory chain supercomplexes assembly and respiratory efficiency. Cell 155:160–171

Crompton M, Costi A (1990) A heart mitochondrial Ca2(+)-dependent pore of possible relevance to re-perfusion-induced injury. Evidence that ADP facilitates pore interconversion between the closed and open states. Biochem J 266:33–39

Crompton M, Ellinger H, Costi A (1988) Inhibition by cyclosporin A of a Ca2+-dependent pore in heart mitochondria activated by inorganic phosphate and oxidative stress. Biochem J 255:357–360

Crompton M, Virji S, Ward JM (1998) Cyclophilin-D binds strongly to complexes of the voltage-dependent anion channel and the adenine nucleotide translocase to form the permeability transition pore. Eur J Biochem 258:729–735

Csordas G, Thomas AP, Hajnoczky G (2001) Calcium signal transmission between ryanodine receptors and mitochondria in cardiac muscle. Trends Cardiovasc Med 11:269–275

Datler C, Pazarentzos E, Mahul-Mellier AL, Chaisaklert W, Hwang MS, Osborne F, Grimm S (2014) CKMT1 regulates the mitochondrial permeability transition pore in a process that provides evidence for alternative forms of the complex. J Cell Sci 127:1816–1828

Davies KM, Strauss M, Daum B, Kief JH, Osiewacz HD, Rycovska A, Zickermann V, Kuhlbrandt W (2011) Macromolecular organization of ATP synthase and complex I in whole mitochondria. Proc Natl Acad Sci U S A 108:14121–14126

DE Marchi E, Bonora M, Giorgi C, Pinton P (2014) The mitochondrial permeability transition pore is a dispensable element for mitochondrial calcium efflux. Cell Calcium 56:1–13

DI Lisa F, Carpi A, Giorgio V, Bernardi P (2011) The mitochondrial permeability transition pore and cyclophilin D in cardioprotection. Biochim Biophys Acta 1813:1316–1322

Dickson VK, Silvester JA, Fearnley IM, Leslie AG, Walker JE (2006) On the structure of the stator of the mitochondrial ATP synthase. EMBO J 25:2911–2918

Dudkina NV, Sunderhaus S, Boekema EJ, Braun HP (2008) The higher level of organization of the oxidative phosphorylation system: mitochondrial supercomplexes. J Bioenerg Biomembr 40:419–424

Eliseev RA, Malecki J, Lester T, Zhang Y, Humphrey J, Gunter TE (2009) Cyclophilin D interacts with Bcl2 and exerts an anti-apoptotic effect. J Biol Chem 284:9692–9699

Elrod JW, Molkentin JD (2013) Physiologic functions of cyclophilin D and the mitochondrial permeability transition pore. Circ J 77:1111–1122

Elrod JW, Wong R, Mishra S, Vagnozzi RJ, Sakthievel B, Goonasekera SA, Karch J, Gabel S, Farber J, Force T, Brown JH, Murphy E, Molkentin JD (2010) Cyclophilin D controls mitochondrial pore-dependent Ca(2+) exchange, metabolic flexibility, and propensity for heart failure in mice. J Clin Invest 120:3680–3687

Elustondo PA, Angelova PR, Kawalec M, Michalak M, Kurcok P, Abramov AY, Pavlov EV (2013) Polyhydroxybutyrate targets mammalian mitochondria and increases permeability of plasmalemmal and mitochondrial membranes. PLoS One 8, e75812

Fournier N, Ducet G, Crevat A (1987) Action of cyclosporine on mitochondrial calcium fluxes. J Bioenerg Biomembr 19:297–303

Gendron MC, Schrantz N, Metivier D, Kroemer G, Maciorowska Z, Sureau F, Koester S, Petit PX (2001) Oxidation of pyridine nucleotides during Fas- and ceramide-induced apoptosis in Jurkat cells: correlation with changes in mitochondria, glutathione depletion, intracellular acidification and caspase 3 activation. Biochem J 353:357–367

Genova ML, Lenaz G (2013) A critical appraisal of the role of respiratory supercomplexes in mitochondria. Biol Chem 394:631–639

Gerle C (2016) On the structural possibility of pore-forming mitochondrial FF ATP synthase. Biochim Biophys Acta 1857:1191–1196

Giorgio V, Bisetto E, Soriano ME, Dabbeni-Sala F, Basso E, Petronilli V, Forte MA, Bernardi P, Lippe G (2009) Cyclophilin D modulates mitochondrial F0F1-ATP synthase by interacting with the lateral stalk of the complex. J Biol Chem 284:33982–33988

Giorgio V, Soriano ME, Basso E, Bisetto E, Lippe G, Forte MA, Bernardi P (2010) Cyclophilin D in mitochondrial pathophysiology. Biochim Biophys Acta 1797:1113–1118

Giorgio V, VON Stockum S, Antoniel M, Fabbro A, Fogolari F, Forte M, Glick GD, Petronilli V, Zoratti M, Szabo I, Lippe G, Bernardi P (2013) Dimers of mitochondrial ATP synthase form the permeability transition pore. Proc Natl Acad Sci U S A 110:5887–5892

Griffiths EJ, Halestrap AP (1993) Protection by Cyclosporin A of ischemia/reperfusion-induced damage in isolated rat hearts. J Mol Cell Cardiol 25:1461–1469

Gutierrez-Aguilar M, Douglas DL, Gibson AK, Domeier TL, Molkentin JD, Baines CP (2014) Genetic manipulation of the cardiac mitochondrial phosphate carrier does not affect permeability transition. J Mol Cell Cardiol 72:316–325

Halestrap AP, Davidson AM (1990) Inhibition of Ca2(+)-induced large-amplitude swelling of liver and heart mitochondria by cyclosporin is probably caused by the inhibitor binding to mitochondrial-matrix peptidyl-prolyl cis-trans isomerase and preventing it interacting with the adenine nucleotide translocase. Biochem J 268:153–160

Harrison M, Durose L, Song CF, Barratt E, Trinick J, Jones R, Findlay JB (2003) Structure and function of the vacuolar H+-ATPase: moving from low-resolution models to high-resolution structures. J Bioenerg Biomembr 35:337–345

Hausenloy D, Wynne A, Duchen M, Yellon D (2004) Transient mitochondrial permeability transition pore opening mediates preconditioning-induced protection. Circulation 109:1714–1717

Havlickova V, Kaplanova V, Nuskova H, Drahota Z, Houstek J (2010) Knockdown of F1 epsilon subunit decreases mitochondrial content of ATP synthase and leads to accumulation of subunit c. Biochim Biophys Acta 1797:1124–1129

Haworth RA, Hunter DR (1979) The Ca2+-induced membrane transition in mitochondria. II. Nature of the Ca2+ trigger site. Arch Biochem Biophys 195:460–467

Haworth RA, Hunter DR (2000) Control of the mitochondrial permeability transition pore by high-affinity ADP binding at the ADP/ATP translocase in permeabilized mitochondria. J Bioenerg Biomembr 32:91–96

He L, Lemasters JJ (2002) Regulated and unregulated mitochondrial permeability transition pores: a new paradigm of pore structure and function? FEBS Lett 512:1–7

Hirst J (2013) Mitochondrial complex I. Annu Rev Biochem 82:551–575

Hom JR, Quintanilla RA, Hoffman DL, de Mesy Bentley KL, Molkentin JD, Sheu SS, Porter GA Jr (2011) The permeability transition pore controls cardiac mitochondrial maturation and myocyte differentiation. Dev Cell 21:469–478

Hunter DR, Haworth RA (1979a) The Ca2+-induced membrane transition in mitochondria. I. The protective mechanisms. Arch Biochem Biophys 195:453–459

Hunter DR, Haworth RA (1979b) The Ca2+-induced membrane transition in mitochondria. III. Transitional Ca2+ release. Arch Biochem Biophys 195:468–477

Hunter DR, Haworth RA, Southard JH (1976) Relationship between configuration, function, and permeability in calcium-treated mitochondria. J Biol Chem 251:5069–5077

Huser J, Blatter LA (1999) Fluctuations in mitochondrial membrane potential caused by repetitive gating of the permeability transition pore. Biochem J 343(Pt 2):311–317

Ichas F, Mazat JP (1998) From calcium signaling to cell death: two conformations for the mitochondrial permeability transition pore. Switching from low- to high-conductance state. Biochim Biophys Acta 1366:33–50

Jonckheere AI, Smeitink JA, Rodenburg RJ (2012) Mitochondrial ATP synthase: architecture, function and pathology. J Inherit Metab Dis 35:211–225

Jones PC, Harrison MA, Kim YI, Finbow ME, Findlay JB (1995) The first putative transmembrane helix of the 16 kDa proteolipid lines a pore in the Vo sector of the vacuolar H(+)-ATPase. Biochem J 312(Pt 3):739–747

Jouaville LS, Ichas F, Mazat JP (1998) Modulation of cell calcium signals by mitochondria. Mol Cell Biochem 184:371–376

Kinnally KW, Campo ML, Tedeschi H (1989) Mitochondrial channel activity studied by patch-clamping mitoplasts. J Bioenerg Biomembr 21:497–506

Kokoszka JE, Waymire KG, Levy SE, Sligh JE, Cai J, Jones DP, Macgregor GR, Wallace DC (2004) The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore. Nature 427:461–465

Kwong JQ, Davis J, Baines CP, Sargent MA, Karch J, Wang X, Huang T, Molkentin JD (2014) Genetic deletion of the mitochondrial phosphate carrier desensitizes the mitochondrial permeability transition pore and causes cardiomyopathy. Cell Death Differ 21:1209–1217

Lapuente-Brun E, Moreno-Loshuertos R, Acin-Perez R, Latorre-Pellicer A, Colas C, Balsa E, Perales-Clemente E, Quiros PM, Calvo E, Rodriguez-Hernandez MA, Navas P, Cruz R, Carracedo A, Lopez-Otin C, Perez-Martos A, Fernandez-Silva P, Fernandez-Vizarra E, Enriquez JA (2013) Supercomplex assembly determines electron flux in the mitochondrial electron transport chain. Science 340:1567–1570

Lenartowicz E, Bernardi P, Azzone GF (1991) Phenylarsine oxide induces the cyclosporin A-sensitive membrane permeability transition in rat liver mitochondria. J Bioenerg Biomembr 23:679–688

Leung AW, Varanyuwatana P, Halestrap AP (2008) The mitochondrial phosphate carrier interacts with cyclophilin D and may play a key role in the permeability transition. J Biol Chem 283:26312–26323

Li B, Chauvin C, de Paulis D, de Oliveira F, Gharib A, Vial G, Lablanche S, Leverve X, Bernardi P, Ovize M, Fontaine E (2012) Inhibition of complex I regulates the mitochondrial permeability transition through a phosphate-sensitive inhibitory site masked by cyclophilin D. Biochim Biophys Acta 1817:1628–1634

Lin DT, Lechleiter JD (2002) Mitochondrial targeted cyclophilin D protects cells from cell death by peptidyl prolyl isomerization. J Biol Chem 277:31134–31141

Maranzana E, Barbero G, Falasca AI, Lenaz G, Genova ML (2013) Mitochondrial respiratory supercomplex association limits production of reactive oxygen species from complex I. Antioxid Redox Signal 19:1469–1480

Masgras I, Rasola A, Bernardi P (2012) Induction of the permeability transition pore in cells depleted of mitochondrial DNA. Biochim Biophys Acta 1817:1860–1866

Mcenery MW, Snowman AM, Trifiletti RR, Snyder SH (1992) Isolation of the mitochondrial benzodiazepine receptor: association with the voltage-dependent anion channel and the adenine nucleotide carrier. Proc Natl Acad Sci U S A 89:3170–3174

Mcgeoch JE, Guidotti G (1997) A 0.1-700 Hz current through a voltage-clamped pore: candidate protein for initiator of neural oscillations. Brain Res 766:188–194

Mcgeoch JE, Mcgeoch MW (2008) Entrapment of water by subunit c of ATP synthase. J R Soc Interface 5:311–318

Mcgeoch JE, Palmer DN (1999) Ion pores made of mitochondrial ATP synthase subunit c in the neuronal plasma membrane and Batten disease. Mol Genet Metab 66:387–392

Mcgeoch JE, Mcgeoch MW, Mao R, Guidotti G (2000) Opposing actions of cGMP and calcium on the conductance of the F(0) subunit c pore. Biochem Biophys Res Commun 274:835–840

Mimaki M, Wang X, McKenzie M, Thorburn DR, Ryan MT (2012) Understanding mitochondrial complex I assembly in health and disease. Biochim Biophys Acta 1817:851–862

Moreno-Lastres D, Fontanesi F, Garcia-Consuegra I, Martin MA, Arenas J, Barrientos A, Ugalde C (2012) Mitochondrial complex I plays an essential role in human respirasome assembly. Cell Metab 15:324–335

Nakagawa T, Shimizu S, Watanabe T, Yamaguchi O, Otsu K, Yamagata H, Inohara H, Kubo T, Tsujimoto Y (2005) Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. Nature 434:652–658

Nicholls DG, Ferguson SJ (2013) The oxygen electrode: monitoring proton current. Bioenergetics 4. Elsevier, Amsterdam

Nickel A, Kohlhaas M, Maack C (2014) Mitochondrial reactive oxygen species production and elimination. J Mol Cell Cardiol 73:26–33

Nicolli A, Petronilli V, Bernardi P (1993) Modulation of the mitochondrial cyclosporin A-sensitive permeability transition pore by matrix pH. Evidence that the pore open-closed probability is regulated by reversible histidine protonation. Biochemistry 32:4461–4465

Pasdois P, Parker JE, Griffiths EJ, Halestrap AP (2013) Hexokinase II and reperfusion injury: TAT-HK2 peptide impairs vascular function in Langendorff-perfused rat hearts. Circ Res 112: e3–e7

Pedersen PL, Greenawalt JW, Reynafarje B, Hullihen J, Decker GL, Soper JW, Bustamente E (1978) Preparation and characterization of mitochondria and submitochondrial particles of rat liver and liver-derived tissues. Methods Cell Biol 20:411–481

Pestana CR, Silva CH, Uyemura SA, Santos AC, Curti C (2010) Impact of adenosine nucleotide translocase (ANT) proline isomerization on Ca2 + -induced cysteine relative mobility/mitochondrial permeability transition pore. J Bioenerg Biomembr 42:329–335

Peters C, Bayer MJ, Buhler S, Andersen JS, Mann M, Mayer A (2001) Trans-complex formation by proteolipid channels in the terminal phase of membrane fusion. Nature 409:581–588

Petit PX, Zamzami N, Vayssiere JL, Mignotte B, Kroemer G, Castedo M (1997) Implication of mitochondria in apoptosis. Mol Cell Biochem 174:185–188

Petronilli V, Szabo I, Zoratti M (1989) The inner mitochondrial membrane contains ion-conducting channels similar to those found in bacteria. FEBS Lett 259:137–143

Petronilli V, Miotto G, Canton M, Brini M, Colonna R, Bernardi P, DI Lisa F (1999) Transient and long-lasting openings of the mitochondrial permeability transition pore can be monitored directly in intact cells by changes in mitochondrial calcein fluorescence. Biophys J 76:725–734

Pogoryelov D, Reichen C, Klyszejko AL, Brunisholz R, Muller DJ, Dimroth P, Meier T (2007) The oligomeric state of c rings from cyanobacterial F-ATP synthases varies from 13 to 15. J Bacteriol 189:5895–5902

Porter GA Jr, Hom J, Hoffman D, Quintanilla R, de Mesy Bentley K, Sheu SS (2011) Bioenergetics, mitochondria, and cardiac myocyte differentiation. Prog Pediatr Cardiol 31:75–81

Quinlan CL, Orr AL, Perevoshchikova IV, Treberg JR, Ackrell BA, Brand MD (2012) Mitochondrial complex II can generate reactive oxygen species at high rates in both the forward and reverse reactions. J Biol Chem 287:27255–27264

Rizzuto R, Pozzan T (2006) Microdomains of intracellular Ca2+: molecular determinants and functional consequences. Physiol Rev 86:369–408

Rizzuto R, Bernardi P, Pozzan T (2000) Mitochondria as all-round players of the calcium game. J Physiol 529(Pt 1):37–47

Rizzuto R, Marchi S, Bonora M, Aguiari P, Bononi A, DE Stefani D, Giorgi C, Leo S, Rimessi A, Siviero R, Zecchini E, Pinton P (2009) Ca(2+) transfer from the ER to mitochondria: when, how and why. Biochim Biophys Acta 1787:1342–1351

Ruck A, Dolder M, Wallimann T, Brdiczka D (1998) Reconstituted adenine nucleotide translocase forms a channel for small molecules comparable to the mitochondrial permeability transition pore. FEBS Lett 426:97–101

Saks V, Kuznetsov AV, Gonzalez-Granillo M, Tepp K, Timohhina N, Karu-Varikmaa M, Kaambre T, DOS Santos P, Boucher F, Guzun R (2012) Intracellular energetic units regulate metabolism in cardiac cells. J Mol Cell Cardiol 52:419–436

Schinzel AC, Takeuchi O, Huang Z, Fisher JK, Zhou Z, Rubens J, Hetz C, Danial NN, Moskowitz MA, Korsmeyer SJ (2005) Cyclophilin D is a component of mitochondrial permeability transition and mediates neuronal cell death after focal cerebral ischemia. Proc Natl Acad Sci U S A 102:12005–12010

Schneggenburger R, Neher E (2005) Presynaptic calcium and control of vesicle fusion. Curr Opin Neurobiol 15:266–274

Seidlmayer LK, Gomez-Garcia MR, Blatter LA, Pavlov E, Dedkova EN (2012) Inorganic polyphosphate is a potent activator of the mitochondrial permeability transition pore in cardiac myocytes. J Gen Physiol 139:321–331

Shanmughapriya S, Rajan S, Hoffman NE, Higgins AM, Tomar D, Nemani N, Hines KJ, Smith DJ, Eguchi A, Vallem S, Shaikh F, Cheung M, Leonard NJ, Stolakis RS, Wolfers MP, Ibetti J, Chuprun JK, Jog NR, Houser SR, Koch WJ, Elrod JW, Madesh M (2015) SPG7 is an essential and conserved component of the mitochondrial permeability transition pore. Mol Cell 60:47–62

Shimizu S, Shinohara Y, Tsujimoto Y (2000) Bax and Bcl-xL independently regulate apoptotic changes of yeast mitochondria that require VDAC but not adenine nucleotide translocator. Oncogene 19:4309–4318

Sileikyte J, Blachly-Dyson E, Sewell R, Carpi A, Menabo R, DI Lisa F, Ricchelli F, Bernardi P, Forte M (2014) Regulation of the mitochondrial permeability transition pore by the outer membrane does not involve the peripheral benzodiazepine receptor (Translocator Protein of 18 kDa (TSPO)). J Biol Chem 289:13769–13781

Sorgato MC, Keller BU, Stuhmer W (1987) Patch-clamping of the inner mitochondrial membrane reveals a voltage-dependent ion channel. Nature 330:498–500

Stotz SC, Scott LO, Drummond-Main C, Avchalumov Y, Girotto F, Davidsen J, Gomez-Garcia MR, Rho JM, Pavlov EV, Colicos MA (2014) Inorganic polyphosphate regulates neuronal excitability through modulation of voltage-gated channels. Mol Brain 7:42

Szabo I, Zoratti M (1991) The giant channel of the inner mitochondrial membrane is inhibited by cyclosporin A. J Biol Chem 266:3376–3379

Szabo I, Zoratti M (2014) Mitochondrial channels: ion fluxes and more. Physiol Rev 94:519–608

Vyssokikh MY, Brdiczka D (2003) The function of complexes between the outer mitochondrial membrane pore (VDAC) and the adenine nucleotide translocase in regulation of energy metabolism and apoptosis. Acta Biochim Pol 50:389–404

Vyssokikh MY, Katz A, Rueck A, Wuensch C, Dorner A, Zorov DB, Brdiczka D (2001) Adenine nucleotide translocator isoforms 1 and 2 are differently distributed in the mitochondrial inner membrane and have distinct affinities to cyclophilin D. Biochem J 358:349–358

Walker JE (2013) The ATP synthase: the understood, the uncertain and the unknown. Biochem Soc Trans 41:1–16

Wallace DC (1999) Mitochondrial diseases in man and mouse. Science 283:1482–1488

Wang W, Fang H, Groom L, Cheng A, Zhang W, Liu J, Wang X, Li K, Han P, Zheng M, Yin J, Wang W, Mattson MP, Kao JP, Lakatta EG, Sheu SS, Ouyang K, Chen J, Dirksen RT, Cheng H (2008) Superoxide flashes in single mitochondria. Cell 134:279–290

Wang X, Jian C, Zhang X, Huang Z, Xu J, Hou T, Shang W, Ding Y, Zhang W, Ouyang M, Wang Y, Yang Z, Zheng M, Cheng H (2012) Superoxide flashes: elemental events of mitochondrial ROS signaling in the heart. J Mol Cell Cardiol 52:940–948

Wittig I, Schagger H (2008) Structural organization of mitochondrial ATP synthase. Biochim Biophys Acta 1777:592–598

Wittig I, Karas M, Schagger H (2007) High resolution clear native electrophoresis for in-gel functional assays and fluorescence studies of membrane protein complexes. Mol Cell Proteomics 6:1215–1225

Woodfield K, Ruck A, Brdiczka D, Halestrap AP (1998) Direct demonstration of a specific interaction between cyclophilin-D and the adenine nucleotide translocase confirms their role in the mitochondrial permeability transition. Biochem J 336(Pt 2):287–290

Zhou A, Rohou A, Schep DG, Bason JV, Montgomery MG, Walker JE, Grigorieff N, Rubinstein JL (2015) Structure and conformational states of the bovine mitochondrial ATP synthase by cryo-EM. Elife 4, e10180

The Roles of Mitochondrial Cation Channels Under Physiological Conditions and in Cancer

Ildikò Szabò and Luigi Leanza

Contents

Abstract

Bioenergetics has become central to our understanding of pathological mechanisms as well as the development of new therapeutic strategies and as a tool for gauging disease progression in neurodegeneration, diabetes, cancer, and cardiovascular disease. The view is emerging that inner mitochondrial membrane (IMM) cation channels have a profound effect on mitochondrial function and, consequently, on the metabolic state and survival of the whole cell. Since disruption of the sustained integrity of mitochondria is strongly linked to human disease, pharmacological intervention offers a new perspective concerning neurodegenerative and cardiovascular diseases as well as cancer. This review summarizes our current knowledge regarding IMM cation channels and their roles

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H. Singh, S.-S. Sheu (eds.), *Pharmacology of Mitochondria*,
Handbook of Experimental Pharmacology 240, DOI 10.1007/164_2016_92

under physiological conditions as well as in cancer, with special emphasis on potassium channels and the mammalian mitochondrial calcium uniporter.

Keywords
Calcium uptake • Cancer • Magnesium channel • Mitochondria • Potassium channels

1 Introduction

Oxidative phosphorylation requires an electrochemical gradient across the inner mitochondrial membrane (IMM). The matrix-negative difference in electrical potential across the IMM (Δψm, ranging between −150 and −180 mV) is maintained by the proton pumps of the respiratory chain (RC). Consequently, cations (K^+, Ca^{2+}, and Mg^{2+}) flow from the intermembrane space (where the concentration of ions is comparable to cytosolic concentrations because the OMM is permeable to these ions via the voltage-dependent anion channels (VDACs)) to the matrix when a permeation pathway opens. To compensate for charge movement, the RC must increase the rate of proton transfer from the matrix to the intermembrane space (IMS). To increase RC activity according to the chemiosmosis model, the transmembrane electrochemical proton gradient ($\Delta\tilde{\mu}_H$, composed of mainly Δψm) must decrease. Thus, passive charge flow and $\Delta\tilde{\mu}_H$ (Δψm) are coupled, and the opening of cation channels in the IMM will lead to depolarization. Vice versa, closing cation channels leads to an increase of $\Delta\tilde{\mu}_H$ (Δψm) (i.e., hyperpolarization). Such alterations may in turn have consequences on the rate of superoxide formation (see, e.g., O-Uchi et al. 2014; Szewczyk et al. 2006; Zorov et al. 2014). Reactive oxygen species (ROS) are emerging as key players in the context of pathological conditions (e.g., during tumorigenesis, neurodegenerative diseases).

In general, due to the lack of molecular identity or to multiple localization in the case of several IMM cation channels (see below), unfortunately, studies in this field must contend with a number of important issues, including difficulties in the generation of genetic models (cells or animals) exclusively lacking the IMM cation channels.

2 Mitochondrial Potassium Channels

IMM K^+ channels recorded by patch clamp include calcium-dependent channels – big-conductance potassium channel (BKCa) (Singh et al. 2012), intermediate-conductance K^+ channel (IKCa) (De Marchi et al. 2009), small-conductance K^+ channel (SKCa) (Dolga et al. 2013), voltage-gated shaker-type K^+ channel Kv1.3 (Szabo et al. 2005), and the ATP-dependent potassium channel (mitoKATP) (Inoue et al. 1991). In addition, biochemical evidence indicates the presence of the two-pore potassium channel TASK-3 (Pocsai et al. 2006), of Kv1.5 (Leanza et al. 2012b), of

ROMK2, and of Kv7.4 (Testai et al. 2015a) (for recent reviews see, e.g., Laskowski et al. 2016; Szabo and Zoratti 2014). Since IMM K^+ channels are encoded by the nuclear genome, the molecular identities of the observed channel activities are still under debate in many (but not all) cases. Basically all above-mentioned IMM K^+ channels identified thus far are considered to be the mitochondrial counterparts of well-known plasma membrane (PM) channels and many of them display even multiple subcellular localizations: (1) SKCa has been found to be located in PM, mitochondria, and ER as well, where it is essential for Ca^{2+} uptake by ER in neurons and cardiomyocytes (Kuum et al. 2012); (2) Kv1.3 is present in the PM, in mitochondrial IMM (Szabo et al. 2005), in the cis-Golgi compartment (Zhu et al. 2014), and in the nuclear membrane system (Jang et al. 2015) (see below); (3) BKCa is present in the PM, Golgi, ER, and mitochondria (Singh et al. 2012; Toro et al. 2014). The role of these channels in the different membranes is only partially established as is (are) the targeting mechanism(s) allowing their localization to different subcellular compartments. The question still waiting for answer in many cases is whether the same protein is sorted to different compartments within the cells or specific mechanisms account for its localizations. As for mitoBKCa, this channel in the heart is encoded by a splice variant of the PM BKCa (*KCNMA1* encoded) and harbors a 50 aa splice insert that is essential for trafficking to the mitochondria (Singh et al. 2013). Instead, the possible mitoKATP component, ROMK2, the renal outer medullary K^+ channel ROMK2 (Kir1.1b), is a short form of ROMK. ROMK2 has been proposed to be the channel-forming subunit (Foster et al. 2012); however the molecular components of mitoKATP are still debated (for recent reviews see Szabo and Zoratti 2014; Testai et al. 2015b). To our knowledge, targeting mechanisms have not been identified for the other mitochondrial K^+ channels, but interestingly, a short sorting signal in the C-terminal transmembrane domain has been reported to determine targeting of a viral potassium channel to mitochondria versus PM when expressed in mammalian cells (von Charpuis et al. 2015). It is important to underline that not all these intracellular channels have been recorded in all tissues, but most of their PM counterparts have relatively wide tissue-expression profiles and the mitochondrial channels were found to be active in healthy tissues as well.

Unfortunately, genetic models, which are fundamental to the investigation of the physiological and pathological roles of mitochondrial K^+ channels, are largely unavailable. In the case of BKCa, *KCNMA1*$^{-/-}$ animals have been used, though these animals also lack the PM BKCa. In *KCNMA1*$^{-/-}$ cardiomyocytes, changes in ROS production and an attenuated oxidative phosphorylation capacity were observed, suggesting a mitochondrial role of BKCa channels in fine-tuning the oxidative state of the cell (Soltysinska et al. 2014). Despite the wide expression and important physiological functions of IKCa, the absence of this channel protein in transgenic KO mice does not result in severe physiological changes, possibly because of developmental compensation (Wulff and Castle 2010). Similarly, in animals lacking Kv1.3, expression of other Kv channels and even of anionic currents has been shown to largely compensate for the lack of Kv1.3 (Koni et al. 2003). ROMK-KO mice have been obtained, although they are short lived (Lu et al. 2002). To the best of our knowledge, no studies have yet been performed on these

animals and in TASK-3 KO mice (Gotter et al. 2011) to assess mitochondrial function. In summary, the available genetic models target both the PM and intracellular forms of the channels and/or are prone to developmental compensation. The generation of adequate cellular/animal models would allow genetic evidence to be obtained regarding the relative importance of these channels (which are often co-expressed in the same tissues) in the regulation of mitochondrial bioenergetics and of the physiological consequences.

Nevertheless, numerous studies applying pharmacological agents point to a crucial role of IMM K^+ channels in the context of energy conversion and cellular protection and provide evidence that K^+ transport modulates the tightness of coupling between mitochondrial respiration and ATP synthesis. As mentioned above, IMM K^+ channels have been proposed to contribute to the regulation of matrix volume, in addition to influencing $\Delta\Psi m$ and ΔpH, calcium transport, production of reactive oxygen species, and mitochondrial dynamics. Activation of a mitochondrial calcium-dependent K^+ channel modulates K^+ uptake and matrix volume while maintaining mitochondrial membrane potential ($\Delta\Psi m$) and confers protection without compromising oxidative phosphorylation during recovery from metabolic stress (Aon et al. 2010). In this case, activation of the channel is proposed to increase bioenergetic efficiency. Other studies instead indicate that a protective mechanism involving the activation of different IMM K^+ channels includes a slight uncoupling effect, i.e., a slight depolarization leading to increased respiration not coupled to ATP production. Such uncoupling would decrease energetic efficiency (Cardoso et al. 2010). Similarly, activity of the evolutionarily conserved ATP-dependent K^+ channel mitoKATP has been linked to ischemic preconditioning, ischemic postconditioning, and cytoprotection in general (Garlid et al. 2009; Lefer et al. 2009), even though in heart mitochondria the increased K^+ influx associated with mitoKATP opening was able to depolarize the membrane by only few millivolts (Kowaltowski et al. 2001). In addition, a recent piece of work demonstrated that the Kv7.2–7.5 activator retigabine depolarized the mitochondrial membrane potential, decreased mitochondrial Ca^{2+} levels, and in vivo largely prevented the functional and morphological changes triggered by global ischemia/reperfusion in Langendorff-perfused rat hearts, even though ROS production was increased (Testai et al. 2015a). The exact basis of K^+ channel openers' (KCO) cytoprotective properties still remains to be elucidated, although it is suggested that (1) attenuation of ROS production in mitochondria may play a significant role; (2) activation of mitoK^+ channels controls matrix volume, preserving a narrow intermembrane space, necessary for efficient oxidative phosphorylation; and (3) opening of mitoK^+ channels produces a mild decrease of membrane potential, thus reducing uptake of Ca^{2+} into the mitochondrial matrix and preventing Ca^{2+} overload and subsequent permeability transition pore opening. As mentioned above, most of these studies employed isolated mitochondria and/or relied on the use of nonspecific inhibitor or activator drugs displaying pleiotropic effects. The second consideration is especially true for the mitochondrial KATP and BKCa channels: the bulk of the evidence supporting the involvement of mitoKATP channel in protection against ischemic/reperfusion damage is pharmacological (Laskowski et al. 2016; Szewczyk et al. 2010). Unfortunately, other cellular targets possibly accounting for the observed effects have been identified

and most, if not all, of the pharmacological agents reportedly activating mitochondrial KATP can behave as membrane-permeable weak acid/base pairs, and thus as uncoupling, $\Delta\tilde{\mu}_H$-dissipating agents, possibly accounting for their protective effects. But specificity issues apply also to the other KCOs. For example, the CGS7184 (ethyl1-[[(4-chlorophenyl)amino]oxo]-2-hydroxy-6-trifluoromethyl-1H-indole-3-carboxy-late), a synthetic BKCa channel opener, directly activates the ryanodine receptor calcium release (RYR2) channel in the sarcoplasmic reticulum (Wrzosek et al. 2012). Furthermore, SERCA, complex I of the respiratory chain, and ATP-synthase inhibition are involved in pleiotropic effects of the BKCa channel activator NS1619 on endothelial cells (Lukasiak et al. 2016).

3 Mitochondrial Potassium Channels and Cancer

Besides a role in cytoprotection (see above), modulation of mitochondrial K^+ channels can also lead to cell death and to regulation of autophagy. Ion channels in general have emerged as "oncogenic" proteins during the last two decades, since they have an aberrant expression in cancers compared to normal tissues and contribute to several hallmarks of cancer, such as metabolic reprogramming, limitless proliferative potential, apoptosis resistance, stimulation of neo-angiogenesis, as well as cell migration and invasiveness. In the latest years, not only plasma membrane but also intracellular channels and transporters (Peruzzo et al. 2016), especially mitochondrial ones (Leanza et al. 2014), have arisen as oncological targets and were proposed to be associated with tumorigenesis. Therefore, the research is currently focusing on understanding the possible role of intracellular ion channels in cancer development and progression on the one hand, and on the other hand on developing new possible drugs able to modulate either expression or activity of these channels. The relevance of studying oncogenic channels (both PM and intracellular ones) is underlined by recent studies demonstrating that their pharmacological modulation can reduce tumor growth even in vivo in preclinical models of brain, lung, stomach, colon, blood, cervix, bladder, skin, prostate, breast, esophagus, and oral cavity cancers (Leanza et al. 2015). Among these examples, efficacy of mitochondrial channel-targeting drugs/molecules in reducing tumors has already been demonstrated even in vivo in preclinical mouse models for VDAC1 (Arif et al. 2014) and Kv1.3 (Leanza et al. 2012a).

Among mitochondrial potassium channels, the example studied in most detail in the context of cell death and cancer is the mitochondrial Kv1.3 channel. MitoKv1.3 mediates an inward potassium flux to the mitochondrial matrix and likely has a role in the organellar K^+ cycle that participates in the modulation of coupling between ATP synthesis and mitochondrial respiration, although a direct proof is missing (Szabo et al. 2012). In vivo evidence has been obtained suggesting that modulation of mitoKv1.3 by pharmacological means represents an unconventional but promising strategy to selectively eliminate cancer cells. Kv1.3 is overexpressed in various cancer tissues/cells and expression of PM-located Kv1.3 seems to correlate with that of the mitochondrial counterpart, mitoKv1.3. MitoKv1.3 was identified as a novel target of Bax: physical interaction between the two proteins via K128 of

Bax took place in apoptotic cells, leading to inhibition of channel activity (Szabo et al. 2008, 2011) and consequent $\Delta\psi m$ changes, increased ROS production, and cytochrome c release, whereas Kv1.3-deficient mitochondria were resistant. In agreement with these results, Psora-4, PAP-1, and clofazimine, three distinct membrane-permeant inhibitors of Kv1.3 (Cahalan and Chandy 2009), induced death in multiple human and mouse cancer cell lines by triggering the same series of events. In contrast, membrane-impermeant, selective, and high-affinity Kv1.3 inhibitors ShK or margatoxin did not trigger apoptosis, suggesting a crucial role for the mitochondrial Kv1.3 versus PM Kv1.3. Genetic deficiency or siRNA-mediated downregulation of Kv1.3 abrogated the effects of the drugs, proving specificity of their action via Kv1.3. In a preclinical mouse model, intraperitoneal injection of clofazimine significantly reduced melanoma tumor size while no adverse effects were observed in several healthy tissues (Leanza et al. 2012a). Furthermore, these drugs induced death of only the pathological primary tumor cells from B-cell chronic lymphocytic leukemia patients (Leanza et al. 2013) and clofazimine efficiently reduced by more than 50% the size of pancreatic ductal adenocarcinoma in an SCID mouse model using Colo357 cells which express high levels of Kv1.3 (Zaccagnino et al. 2016). Even though following silencing of Kv1.3 clofazimine-induced death does not take place, in vivo tumor reduction by clofazimine due to some pleiotropic effect cannot be fully excluded. Clofazimine has been in clinical use since the 1960s and is well tolerated, although bioaccumulation of the drug can lead to visible (but reversible) changes in skin pigmentation. It possesses anti-inflammatory and immunosuppressive activities; therefore it is used in various cutaneous, nonmicrobial, and chronic inflammatory disorders but more recent studies suggested an expansion of the clinical use of clofazimine to non-cutaneous inflammatory disorders such as multiple sclerosis, rheumatoid arthritis, and type I diabetes mellitus (Cholo et al. 2012). Clofazimine has recently been identified in a large-scale screening as an inhibitor of Wnt signaling (Koval et al. 2014). In addition, clofazimine has been shown to exert an inhibitory action on ABCB1/MDR1/P-glycoproteins (Pgp) (Van Rensburg et al. 1994) and to have the potential to inhibit p53–MDM2 interaction leading in turn to stabilization and activation of the tumor suppressor p53 (Casey et al. 2009; Patil 2013). Therefore, a more specific drug, acting exclusively on the mitochondrial counterpart of Kv1.3, would be required to more efficiently and specifically target this channel.

The intermediate-conductance potassium channel (IKCa or KCa3.1) is expressed in various tissues, such as epithelial and endothelial tissues, immune system, sensory neurons, and microglia but not in excitable tissues. PM IKCa is involved in several physiological processes modulating membrane potential and calcium signaling, including cell proliferation and differentiation in numerous cell types, and it shows a differential expression in cancer cells with respect to normal cells. An intracellular localization for the IKCa (mitoIKCa) in the inner mitochondrial membrane of human colon carcinoma and HeLa cells, as well as in mouse embryonic fibroblasts, has been demonstrated by electrophysiology and Western blotting (De Marchi et al. 2009; Sassi et al. 2010). The plasma membrane channel and the mitochondrial one seem to have the same pharmacological properties, since both are inhibited by TRAM-34 and clotrimazole (De Marchi et al. 2009). A connection between IKCa channels and the

intrinsic apoptotic pathway has been observed in a human glioblastoma cell line (McFerrin et al. 2012) and inhibition of IKCa by TRAM-34 increased the sensitivity of melanoma cells to TRAIL treatment (Quast et al. 2012). In addition, clotrimazole reduced cell viability of chronic leukemic B lymphocytes (Grossinger et al. 2014), even though, surprisingly, TRAM-34 did not exert the same effect. These examples raise the exciting possibility that the mitochondrial IKCa might also become an oncological target.

Mitochondrial TASK-3 channels are also likely to contribute to the regulation of apoptosis since silencing of TASK-3 in melanoma cells resulted in compromised mitochondrial function, i.e., mitochondrial membrane depolarization, and reduced cell survival inducing apoptotic cell death (Nagy et al. 2014). The tandem of P-domains (K2P) in weakly inward rectifying K^+ (TWIK)-related acid-sensitive K^+ channel 3 (TASK-3) is the most recently identified group among the K^+ channels. K2P channels are "leak" K^+ channels that set resting membrane potential and regulate cell excitability (Enyedi and Czirjak 2010). PM TASK-3 is normally found in the adrenal cortex, gastrointestinal tract, neuronal tissue, and salivary glands and is modulated by alterations in extracellular pH and by anesthetic agents, and plays a role in aldosterone secretion (Enyedi and Czirjak 2010). Altered PM/intracellular TASK-3 expression has already been defined in several types of cancer, i.e., in breast cancer, and the gene encoding TASK-3 (KCNK9) was found to be overexpressed by five- to >100-fold in 44% of tumors: in lung, colon, and ovarian cancers (Peruzzo et al. 2016), as well as in melanoma (Pocsai et al. 2006). Unfortunately, no highly specific mtTASK-3 modulators are available but dihydropyrrolo[2,1-α]isoquinoline (DPI) compounds, that are able to inhibit TASK channels, could become possible candidates for developing new, selective inhibitors (Noriega-Navarro et al. 2014).

Finally, sporadic information exist regarding a link between mitoBKCa function and cell death. The large-conductance calcium- and voltage-activated K^+ channel BKCa (KCa1.1) is expressed at the PM of both excitable and non-excitable cells, including sensory and epithelial cells, smooth muscle, and cardiac muscle, where it is involved in muscle contraction but also in cytoprotection during ischemia/reperfusion. MitoBKCa has been observed in mitochondria of glioma cell lines, in astrocytes, as well as in ventricular cells, skeletal muscle, brain, and endothelial cells (Szabo and Zoratti 2014). The known modulators of the plasma membrane channel act also on the mitoBKCa. These compounds can be divided into activators, such as calcium, diCl-DHAA, NS1619, 17-estradiol, and hypoxia, or inhibitors like charybdotoxin, iberiotoxin, and paxillin. To our knowledge, none of these drugs act exclusively on the mitochondrial channel (Szewczyk et al. 2009), and as mentioned above, they may also exert pleiotropic effects. MitoBKCa has been associated with cell death, since Bax can inhibit the channel leading to the activation of the MPTP (Cheng et al. 2010). Recently a modified channel function of mitochondrial BKCa has been linked to amyloid-beta (Aβ)-induced neuronal toxicity (Jafari et al. 2015). Thus, even though alteration of mitoBKCa function seems to be linked to cell death, to our knowledge, no direct evidence for involvement of the mitoBKCa in cancer development and/or progression exists to date.

In summary, the relationship between mitochondrial potassium transport and diseases linked to altered mitochondrial function is still only partially explored, but the so-far available data point to IMM K^+ channels as possible targets for therapeutic application against various pathologies, including cancer.

4 Mitochondrial Calcium Channels

Mitochondria play a crucial role in intracellular Ca^{2+} regulation by shaping, remodeling, relaying, and decoding Ca^{2+} signals, due to their ability to rapidly and transiently accumulate Ca^{2+} (Drago et al. 2011). A membrane potential of −180 mV (negative inside) generated by the respiratory chain would theoretically lead to a 1,000,000-fold accumulation of matrix Ca^{2+} if electrophoretic Ca^{2+} passage was unrestricted. Accordingly, the protonophore CCCP does not only collapse the membrane potential but also the Ca^{2+} transport (Selwyn et al. 1970). Ca^{2+} uptake into mammalian mitochondria is additionally blocked by low concentrations of ruthenium red and Ru360, which leads to a direct inhibition of the uniporter (Moore 1971; Reed and Bygrave 1974; Vasington et al. 1972). In animal cells mitochondria were the first intracellular organelles to be associated with Ca^{2+} handling, and well before the identification of the mitochondrial calcium uniporter complex (MCUC) components, its ability to rapidly sense Ca^{2+} signals and to act as localized buffers with high capacity in proximity of PM and ER Ca^{2+} channels/transporters has been proven (Rizzuto et al. 2012). Indeed, mitochondrial Ca^{2+} uptake, by impacting on local calcium concentrations and on calcium-mediated feedback mechanism known to modulate the activity of Ca^{2+} channels, influences frequency and amplitude of cytosolic calcium signals. For example, calcium flux across both the PM/ER-located calcium release-induced calcium channel CRAC (Orai1/Stim1) and the ER-located inositol-1,4,5-trisphosphate receptor is influenced by physical vicinity of mitochondria. This proximity, sustained by specific contacts, the so-called mitochondria-associated membranes (MAMs) via chaperons such as sigma receptor 1, in turn sets the extent and duration of mitochondrial calcium increase (Rizzuto et al. 2012). In addition, especially in large cells, recruitment of mitochondria to specific regions seems to be important for constraining Ca^{2+} signals to defined cell domains. As a result, mitochondrial calcium uptake has been shown at least in vitro to govern numerous pathophysiological processes ranging from insulin secretion, neuronal excitotoxicity, and cardiomyocyte function to tumorigenesis. The reader is advised to consult excellent, recent reviews on this topic (e.g., De Stefani et al. 2016; Foskett and Philipson 2015; Rizzuto et al. 2012).

Significant advances in identifying the molecular players of the mitochondrial Ca^{2+}-handling machinery have been achieved only during the last decade. The finding that a highly Ca^{2+}-selective ion channel, displaying a very small conductance of only 5 pS in 100 mM Ca^{2+} in vitro, recapitulated the key characteristics observed for the mammalian mitochondrial uniporter in classical bioenergetic experiments represented a milestone toward the molecular identification of the uniporter (Kirichok et al. 2004). The MitoCarta database, containing more than a thousand of mitochondrial proteins

(Pagliarini et al. 2008), then provided the basis for the identification of several mitochondrial calcium uniporter complex (MCUC) components in mammals, including the central pore-forming protein mitochondrial calcium uniporter (MCU) (Baughman et al. 2011; De Stefani et al. 2011). At the current stage MCUC appears to include at least the pore-forming protein MCU, an MCU paralog (MCUb), the essential MCU regulator (EMRE), the regulatory MICU proteins, and possibly the mitochondrial calcium uniport regulator 1 (MCUR1) in mammals, unlike in other systems containing a more simplified MCUC (Wagner et al. 2016). MCU is a 40 kDa protein that is inserted into the IMM via two transmembrane domains and oligomerizes into multimers (penta- or tetramers) (Oxenoid et al. 2016; Raffaello et al. 2013) to form a pore that allows Ca^{2+} entry into the mitochondrial matrix driven by the electrical membrane gradient (Baughman et al. 2011; De Stefani et al. 2011; Raffaello et al. 2013). Recombinant MCU protein, when incorporated into an artificial membrane, mediates Ca^{2+}-permeable activity, resembling the electrophysiological characteristics of the mitochondrial uniporter (De Stefani et al. 2011; Kirichok et al. 2004). Mammalian MCU activity can be regulated through its paralog MCUb. MCU and MCUb share 50% sequence similarity and both proteins physically interact. MCUb carries two conserved amino acid exchanges in the IMS-exposed loop of MCU which is necessary to permit Ca^{2+} transport through MCU in lipid bilayer experiments (Raffaello et al. 2013). In cultured cells, MCUb forms heterooligomers with MCU and likely constitutes a dominant-negative regulator of MCU transport activity. The mammalian MICU protein family consists of three members that share more than 40% sequence identity. MICU1, the first uniporter component identified (Perocchi et al. 2010), is a 50 kDa protein with two functional and two pseudo EF-hands that resides in the mitochondrial intermembrane space (Csordas et al. 2013; Hung et al. 2014; Patron et al. 2014; Petrungaro et al. 2015; Wang et al. 2014). It was quickly referred to as the uniporter "gatekeeper" that sets a threshold for mitochondrial Ca^{2+} uptake through MCU at low extramitochondrial Ca^{2+} concentrations but activates the channel when surrounding Ca^{2+} concentrations are high (Csordas et al. 2013; Mallilankaraman et al. 2012). Recent findings showing that elevations in cytosolic Ca^{2+} are sufficient (EC_{50} of 4.4 μM) to induce rearrangement of MICU1 multimers and to trigger activation of mitochondrial Ca^{2+} uptake are in agreement with this concept (Waldeck-Weiermair et al. 2015). Interestingly, a splice variant with much higher affinity for calcium has been identified in skeletal muscle (Vecellio Reane et al. 2016). The initial model for MICU function was further refined after the identification of two MICU1 isoforms, MICU2 and MICU3 (Plovanich et al. 2013). As MICU3 was found to be almost exclusively expressed in neural tissues (Plovanich et al. 2013), functional characterization focused on ubiquitously expressed MICU2. MICU2 forms a heterodimer with MICU1 through an intermolecular disulfide bond and closes the channel at low extramitochondrial Ca^{2+} concentrations (Patron et al. 2014; Petrungaro et al. 2015). The stability of MICU2 depends on MICU1 (Patron et al. 2014; Plovanich et al. 2013) and loss of MICU2 in MICU1-silenced cells complicates assignment of individual MICU1 and MICU2 functions. However, in electrophysiological experiments MICU2 inhibits the channel activity in the absence of Ca^{2+}, while MICU1 activates the channel in the presence of calcium, in accordance with the proposed model of

MICU2 and MICU2 being gatekeeper or activator, respectively (Patron et al. 2014). Currently, two models coexist that find MICU1 (1) to act exclusively as a uniporter activator at high cytosolic Ca^{2+} concentrations (Patron et al. 2014) or (2) to gradually disinhibit the uniporter with increasing Ca^{2+} concentrations in the cytosol (Csordas et al. 2013).

Another core component of the mammalian MCUC is EMRE, a 10 kDa protein that spans the inner mitochondrial membrane with only one transmembrane motif. The proposed role of EMRE in mammals is that of mediating the physical interaction between MCU and a MICU1/MICU2 dimer and regulating MCU channel activity depending on the matrix Ca^{2+} concentration (Sancak et al. 2013; Vais et al. 2016). In vitro MCU protein alone is sufficient to form a functional channel (De Stefani et al. 2011) even though structural studies allowed to forward the hypothesis that the outer and inner juxtamembrane helices as well as a loop region in MCU are unstable regions which may undergo conformational changes upon activation by EMRE in order to create the lateral exit path for Ca^{2+} in the MCU channel (Oxenoid et al. 2016). EMRE is a metazoan-specific protein and its role is supported by reconstitution of a functional uniporter in budding yeast as heterologous system without a endogenous mitochondrial Ca^{2+} uniporter: while expression of MCU from the slime mold *Dictyostelium* alone was sufficient to import Ca^{2+} into yeast mitochondria, human EMRE needed to be expressed alongside MCU to form an active Ca^{2+} uniporter system (Kovacs-Bogdan et al. 2014). A recent study, using mammalian MCU, suggests that both regulators, MICU1 and the splice variant of MICU1, are able to interact with immobilized MCU or with MCU/EMRE complex displaying very similar affinity, suggesting that the presence of EMRE is not (or only poorly) involved in MCU-MICU1 (Vecellio Reane et al. 2016). In summary, the role of EMRE is far from being clarified.

As to the pathophysiological importance of mitochondrial calcium homeostasis, Ca^{2+} elevations in the mitochondrial matrix are known to stimulate respiration and ATP synthesis to cover temporarily high energy needs of cells (Denton 2009). Ca^{2+} overload, by contrast, can trigger cell death (Duchen 2000). Increased biosynthesis rates of ATP rely on the activation of three mitochondrial dehydrogenases by Ca^{2+} (McCormack et al. 1990). In addition, increasing evidence suggests that cross talk between mitochondrial calcium and ROS signaling systems promotes fine-tuning of the cellular signaling networks and of metabolism (Gorlach et al. 2015). For example, the mammalian pyruvate and 2-oxoglutarate dehydrogenases are a source of mitochondrial O_2^- and H_2O_2, are regulated by Ca^{2+}, and contain a dihydrolipoyl dehydrogenase subunit that is inhibited by H_2O_2-induced glutathionylation (McLain et al. 2013; Nulton-Persson et al. 2003; Yan et al. 2013). Besides the pyruvate dehydrogenase (PDH) (Denton et al. 1972) and oxoglutarate dehydrogenase (OGDH) (McCormack and Denton 1979), NAD-isocitrate dehydrogenase (NAD-ICDH) (Denton et al. 1978) is also activated by physiologically relevant Ca^{2+} concentrations (100 nM and 1 μM) in mitochondria isolated from mammalian tissues (Denton and McCormack 1980; Denton et al. 1980) and phosphorylation of PDH is thought to be modulated by the calcium-sensitive phosphatase PDP1 (Holness and Sugden 2003). Ca^{2+} elevations in intact cells result in NAD(P) reduction (Duchen 1992; Pralong et al. 1992), supporting a central role for Ca^{2+}-dependent regulation of

mitochondrial metabolism. Knockout of the MCUC regulator MICU1 that results in an increased resting-state level of Ca^{2+} in the mitochondrial matrix accordingly alters the PDH phosphorylation state in cultured cells (Mallilankaraman et al. 2012). In addition, lower levels of basal matrix calcium in the *MCU*$^{-/-}$ mice led to markedly increased levels of PDH phosphorylation in these animals (Pan et al. 2013), although the animal model used in this study is highly debated especially in view of the modest phenotype and of the fact that viable mice could be obtained only in a mixed genetic background (see, e.g., Pendin et al. 2014). Indeed, the consensus view is that conditional and inducible, tissue-specific knockout models, as well as viral based gene-delivery systems, will be needed to conclusively assess the real physiological impact of mitochondrial calcium homeostasis in vivo. A few recent in vivo studies in fact demonstrate that mitochondrial calcium homeostasis is crucial for regulation of metabolism and its alterations are linked to pathologies. Genetic manipulation of MCU in lower organisms such as zebrafish (Prudent et al. 2013) and *Trypanosoma brucei* (Huang et al. 2013) resulted in major developmental and energetic defects. Postnatal manipulation of MCU levels in mice (by using adeno-associated virus-mediated gene transfer) demonstrated the contribution of MCUC to the regulation of skeletal muscle tropism. MCU overexpression or downregulation caused muscular hypertrophy or atrophy, respectively. These processes were likely independent of metabolic alterations, but were linked to a novel Ca^{2+}-dependent mitochondria-to-nucleus signaling pathway via transcriptional regulators (Mammucari et al. 2015). Finally, in mice with myocardial MCU inhibition by transgenic expression of a dominant-negative (DN) MCU, a strong correlation between MCU function, MCU-enhanced oxidative phosphorylation, and correct pacemaker cell function has been found (Wu et al. 2015).

The importance of the correct calcium handling is illustrated by the finding that homozygous patients carrying a loss-of-function mutation of MICU1 are characterized by myopathy, cognitive impairment, and extrapyramidal movement disorder (Logan et al. 2014), along with an increased agonist-induced mitochondrial Ca^{2+} uptake at low cytosolic Ca^{2+} concentrations and a decreased cytosolic Ca^{2+} signal. However, at least under resting conditions, the fibroblasts from affected individuals do not display defects in overall cellular metabolic function, but chronic elevation of the mitochondrial matrix Ca^{2+} load seems to lead to moderate mitochondrial stress, resulting in fragmentation of the mitochondrial network. Furthermore, MICU1 has recently been shown to be vital for adaptation to postnatal life and for tissue repair after injury of liver (Antony et al. 2016). Ca^{2+} overload-induced mitochondrial permeability transition pore (PTP) opening was accelerated in MICU1-deficient hepatocytes. PTP inhibition prevented necrosis and rescued regeneration. This work further highlights the importance of regulating MCU under stress conditions when the risk of Ca^{2+} overload is elevated.

Other mitochondrial Ca^{2+} uptake modes (e.g., Ca^{2+}-selective conductance (mCa) 1 and 2 and rapid mode of uptake (RaM)) have also been observed in animals. These uptake modes were proposed to differ from MCUC-mediated Ca^{2+} uptake in terms of Ca^{2+} affinity, uptake kinetics, and pharmacological control. These modes have been studied in heart cells and are – similar to MCU – inhibited by ruthenium red

and activated by spermine (Sparagna et al. 1995), yet at higher concentrations. The relative and/or tissue-dependent contribution of these other uptake pathways with respect to MCU still has to be elucidated. *MCU* knockdown very efficiently abolishes Ca^{2+} transients in mammalian cell culture (Baughman et al. 2011; Bondarenko et al. 2014; De Stefani et al. 2011), indicating that the MCUC can have a dominating role among uptake mechanisms. In accordance, Ca^{2+} uptake into mitochondria was almost completely abolished in the liver of $MCU^{-/-}$ animals (Pan et al. 2013). On the other hand, this does not rule out the possibility that other mechanisms make major contributions to Ca^{2+} uptake, particularly in specialized tissues. Potential candidates include the TRPC3 channel (Feng et al. 2013; Wang et al. 2015) and the mitochondrial ryanodine receptor (mRyR1). A low level of RyR1 is detectable in heart mitochondria and provides rapid transport of Ca^{2+} that is insensitive to ruthenium red (Beutner et al. 2001, 2005). The availability of several animal model systems in which MCU is genetically knocked out should help to test the hypothesis of MCUC being responsible for different uptake modes and clarify the presence and kinetics of coexisting uptake mechanisms.

5 Mitochondrial Calcium Homeostasis and Cancer

Besides its physiological role, MCU has been implicated also in cancer-related processes, in particular in the control of metastasis. Recently, Hall and colleagues (Hall et al. 2014) described that breast cancer patient outcomes negatively correlated with increased MCU Ca^{2+}-conducting pore subunit expression and decreased MICU1 regulatory subunit expression. However, they showed that a widely used breast cancer cell line did not require MCU or MICU1 activity for survival in contrast to cervical, colon, and prostate cancer-derived cells. Our research in a publically available database (Peruzzo et al. 2016) suggests that indeed expression of MCU is often altered only slightly in tumoral tissues, in accordance with previous findings (Davis et al. 2013). On the other hand, Tang and colleagues revealed that MCU expression correlates with metastasis and invasiveness of breast cancer. MCU inhibition by ruthenium red or MCU silencing by siRNA abolished migration of breast cancer cells and reduced serum- or thapsigargin (TG)-induced store-operated Ca^{2+} entry (SOCE). Serum-induced migrations in these MDA-MB-231 cells were blocked by SOCE inhibitors, suggesting that MCU plays a critical role in breast cancer cell migration by regulating SOCE (Tang et al. 2015). In an independent study, MCU expression has been related to breast tumor size and lymph node infiltration (Tosatto et al. 2016). In a triple-negative xenograft model using MDA-MB-231 cells, ablation of MCU induced a reduction in tumor growth and metastasis formation. MCU downregulation hampered cell motility and invasiveness and reduced tumor growth, lymph node infiltration, and lung metastasis. In MCU-silenced cells, production of mitochondrial ROS was blunted and expression of the hypoxia-inducible factor-1α (HIF-1α) was found to be reduced, suggesting a signaling role for mitochondrial ROS and HIF-1α, downstream of mitochondrial Ca^{2+}. HIF-1α has been demonstrated to be a major effector of MCU, since rescuing HIF-1α expression the tumor cells restored the ability to migrate. In addition, in breast cancer

mRNA samples, a positive correlation of MCU expression with HIF-1α signaling route was present (Tosatto et al. 2016). Finally, it has been proposed that a small molecule, AG311 (5-[(4-methylphenyl)thio]-9H-pyrimido[4,5-b]indole-2,4-diamine), shown to retard tumor growth and to reduce lung metastases, might induce breast cancer cell death by activating MCU, although direct proof is missing (Bastian et al. 2015).

In summary, there is an urgent need to identify pharmacological agents able to impact mitochondrial calcium uptake via their specific action on MCUC components, since the so-far used ruthenium red and lanthanides are wide-spectrum modulators. This task could be much greatly helped by structure–activity relationship (SAR) studies, taking advantage of the recently resolved 3D structure of MCU (Oxenoid et al. 2016).

6 Mitochondrial Sodium Homeostasis in Cellular Physiology

As other cations, Na^+ is expected to be strongly attracted into mitochondria because of the highly negative electrical potential inside the mitochondrial matrix. Classical bioenergetics studies suggest that mitochondria possess a very active $Na^+(Li^+)$-selective H^+/Na^+ antiporter (NHE) that does not transport K^+ (Cockrell 1973; Mitchell and Moyle 1969). This antiporter is readily inhibited by amiloride analogs (Brierley et al. 1989) and its activity declines at pH values higher than 7.0 (Douglas and Cockrell 1974). Unlike the K^+/H^+ antiporter, NHE is not regulated by Mg^{2+} and is insensitive to quinine (Nakashima and Garlid 1982). NHE function is probably linked to the setting of the steady-state Ca^{2+} cycling in energized mitochondria, which largely occurs through coupling of electrophoretic influx via the Ca^{2+} uniporter and efflux via the Na^+/Ca^{2+} antiporter (NCLX) (Crompton and Heid 1978). The current view is that fluxes of Ca^{2+}, Na^+, and H^+ during the Na^+-induced efflux of Ca^{2+} support a series of events in which the Na^+–H^+ exchange enables unidirectional Ca^{2+} fluxes via the uniport and antiport systems to be integrated into a cycle (Crompton and Heid 1978; Drago et al. 2011). The main Na^+ efflux mechanism is represented by the mitochondrial NHE, while the main influx pathway is the NCLX (Murphy and Eisner 2009). Whether the electrogenic NCLX which mediates the flux of three Na^+ ions against one Ca^{2+} ion might, at least under some conditions, be linked to the previously described sodium uniport activity is still unclear. It has been shown that addition of EDTA to mitochondria is followed by swelling in NaCl and LiCl but not in KCl solution, suggesting the existence of an inner membrane channel with selectivity for Na^+ and Li^+ (Bernardi 1999). Considering that this Mg^{2+} depletion-induced Na^+ flux is finely regulated by Mg^{2+}, ruthenium red, pH, and glibenclamide (Bernardi et al. 1990; Kapus et al. 1990; Szewczyk et al. 1996), it is likely not to occur through an unspecific leak, but rather via a specific channel in the inner membrane. However, to our knowledge no electrophysiological study identified an activity with the above characteristics and the molecular identity of the uniport observed by classical bioenergetics still awaits clarification.

Cytosolic Na^+ changes are transmitted to the mitochondria by the NCLX and Ca^{2+} plays an important role in regulating Na^+ flux, suggesting the existence of a Na^+ sensor

in the mitochondria: indeed, a decrease in intracellular Ca^{2+} was observed to lead to a reduced mitochondrial Na^+ uptake, while an increase in Ca^{2+} availability enhanced mitochondrial Na^+ influx (Bernardi 1999). Since cytosolic Na^+ oscillations may influence intracellular (mitochondrial and ER) Ca^{2+} variations, which in turn intimately regulate mitochondrial metabolism and are implicated in several diseases, the correlation existing between Na^+ and Ca^{2+} fluxes (see above) might acquire importance in pathological settings. Indeed, in melanoma cells, an increase in cytosolic Na^+ mediated by a splice variant of the Na^+ channel Nav 1.6 has been shown to promote mitochondrial Ca^{2+} release (indirectly, via the Na^+/Ca^{2+} exchanger), which enhanced the invasiveness of these tumor cells (Carrithers et al. 2009). Activation of mitochondrial Na^+/Ca^{2+} exchange by an increase in intracellular Na^+ is also a prime suspect in several cellular pathologies such as heart failure, cancer, and hypertension (Murphy and Eisner 2009). For example, in cardiomyocytes from failing hearts, insufficient mitochondrial Ca^{2+} accumulation (as a consequence of cytoplasmic Na^+ overload and activation of the mitochondrial Na^+/Ca^{2+} exchanger) decreases the NAD(P)H/NAD(P)(+) redox potential and increases oxidative stress upon increase of the workload. These effects were shown to be abolished by acute treatment with CGP-37157 (CGP), an inhibitor of the mitochondrial NCLX (Liu et al. 2014). In another study however, knocking down of NCLX resulted in downregulation of MCU-dependent mitochondrial Ca^{2+} uptake (Nita et al. 2016). CGP-37157 has been exploited also in the context of cancer and shown to induce calcium overload-linked cell death of malignant cells in vitro (Choudhary et al. 2014; Yoon et al. 2012).

7 The Mitochondrial Magnesium Channel Mrs2 in Physiology and in Cancer

Mitochondria do not only store calcium, but also take up magnesium to maintain the optimal cytosolic Mg^{2+} concentration (0.5–0.7 mM) (Kubota et al. 2005). Mg^{2+} can be accumulated inside the mitochondria via the Mg^{2+}-selective channel (Kolisek et al. 2003) that takes advantage of the driving force produced by the mitochondrial membrane potential and is feedback regulated by increasing Mg^{2+} concentration in the matrix. Human mitochondrial Mrs2 protein (hMrs2) is a magnesium-transporting channel in the IMM. It was identified as an upregulated gene in a multidrug-resistant (MDR) gastric cancer cell line compared to its parental cells by subtractive hybridization. Expression of hMrs2 in mrs2-1 knockout mutant yeast partly restored mitochondrial magnesium concentrations that were significantly reduced in this mutant (Zsurka et al. 2001). Knockdown of Mrs2 caused cell death by inducing loss of respiratory complex I and by triggering mitochondrial membrane depolarization (Piskacek et al. 2009). On the contrary, an upregulation of Mrs2 has been observed in parental human gastric adenocarcinoma cell lines, indicating that high expression of Mrs2 may protect against death (Wolf and Trapani 2009). hMrs2 expression positively regulated adriamycin resistance of gastric cancer cells both in vitro and in vivo (Chen et al. 2009). Additionally, hMrs2 promoted cell growth and cells with decreased expression exhibited significant inhibition of cell growth with G(1) cell

cycle arrest. Upon hMrs2 overexpression, p27 was downregulated whereas cyclinD1 was upregulated. Interestingly, an augmented expression of *MRS2* gene has been reported by comparing normal and cancer organs with a general increase of around two- to threefold (but in testis tumor a 17-fold increase), which has been observed in blood, skin, ovarian, kidney, breast, lung, and bladder tumors when compared to the normal tissues (Peruzzo et al. 2016). Thus, it would be worthwhile to further deepen our understanding on the possible role of mitochondrial Mg^{2+} fluxes in cancer development as well as to gain insight into the complete structure of the Mrs2 proteins (so far only the N-terminal part has been resolved) (Khan et al. 2013) in order to exploit SAR strategies to design specific drugs.

8 Future Outlook

The most critical question that will have to be addressed in future studies is related to the organelle-specific targeting and determination of the molecular identity of some of the above-mentioned channels (e.g., mitoKATP), in order to fully appreciate the physiological functions of these ion channels. Hopefully, technical improvement in proteomic techniques (increase of sensibility, preparation of highly purified membranes) will help us to identify more and more organelle-located channel proteins. Once the localization of a given protein is validated also in intact cells (e.g., using tagged proteins), its channel function can be demonstrated by (1) exploiting the innovative in vitro translation/transcription system for expression and reconstitution of active membrane proteins (Berrier et al. 2004; Ezure et al. 2014); (2) using purified membrane vesicles; (3) direct patch clamping of mitoplasts (mitochondria without outer membrane). An indication for the in vivo function and physiological role of a given channel might be obtained by comparison of WT and mutant organisms lacking a given protein that likely forms the channel. The above approach however might lead to misleading results, since function of a missing channel can be overtaken by, e.g., another member of the same family. This problem, arising from redundancy of proteins with structural similarities and likely fulfilling the same task, should not be underestimated. Emerging techniques allowing multiple genetic manipulation (e.g., CRISPR-Cas9) in diverse organisms might be of great help in this respect. Elucidation of the regulation of mitochondrial ion channels by posttranslational modifications is also a major challenge for the future as is the design and synthesis of specific drugs able to act exclusively on the mitochondrial counterparts of the channels that are present at multiple sites within the cells. It also has to be mentioned that in addition to ion channels, a plethora of cation-transporting carriers have been described in mitochondrial membranes, whose function is likely to be well coordinated with that of the channels. Such coordinated action, which might be fully appreciated by using multiple knockout organisms, is expected to allow fine-tuning of bioenergetic efficiency. In conclusion, a combination of diversified approach will certainly lead to a considerable increase in our knowledge of the mechanisms of organelle function mediated by the

ion channels and will also improve our understanding of the importance of mitochondrial channels for tumorigenesis and/or tumor progression.

Acknowledgements We are grateful to the Italian Association for Cancer Research (AIRC grant IG 11814 to I.S.) and to the Italian Ministry of Education (PRIN project 2010CSJX4F and 2015795S5W to I.S.), for financial support.

References

Antony AN, Paillard M, Moffat C, Juskeviciute E, Correnti J, Bolon B, Rubin E, Csordas G, Seifert EL, Hoek JB et al (2016) MICU1 regulation of mitochondrial Ca(2+) uptake dictates survival and tissue regeneration. Nat Commun 7:10955

Aon MA, Cortassa S, Wei AC, Grunnet M, O'Rourke B (2010) Energetic performance is improved by specific activation of K+ fluxes through K(Ca) channels in heart mitochondria. Biochim Biophys Acta 1797:71–80

Arif T, Vasilkovsky L, Refaely Y, Konson A, Shoshan-Barmatz V (2014) Silencing VDAC1 expression by siRNA inhibits cancer cell proliferation and tumor growth in vivo. Mol Ther Nucleic Acids 3:e159

Bastian A, Thorpe JE, Disch BC, Bailey-Downs LC, Gangjee A, Devambatla RK, Henthorn J, Humphries KM, Vadvalkar SS, Ihnat MA (2015) A small molecule with anticancer and antimetastatic activities induces rapid mitochondrial-associated necrosis in breast cancer. J Pharmacol Exp Ther 353(2):392–404. doi:10.1124/jpet.114.220335, Epub 2015 Feb 26

Baughman JM, Perocchi F, Girgis HS, Plovanich M, Belcher-Timme CA, Sancak Y, Bao XR, Strittmatter L, Goldberger O, Bogorad RL et al (2011) Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter. Nature 476:341–345

Bernardi P (1999) Mitochondrial transport of cations: channels, exchangers, and permeability transition. Physiol Rev 79:1127–1155

Bernardi P, Angrilli A, Azzone GF (1990) A gated pathway for electrophoretic Na+ fluxes in rat liver mitochondria. Regulation by surface Mg2+. Eur J Biochem 188:91–97

Berrier C, Park KH, Abes S, Bibonne A, Betton JM, Ghazi A (2004) Cell-free synthesis of a functional ion channel in the absence of a membrane and in the presence of detergent. Biochemistry 43:12585–12591

Beutner G, Sharma VK, Giovannucci DR, Yule DI, Sheu SS (2001) Identification of a ryanodine receptor in rat heart mitochondria. J Biol Chem 276:21482–21488

Beutner G, Sharma VK, Lin L, Ryu SY, Dirksen RT, Sheu SS (2005) Type 1 ryanodine receptor in cardiac mitochondria: transducer of excitation-metabolism coupling. Biochim Biophys Acta 1717:1–10

Bondarenko AI, Jean-Quartier C, Parichatikanond W, Alam MR, Waldeck-Weiermair M, Malli R, Graier WF (2014) Mitochondrial Ca(2+) uniporter (MCU)-dependent and MCU-independent Ca(2+) channels coexist in the inner mitochondrial membrane. Pflugers Arch 466:1411–1420

Brierley GP, Davis MH, Cragoe EJ Jr, Jung DW (1989) Kinetic properties of the Na+/H+ antiport of heart mitochondria. Biochemistry 28:4347–4354

Cahalan MD, Chandy KG (2009) The functional network of ion channels in T lymphocytes. Immunol Rev 231:59–87

Cardoso AR, Queliconi BB, Kowaltowski AJ (2010) Mitochondrial ion transport pathways: role in metabolic diseases. Biochim Biophys Acta 1797:832–838

Carrithers MD, Chatterjee G, Carrithers LM, Offoha R, Iheagwara U, Rahner C, Graham M, Waxman SG (2009) Regulation of podosome formation in macrophages by a splice variant of the sodium channel SCN8A. J Biol Chem 284:8114–8126

Casey FP, Pihan E, Shields DC (2009) Discovery of small molecule inhibitors of protein-protein interactions using combined ligand and target score normalization. J Chem Inf Model 49:2708–2717

Chen Y, Wei X, Yan P, Han Y, Sun S, Wu K, Fan D (2009) Human mitochondrial Mrs2 protein promotes multidrug resistance in gastric cancer cells by regulating p27, cyclin D1 expression and cytochrome C release. Cancer Biol Ther 8:607–614

Cheng Y, Debska-Vielhaber G, Siemen D (2010) Interaction of mitochondrial potassium channels with the permeability transition pore. FEBS Lett 584:2005–2012

Cholo MC, Steel HC, Fourie PB, Germishuizen WA, Anderson R (2012) Clofazimine: current status and future prospects. J Antimicrob Chemother 67:290–298

Choudhary V, Kaddour-Djebbar I, Alaisami R, Kumar MV, Bollag WB (2014) Mitofusin 1 degradation is induced by a disruptor of mitochondrial calcium homeostasis, CGP37157: a role in apoptosis in prostate cancer cells. Int J Oncol 44:1767–1773

Cockrell RS (1973) Energy-linked ion translocation in submitochondrial particles. 3. Transport of monovalent cations by submitochondrial particles. J Biol Chem 248:6828–6833

Crompton M, Heid I (1978) The cycling of calcium, sodium, and protons across the inner membrane of cardiac mitochondria. Eur J Biochem 91:599–608

Csordas G, Golenar T, Seifert EL, Kamer KJ, Sancak Y, Perocchi F, Moffat C, Weaver D, de la Fuente Perez S, Bogorad R et al (2013) MICU1 controls both the threshold and cooperative activation of the mitochondrial Ca(2)(+) uniporter. Cell Metab 17:976–987

Davis FM, Parsonage MT, Cabot PJ, Parat MO, Thompson EW, Roberts-Thomson SJ, Monteith GR (2013) Assessment of gene expression of intracellular calcium channels, pumps and exchangers with epidermal growth factor-induced epithelial-mesenchymal transition in a breast cancer cell line. Cancer Cell Int 13:76

De Marchi U, Sassi N, Fioretti B, Catacuzzeno L, Cereghetti GM, Szabo I, Zoratti M (2009) Intermediate conductance Ca2+-activated potassium channel (KCa3.1) in the inner mitochondrial membrane of human colon cancer cells. Cell Calcium 45:509–516

De Stefani D, Raffaello A, Teardo E, Szabo I, Rizzuto R (2011) A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter. Nature 476:336–340

De Stefani D, Rizzuto R, Pozzan T (2016) Enjoy the trip: calcium in mitochondria back and forth. Annu Rev Biochem 85:161–192

Denton RM (2009) Regulation of mitochondrial dehydrogenases by calcium ions. Biochim Biophys Acta 1787:1309–1316

Denton RM, McCormack JG (1980) On the role of the calcium transport cycle in heart and other mammalian mitochondria. FEBS Lett 119:1–8

Denton RM, Randle PJ, Martin BR (1972) Stimulation by calcium ions of pyruvate dehydrogenase phosphate phosphatase. Biochem J 128:161–163

Denton RM, Richards DA, Chin JG (1978) Calcium ions and the regulation of NAD+-linked isocitrate dehydrogenase from the mitochondria of rat heart and other tissues. Biochem J 176:899–906

Denton RM, McCormack JG, Edgell NJ (1980) Role of calcium ions in the regulation of intramitochondrial metabolism. Effects of Na+, Mg2+ and ruthenium red on the Ca2+-stimulated oxidation of oxoglutarate and on pyruvate dehydrogenase activity in intact rat heart mitochondria. Biochem J 190:107–117

Dolga AM, Netter MF, Perocchi F, Doti N, Meissner L, Tobaben S, Grohm J, Zischka H, Plesnila N, Decher N et al (2013) Mitochondrial small conductance SK2 channels prevent glutamate-induced oxytosis and mitochondrial dysfunction. J Biol Chem 288:10792–10804

Douglas MG, Cockrell RS (1974) Mitochondrial cation-hydrogen ion exchange. Sodium selective transport by mitochondria and submitochondrial particles. J Biol Chem 249:5464–5471

Drago I, Pizzo P, Pozzan T (2011) After half a century mitochondrial calcium in- and efflux machineries reveal themselves. EMBO J 30:4119–4125

Duchen MR (1992) Ca(2+)-dependent changes in the mitochondrial energetics in single dissociated mouse sensory neurons. Biochem J 283(Pt 1):41–50

Duchen MR (2000) Mitochondria and calcium: from cell signalling to cell death. J Physiol 529 (Pt 1):57–68

Enyedi P, Czirjak G (2010) Molecular background of leak K+ currents: two-pore domain potassium channels. Physiol Rev 90:559–605

Ezure T, Nanatani K, Sato Y, Suzuki S, Aizawa K, Souma S, Ito M, Hohsaka T, von Heijine G, Utsumi T et al (2014) A cell-free translocation system using extracts of cultured insect cells to yield functional membrane proteins. PLoS One 9:e112874

Feng S, Li H, Tai Y, Huang J, Su Y, Abramowitz J, Zhu MX, Birnbaumer L, Wang Y (2013) Canonical transient receptor potential 3 channels regulate mitochondrial calcium uptake. Proc Natl Acad Sci U S A 110:11011–11016

Foskett JK, Philipson B (2015) The mitochondrial Ca(2+) uniporter complex. J Mol Cell Cardiol 78:3–8

Foster DB, Ho AS, Rucker J, Garlid AO, Chen L, Sidor A, Garlid KD, O'Rourke B (2012) Mitochondrial ROMK channel is a molecular component of mitoK(ATP). Circ Res 111:446–454

Garlid KD, Costa AD, Quinlan CL, Pierre SV, Dos Santos P (2009) Cardioprotective signaling to mitochondria. J Mol Cell Cardiol 46:858–866

Gorlach A, Bertram K, Hudecova S, Krizanova O (2015) Calcium and ROS: a mutual interplay. Redox Biol 6:260–271

Gotter AL, Santarelli VP, Doran SM, Tannenbaum PL, Kraus RL, Rosahl TW, Meziane H, Montial M, Reiss DR, Wessner K et al (2011) TASK-3 as a potential antidepressant target. Brain Res 1416:69–79

Grossinger EM, Weiss L, Zierler S, Rebhandl S, Krenn PW, Hinterseer E, Schmolzer J, Asslaber D, Hainzl S, Neureiter D et al (2014) Targeting proliferation of chronic lymphocytic leukemia (CLL) cells through KCa3.1 blockade. Leukemia 28:954–958

Hall DD, Wu Y, Domann FE, Spitz DR, Anderson ME (2014) Mitochondrial calcium uniporter activity is dispensable for MDA-MB-231 breast carcinoma cell survival. PLoS One 9:e96866

Holness MJ, Sugden MC (2003) Regulation of pyruvate dehydrogenase complex activity by reversible phosphorylation. Biochem Soc Trans 31:1143–1151

Huang G, Vercesi AE, Docampo R (2013) Essential regulation of cell bioenergetics in Trypanosoma brucei by the mitochondrial calcium uniporter. Nat Commun 4:2865

Hung V, Zou P, Rhee HW, Udeshi ND, Cracan V, Svinkina T, Carr SA, Mootha VK, Ting AY (2014) Proteomic mapping of the human mitochondrial intermembrane space in live cells via ratiometric APEX tagging. Mol Cell 55:332–341

Inoue I, Nagase H, Kishi K, Higuti T (1991) ATP-sensitive K+ channel in the mitochondrial inner membrane. Nature 352:244–247

Jafari A, Noursadeghi E, Khodagholi F, Saghiri R, Sauve R, Aliaghaei A, Eliassi A (2015) Brain mitochondrial ATP-insensitive large conductance Ca(+)(2)-activated K(+) channel properties are altered in a rat model of amyloid-beta neurotoxicity. Exp Neurol 269:8–16

Jang SH, Byun JK, Jeon WI, Choi SY, Park J, Lee BH, Yang JE, Park JB, O'Grady SM, Kim DY et al (2015) Nuclear localization and functional characteristics of voltage-gated potassium channel Kv1.3. J Biol Chem 290:12547–12557

Kapus A, Szaszi K, Kaldi K, Ligeti E, Fonyo A (1990) Ruthenium red inhibits mitochondrial Na+ and K+ uniports induced by magnesium removal. J Biol Chem 265:18063–18066

Khan MB, Sponder G, Sjoblom B, Svidova S, Schweyen RJ, Carugo O, Djinovic-Carugo K (2013) Structural and functional characterization of the N-terminal domain of the yeast Mg2+ channel Mrs2. Acta Crystallogr D Biol Crystallogr 69:1653–1664

Kirichok Y, Krapivinsky G, Clapham DE (2004) The mitochondrial calcium uniporter is a highly selective ion channel. Nature 427:360–364

Kolisek M, Zsurka G, Samaj J, Weghuber J, Schweyen RJ, Schweigel M (2003) Mrs2p is an essential component of the major electrophoretic Mg2+ influx system in mitochondria. EMBO J 22:1235–1244

Koni PA, Khanna R, Chang MC, Tang MD, Kaczmarek LK, Schlichter LC, Flavella RA (2003) Compensatory anion currents in Kv1.3 channel-deficient thymocytes. J Biol Chem 278:39443–39451

Kovacs-Bogdan E, Sancak Y, Kamer KJ, Plovanich M, Jambhekar A, Huber RJ, Myre MA, Blower MD, Mootha VK (2014) Reconstitution of the mitochondrial calcium uniporter in yeast. Proc Natl Acad Sci U S A 111:8985–8990

Koval AV, Vlasov P, Shichkova P, Khunderyakova S, Markov Y, Panchenko J, Volodina A, Kondrashov FA, Katanaev VL (2014) Anti-leprosy drug clofazimine inhibits growth of triple-

negative breast cancer cells via inhibition of canonical Wnt signaling. Biochem Pharmacol 87:571–578

Kowaltowski AJ, Seetharaman S, Paucek P, Garlid KD (2001) Bioenergetic consequences of opening the ATP-sensitive K(+) channel of heart mitochondria. Am J Physiol Heart Circ Physiol 280: H649–H657

Kubota T, Shindo Y, Tokuno K, Komatsu H, Ogawa H, Kudo S, Kitamura Y, Suzuki K, Oka K (2005) Mitochondria are intracellular magnesium stores: investigation by simultaneous fluorescent imagings in PC12 cells. Biochim Biophys Acta 1744:19–28

Kuum M, Veksler V, Liiv J, Ventura-Clapier R, Kaasik A (2012) Endoplasmic reticulum potassium-hydrogen exchanger and small conductance calcium-activated potassium channel activities are essential for ER calcium uptake in neurons and cardiomyocytes. J Cell Sci 125:625–633

Laskowski M, Augustynek B, Kulawiak B, Koprowski P, Bednarczyk P, Jarmuszkiewicz W, Szewczyk A (2016) What do we not know about mitochondrial potassium channels? Biochim Biophys Acta 1857:1247–1257

Leanza L, Henry B, Sassi N, Zoratti M, Chandy KG, Gulbins E, Szabo I (2012a) Inhibitors of mitochondrial Kv1.3 channels induce Bax/Bak-independent death of cancer cells. EMBO Mol Med 4:577–593

Leanza L, Zoratti M, Gulbins E, Szabo I (2012b) Induction of apoptosis in macrophages via Kv1.3 and Kv1.5 potassium channels. Curr Med Chem 19:5394–5404

Leanza L, Trentin L, Becker KA, Frezzato F, Zoratti M, Semenzato G, Gulbins E, Szabo I (2013) Clofazimine, Psora-4 and PAP-1, inhibitors of the potassium channel Kv1.3, as a new and selective therapeutic strategy in chronic lymphocytic leukemia. Leukemia 27:1782–1785

Leanza L, Zoratti M, Gulbins E, Szabo I (2014) Mitochondrial ion channels as oncological targets. Oncogene 33:5569–5581

Leanza L, Manago A, Zoratti M, Gulbins E, Szabo I (2015) Pharmacological targeting of ion channels for cancer therapy: in vivo evidences. Biochim Biophys Acta 1863(6 Pt B):1385–1397

Lefer DJ, Nichols CG, Coetzee WA (2009) Sulfonylurea receptor 1 subunits of ATP-sensitive potassium channels and myocardial ischemia/reperfusion injury. Trends Cardiovasc Med 19:61–67

Liu T, Takimoto E, Dimaano VL, DeMazumder D, Kettlewell S, Smith G, Sidor A, Abraham TP, O'Rourke B (2014) Inhibiting mitochondrial Na+/Ca2+ exchange prevents sudden death in a Guinea pig model of heart failure. Circ Res 115:44–54

Logan CV, Szabadkai G, Sharpe JA, Parry DA, Torelli S, Childs AM, Kriek M, Phadke R, Johnson CA, Roberts NY et al (2014) Loss-of-function mutations in MICU1 cause a brain and muscle disorder linked to primary alterations in mitochondrial calcium signaling. Nat Genet 46:188–193

Lu M, Wang T, Yan Q, Yang X, Dong K, Knepper MA, Wang W, Giebisch G, Shull GE, Hebert SC (2002) Absence of small conductance K+ channel (SK) activity in apical membranes of thick ascending limb and cortical collecting duct in ROMK (Bartter's) knockout mice. J Biol Chem 277:37881–37887

Lukasiak A, Skup A, Chlopicki S, Lomnicka M, Kaczara P, Proniewski B, Szewczyk A, Wrzosek A (2016) SERCA, complex I of the respiratory chain and ATP-synthase inhibition are involved in pleiotropic effects of NS1619 on endothelial cells. Eur J Pharmacol 786:137–147

Mallilankaraman K, Doonan P, Cardenas C, Chandramoorthy HC, Muller M, Miller R, Hoffman NE, Gandhirajan RK, Molgo J, Birnbaum MJ et al (2012) MICU1 is an essential gatekeeper for MCU-mediated mitochondrial Ca(2+) uptake that regulates cell survival. Cell 151:630–644

Mammucari C, Gherardi G, Zamparo I, Raffaello A, Boncompagni S, Chemello F, Cagnin S, Braga A, Zanin S, Pallafacchina G et al (2015) The mitochondrial calcium uniporter controls skeletal muscle trophism in vivo. Cell Rep 10:1269–1279

McCormack JG, Denton RM (1979) The effects of calcium ions and adenine nucleotides on the activity of pig heart 2-oxoglutarate dehydrogenase complex. Biochem J 180:533–544

McCormack JG, Halestrap AP, Denton RM (1990) Role of calcium ions in regulation of mammalian intramitochondrial metabolism. Physiol Rev 70:391–425

McFerrin MB, Turner KL, Cuddapah VA, Sontheimer H (2012) Differential role of IK and BK potassium channels as mediators of intrinsic and extrinsic apoptotic cell death. Am J Physiol Cell Physiol 303:C1070–C1078

McLain AL, Cormier PJ, Kinter M, Szweda LI (2013) Glutathionylation of alpha-ketoglutarate dehydrogenase: the chemical nature and relative susceptibility of the cofactor lipoic acid to modification. Free Radic Biol Med 61:161–169

Mitchell P, Moyle J (1969) Translocation of some anions cations and acids in rat liver mitochondria. Eur J Biochem 9:149–155

Moore CL (1971) Specific inhibition of mitochondrial Ca++ transport by ruthenium red. Biochem Biophys Res Commun 42:298–305

Murphy E, Eisner DA (2009) Regulation of intracellular and mitochondrial sodium in health and disease. Circ Res 104:292–303

Nagy D, Gonczi M, Dienes B, Szoor A, Fodor J, Nagy Z, Toth A, Fodor T, Bai P, Szucs G et al (2014) Silencing the KCNK9 potassium channel (TASK-3) gene disturbs mitochondrial function, causes mitochondrial depolarization, and induces apoptosis of human melanoma cells. Arch Dermatol Res 306:885–902

Nakashima RA, Garlid KD (1982) Quinine inhibition of Na+ and K+ transport provides evidence for two cation/H+ exchangers in rat liver mitochondria. J Biol Chem 257:9252–9254

Nita II, Caspi Y, Gudes S, Fishman D, Lev S, Hersfinkel M, Sekler I, Binshtok AM (2016) Privileged crosstalk between TRPV1 channels and mitochondrial calcium shuttling machinery controls nociception. Biochim Biophys Acta 1863:2868–2880

Noriega-Navarro R, Lopez-Charcas O, Hernandez-Enriquez B, Reyes-Gutierrez PE, Martinez R, Landa A, Moran J, Gomora JC, Garcia-Valdes J (2014) Novel TASK channels inhibitors derived from dihydropyrrolo[2,1-a]isoquinoline. Neuropharmacology 79:28–36

Nulton-Persson AC, Starke DW, Mieyal JJ, Szweda LI (2003) Reversible inactivation of alpha-ketoglutarate dehydrogenase in response to alterations in the mitochondrial glutathione status. Biochemistry 42:4235–4242

O-Uchi J, Ryu SY, Jhun BS, Hurst S, Sheu SS (2014) Mitochondrial ion channels/transporters as sensors and regulators of cellular redox signaling. Antioxid Redox Signal 21:987–1006

Oxenoid K, Dong Y, Cao C, Cui T, Sancak Y, Markhard AL, Grabarek Z, Kong L, Liu Z, Ouyang B et al (2016) Architecture of the mitochondrial calcium uniporter. Nature 533:269–273

Pagliarini DJ, Calvo SE, Chang B, Sheth SA, Vafai SB, Ong S-E, Walford GA, Sugiana C, Boneh A, Chen WK et al (2008) A mitochondrial protein compendium elucidates complex I disease biology. Cell 134:112–123

Pan X, Liu J, Nguyen T, Liu C, Sun J, Teng Y, Fergusson MM, Rovira II, Allen M, Springer DA et al (2013) The physiological role of mitochondrial calcium revealed by mice lacking the mitochondrial calcium uniporter. Nat Cell Biol 15:1464–1472

Patil SP (2013) FOLICation: engineering approved drugs as potential p53-MDM2 interaction inhibitors for cancer therapy. Med Hypotheses 81:1104–1107

Patron M, Checchetto V, Raffaello A, Teardo E, Vecellio Reane D, Mantoan M, Granatiero V, Szabo I, De Stefani D, Rizzuto R (2014) MICU1 and MICU2 finely tune the mitochondrial Ca2+ uniporter by exerting opposite effects on MCU activity. Mol Cell 53:726–737

Pendin D, Greotti E, Pozzan T (2014) The elusive importance of being a mitochondrial Ca(2+) uniporter. Cell Calcium 55:139–145

Perocchi F, Gohil VM, Girgis HS, Bao XR, McCombs JE, Palmer AE, Mootha VK (2010) MICU1 encodes a mitochondrial EF hand protein required for Ca(2+) uptake. Nature 467:291–296

Peruzzo R, Biasutto L, Szabo I, Leanza L (2016) Impact of intracellular ion channels on cancer development and progression. Eur Biophys J 45(7):685–707

Petrungaro C, Zimmermann KM, Kuttner V, Fischer M, Dengjel J, Bogeski I, Riemer J (2015) The Ca(2+)-dependent release of the Mia40-induced MICU1-MICU2 dimer from MCU regulates mitochondrial Ca(2+) uptake. Cell Metab 22:721–733

Piskacek M, Zotova L, Zsurka G, Schweyen RJ (2009) Conditional knockdown of hMRS2 results in loss of mitochondrial Mg(2+) uptake and cell death. J Cell Mol Med 13:693–700

Plovanich M, Bogorad RL, Sancak Y, Kamer KJ, Strittmatter L, Li AA, Girgis HS, Kuchimanchi S, De Groot J, Speciner L et al (2013) MICU2, a paralog of MICU1, resides within the mitochondrial uniporter complex to regulate calcium handling. PLoS One 8:e55785

Pocsai K, Kosztka L, Bakondi G, Gonczi M, Fodor J, Dienes B, Szentesi P, Kovacs I, Feniger-Barish R, Kopf E et al (2006) Melanoma cells exhibit strong intracellular TASK-3-specific immunopositivity in both tissue sections and cell culture. Cell Mol Life Sci 63:2364–2376

Pralong WF, Hunyady L, Varnai P, Wollheim CB, Spat A (1992) Pyridine nucleotide redox state parallels production of aldosterone in potassium-stimulated adrenal glomerulosa cells. Proc Natl Acad Sci U S A 89:132–136

Prudent J, Popgeorgiev N, Bonneau B, Thibaut J, Gadet R, Lopez J, Gonzalo P, Rimokh R, Manon S, Houart C et al (2013) Bcl-wav and the mitochondrial calcium uniporter drive gastrula morphogenesis in zebrafish. Nat Commun 4:2330

Quast SA, Berger A, Buttstadt N, Friebel K, Schonherr R, Eberle J (2012) General sensitization of melanoma cells for TRAIL-induced apoptosis by the potassium channel inhibitor TRAM-34 depends on release of SMAC. PLoS One 7:e39290

Raffaello A, De Stefani D, Sabbadin D, Teardo E, Merli G, Picard A, Checchetto V, Moro S, Szabo I, Rizzuto R (2013) The mitochondrial calcium uniporter is a multimer that can include a dominant-negative pore-forming subunit. EMBO J 32:2362–2376

Reed KC, Bygrave FL (1974) The inhibition of mitochondrial calcium transport by lanthanides and ruthenium red. Biochem J 140:143–155

Rizzuto R, De Stefani D, Raffaello A, Mammucari C (2012) Mitochondria as sensors and regulators of calcium signalling. Nat Rev Mol Cell Biol 13:566–578

Sancak Y, Markhard AL, Kitami T, Kovacs-Bogdan E, Kamer KJ, Udeshi ND, Carr SA, Chaudhuri D, Clapham DE, Li AA et al (2013) EMRE is an essential component of the mitochondrial calcium uniporter complex. Science 342:1379–1382

Sassi N, De Marchi U, Fioretti B, Biasutto L, Gulbins E, Franciolini F, Szabo I, Zoratti M (2010) An investigation of the occurrence and properties of the mitochondrial intermediate-conductance Ca2+-activated K+ channel mtKCa3.1. Biochim Biophys Acta 1797:1260–1267

Selwyn MJ, Dawson AP, Dunnett SJ (1970) Calcium transport in mitochondria. FEBS Lett 10:1–5

Singh H, Stefani E, Toro L (2012) Intracellular BK(Ca) (iBK(Ca)) channels. J Physiol 590:5937–5947

Singh H, Lu R, Bopassa JC, Meredith AL, Stefani E, Toro L (2013) MitoBK(Ca) is encoded by the Kcnma1 gene, and a splicing sequence defines its mitochondrial location. Proc Natl Acad Sci U S A 110:10836–10841

Soltysinska E, Bentzen BH, Barthmes M, Hattel H, Thrush AB, Harper ME, Qvortrup K, Larsen FJ, Schiffer TA, Losa-Reyna J et al (2014) KCNMA1 encoded cardiac BK channels afford protection against ischemia-reperfusion injury. PLoS One 9:e103402

Sparagna GC, Gunter KK, Sheu SS, Gunter TE (1995) Mitochondrial calcium uptake from physiological-type pulses of calcium. A description of the rapid uptake mode. J Biol Chem 270:27510–27515

Szabo I, Zoratti M (2014) Mitochondrial channels: ion fluxes and more. Physiol Rev 94:519–608

Szabo I, Bock J, Jekle A, Soddemann M, Adams C, Lang F, Zoratti M, Gulbins E (2005) A novel potassium channel in lymphocyte mitochondria. J Biol Chem 280:12790–12798

Szabo I, Bock J, Grassme H, Soddemann M, Wilker B, Lang F, Zoratti M, Gulbins E (2008) Mitochondrial potassium channel Kv1.3 mediates Bax-induced apoptosis in lymphocytes. Proc Natl Acad Sci U S A 105:14861–14866

Szabo I, Soddemann M, Leanza L, Zoratti M, Gulbins E (2011) Single-point mutations of a lysine residue change function of Bax and Bcl-xL expressed in Bax- and Bak-less mouse embryonic fibroblasts: novel insights into the molecular mechanisms of Bax-induced apoptosis. Cell Death Differ 18:427–438

Szabo I, Leanza L, Gulbins E, Zoratti M (2012) Physiology of potassium channels in the inner membrane of mitochondria. Pflugers Arch 463:231–246

Szewczyk A, Pikula S, Wojcik G, Nalecz MJ (1996) Glibenclamide inhibits mitochondrial K+ and Na+ uniports induced by magnesium depletion. Int J Biochem Cell Biol 28:863–871

Szewczyk A, Skalska J, Glab M, Kulawiak B, Malinska D, Koszela-Piotrowska I, Kunz WS (2006) Mitochondrial potassium channels: from pharmacology to function. Biochim Biophys Acta 1757:715–720

Szewczyk A, Jarmuszkiewicz W, Kunz WS (2009) Mitochondrial potassium channels. IUBMB Life 61:134–143

Szewczyk A, Kajma A, Malinska D, Wrzosek A, Bednarczyk P, Zablocka B, Dolowy K (2010) Pharmacology of mitochondrial potassium channels: dark side of the field. FEBS Lett 584:2063–2069

Tang S, Wang X, Shen Q, Yang X, Yu C, Cai C, Cai G, Meng X, Zou F (2015) Mitochondrial Ca(2)(+) uniporter is critical for store-operated Ca(2)(+) entry-dependent breast cancer cell migration. Biochem Biophys Res Commun 458:186–193

Testai L, Barrese V, Soldovieri MV, Ambrosino P, Martelli A, Vinciguerra I, Miceli F, Greenwood I, Curtis MJ, Breschi MC et al (2015a) Expression and function of Kv7.4 channels in Rat cardiac mitochondria: possible targets for cardioprotection. Cardiovasc Res 110(1):40–50

Testai L, Rapposelli S, Martelli A, Breschi MC, Calderone V (2015b) Mitochondrial potassium channels as pharmacological target for cardioprotective drugs. Med Res Rev 35:520–553

Toro L, Li M, Zhang Z, Singh H, Wu Y, Stefani E (2014) MaxiK channel and cell signalling. Pflugers Arch 466:875–886

Tosatto A, Sommaggio R, Kummerow C, Bentham RB, Blacker TS, Berecz T, Duchen MR, Rosato A, Bogeski I, Szabadkai G et al (2016) The mitochondrial calcium uniporter regulates breast cancer progression via HIF-1alpha. EMBO Mol Med 8:569–585

Vais H, Mallilankaraman K, Mak DO, Hoff H, Payne R, Tanis JE, Foskett JK (2016) EMRE is a matrix Ca(2+) sensor that governs gatekeeping of the mitochondrial Ca(2+) uniporter. Cell Rep 14:403–410

Van Rensburg CE, Anderson R, Myer MS, Joone GK, O'Sullivan JF (1994) The riminophenazine agents clofazimine and B669 reverse acquired multidrug resistance in a human lung cancer cell line. Cancer Lett 85:59–63

Vasington FD, Gazzotti P, Tiozzo R, Carafoli E (1972) The effect of ruthenium red on Ca2+ transport and respiration in rat liver mitochondria. Biochim Biophys Acta 256:43–54

Vecellio Reane D, Vallese F, Checchetto V, Acquasaliente L, Butera G, De Filippis V, Szabò I, Zanotti G, Rizzuto R, Raffaello A (2016) A MICU1 splice variant confers high sensitivity to the mitochondrial Ca2+ uptake machinery of skeletal muscle. Mol Cell 64(4):760–773

von Charpuis C, Meckel T, Moroni A, Thiel G (2015) The sorting of a small potassium channel in mammalian cells can be shifted between mitochondria and plasma membrane. Cell Calcium 58:114–121

Wagner S, De Bortoli S, Schwarzlander M, Szabo I (2016) Regulation of mitochondrial calcium in plants versus animals. J Exp Bot 67(13):3809–3829

Waldeck-Weiermair M, Malli R, Parichatikanond W, Gottschalk B, Madreiter-Sokolowski CT, Klec C, Rost R, Graier WF (2015) Rearrangement of MICU1 multimers for activation of MCU is solely controlled by cytosolic Ca(2.). Sci Rep 5:15602

Wang L, Yang X, Li S, Wang Z, Liu Y, Feng J, Zhu Y, Shen Y (2014) Structural and mechanistic insights into MICU1 regulation of mitochondrial calcium uptake. EMBO J 33:594–604

Wang L, Yang X, Shen Y (2015) Molecular mechanism of mitochondrial calcium uptake. Cell Mol Life Sci 72:1489–1498

Wolf FI, Trapani V (2009) Multidrug resistance phenotypes and MRS2 mitochondrial magnesium channel: two players from one stemness? Cancer Biol Ther 8:615–617

Wrzosek A, Tomaskova Z, Ondrias K, Lukasiak A, Szewczyk A (2012) The potassium channel opener CGS7184 activates Ca(2)(+) release from the endoplasmic reticulum. Eur J Pharmacol 690:60–67

Wu Y, Rasmussen TP, Koval OM, Joiner ML, Hall DD, Chen B, Luczak ED, Wang Q, Rokita AG, Wehrens XH et al (2015) The mitochondrial uniporter controls fight or flight heart rate increases. Nat Commun 6:6081

Wulff H, Castle NA (2010) Therapeutic potential of KCa3.1 blockers: recent advances and promising trends. Expert Rev Clin Pharmacol 3:385–396

Yan LJ, Sumien N, Thangthaeng N, Forster MJ (2013) Reversible inactivation of dihydrolipoamide dehydrogenase by mitochondrial hydrogen peroxide. Free Radic Res 47:123–133

Yoon MJ, Kim EH, Kwon TK, Park SA, Choi KS (2012) Simultaneous mitochondrial Ca(2+) overload and proteasomal inhibition are responsible for the induction of paraptosis in malignant breast cancer cells. Cancer Lett 324:197–209

Zaccagnino A, Managò A, Leanza L, Gontarewitz A, Linder B, Azzolini M, Biasutto L, Zoratti M, Peruzzo R, Legler K, Trauzold A, Kalthoff H, Szabo I (2016) Tumor-reducing effect of the clinically used drug clofazimine in a SCID mouse model of pancreatic ductal adenocarcinoma. Oncotarget. doi:10.18632/oncotarget.11299

Zhu J, Yan J, Thornhill WB (2014) The Kv1.3 potassium channel is localized to the cis-Golgi and Kv1.6 is localized to the endoplasmic reticulum in rat astrocytes. FEBS J 281:3433–3445

Zorov DB, Juhaszova M, Sollott SJ (2014) Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. Physiol Rev 94:909–950

Zsurka G, Gregan J, Schweyen RJ (2001) The human mitochondrial Mrs2 protein functionally substitutes for its yeast homologue, a candidate magnesium transporter. Genomics 72:158–168

Anion Channels of Mitochondria

Devasena Ponnalagu and Harpreet Singh

Contents

Abstract

Mitochondria are the "power house" of a cell continuously generating ATP to ensure its proper functioning. The constant production of ATP via oxidative phosphorylation demands a large electrochemical force that drives protons across the highly selective and low-permeable mitochondrial inner membrane. Besides the conventional role of generating ATP, mitochondria also play an active role in calcium signaling, generation of reactive oxygen species (ROS), stress responses, and regulation of cell-death pathways. Deficiencies in these functions result in several pathological disorders like aging, cancer, diabetes, neurodegenerative and cardiovascular diseases. A plethora of ion channels and

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H. Singh, S.-S. Sheu (eds.), *Pharmacology of Mitochondria*,
Handbook of Experimental Pharmacology 240, DOI 10.1007/164_2016_39

transporters are present in the mitochondrial inner and outer membranes which work in concert to preserve the ionic equilibrium of a cell for the maintenance of cell integrity, in physiological as well as pathophysiological conditions. For, e.g., mitochondrial cation channels K_{ATP} and BK_{Ca} play a significant role in cardioprotection from ischemia–reperfusion injury. In addition to the cation channels, mitochondrial anion channels are equally essential, as they aid in maintaining electro-neutrality by regulating the cell volume and pH. This chapter focusses on the information on molecular identity, structure, function, and physiological relevance of mitochondrial chloride channels such as voltage dependent anion channels (VDACs), uncharacterized mitochondrial inner membrane anion channels (IMACs), chloride intracellular channels (CLIC) and the aspects of forthcoming chloride channels.

Keywords
Anion channels • Chloride intracellular channels (CLICs) • Inner membrane anion channel (IMAC) • Mitochondria • Mitochondrial permeability transition pore (mPTP) • Uncoupling protein (UCP) • Voltage dependent anion channel (VDAC)

1 Introduction

Typical ionic concentration and composition of the cytosol dramatically differ from the extracellular environments. All the cells from prokaryotes to eukaryotes maintain a cytosolic pH of ~7.2, with concentration of potassium ions (K^+) many fold higher than sodium (Na^+) and calcium (Ca^{2+}) ions (Bose et al. 2015; Prindle et al. 2015). In addition to cations, several organic and inorganic anions such as halides exist in cytosol to counter cations and also play an active functional role in cells. Intracellular ions exist in either free or remain bound to cytoplasmic proteins; however, they are classically divided by intracellular membranes, and are enclosed in intracellular organelles. The distribution of intracellular ions in different cellular compartments as well as cytosol is chiefly governed by Gibbs–Donnan effect (Sollner 1955), and is responsible for maintaining physiological functions of the cell.

Cell membranes possess a wide array of ion transporters and channel proteins in order to maintain physiological ionic concentrations (Jentsch et al. 2004). The ionic gradients and membrane potentials across cellular membrane (plasma and intracellular organelle) are key drivers of several physiological processes such as neurotransmission, cardiac conduction, and muscle contraction and relaxation (Jentsch et al. 2004). Intracellular ion transport is important for cell cycle-regulation, energy production, cellular signaling, and apoptosis (Leanza et al. 2013). Ion transport across the plasma membrane has been discussed in several reviews (Feske et al. 2015; Bates 2015; Singh et al. 2012a; Toro et al. 2014), and therefore in this chapter, emphasis is on the ion channels across mitochondrial membranes.

Several models of energy production in the mitochondrion indicate that the presence of an ion channel on mitochondrial membrane would lower efficiency and effectiveness of the process (O'Rourke 2007). In addition, theoretical opening of any ion channels, for example, a mitoBK$_{Ca}$ will readily depolarize mitochondrial membrane within few nanoseconds, hence supporting the earlier hypothesis that mitochondria should not possess ion channels (Singh et al. 2012a; O'Rourke 2007). At the same time, it is well-recognized that membrane potential of inner mitochondrial membrane (IMM) is ~ −180 mV (mV) (Colombini 2012; Colombini and Mannella 1818; O'Rourke et al. 2005). However, when compared to the electric potential of plasma membrane (~ − 70 mV), mitochondrial membrane seems to be a better capacitor in storing charge. To further signify the effectiveness of mitochondrial membrane potential (ψ_{mito}), we can precisely calculate the charge stored by these cellular capacitors. It is known that the IMM is ~7.0 nm thick (Perkins et al. 1997) thus the voltage gradient across the IMM is 0.180 V per 7.0×10^{-7} cm. This is equivalent to 257,142.90 V per cm which is significantly higher than high-voltage transmission electricity supply lines employing voltage gradient of about 200,000 V per km (and higher to the plasma membrane voltage gradient of 200,000 V per cm as well) (Lodish et al. 2000). Majority of the membrane potential across the mitochondrial inner membrane is generated by proton transport which is directly coupled with ATP production.

Increasing evidence indicate that both outer mitochondrial membrane (OMM) and IMM possess ion channels (Szabo and Zoratti 2014). As stated earlier, until 1980s, scientific community was highly skeptical and divided over existence of typical ion channels in mitochondria (Colombini and Mannella 1818; O'Rourke 2000; Colombini 1979). However, isolation of voltage dependent anion channel (VDAC) from paramecium, and reconstitution in lipid bilayers in 1976 set the ground for mitochondrial ion channels (Schein et al. 1976). This was immediately followed by characterization of permeability transition pore (PTP) (Hunter et al. 1976), and in the last 40 years several ion channels of OMM and IMM were established (Szabo and Zoratti 2014). More recently, mitochondrial ion channels are shown to be the key modulators involved in protection from ischemia–reperfusion (IR) injury, apoptosis, and several other pathophysiological disorders (O'Rourke 2007; O'Rourke et al. 2005; Szabo and Zoratti 2014; O'Rourke 2000). Even though anion channels (VDAC) were the first one to be described in mitochondria (Colombini 1979), majority of the physiological functions are attributed to the cation channels (Szabo and Zoratti 2014). This is chiefly due to non-availability of specific pharmacological agents for anion channels and lack of molecular identity of mitochondrial anion channels residing in IMMs.

Much like plasma membrane anion channels, mitochondrial anion channels are proteinaceous pores which allow passive diffusion of negatively charged ions down their electrochemical gradient. Anion channels may conduct other anions (F^-, Br^-, I^-, or NO^-_3) better than Cl^- but they are often referred as Cl^- channels, as Cl^- is the most abundant anion, predominantly permeating under physiological conditions (Ashley 2003). Apart from VDAC which resides in the OMM (Colombini and Mannella 1818; Colombini 1979), chloride intracellular channel 4 (CLIC4), and

CLIC5 (Ponnalagu et al. 2016a), molecular identity of other inner mitochondrial anion channels is not known. Also classification of anion channels is still ambiguous (Roelfsema et al. 2012). Previous attempts to classify them on the basis of their localization (plasma membrane vs. intracellular membranes), single-channel conductance, and/or regulatory mechanism could not comprehend the diversity of anion channels (Verkman and Galietta 2009). We believe that mitochondrial anion channels can be systematically classified on the basis of their localization, outer membrane anion channels and inner membrane anion channels (IMACs), as well as on the basis of their selectivity. As anion channels are known to be permeable for other ions, we propose to classify them as anion-selective anion channels [ASACs, non-selective for anions], cation-selective anion channels (CSACs, prefer cation over anions), and non-selective anion channels [NSACs, not selective for cations or anions]. These channels will be discussed in detail in following sections.

In this chapter, we have presented an overview of mitochondrial anion channels, investigated their mitochondrial localization as well as distribution, and also show how some of the known anion channel inhibitors affect mitochondrial reactive oxygen species (ROS) generation.

2 Anion Channels of Outer Mitochondrial Membrane

2.1 Voltage Dependent Anion Channels

VDAC, an intrinsic membrane channel, is the first channel to be reconstituted and studied at the single-channel level (Colombini and Mannella 1818; Schein et al. 1976). It is a highly conserved protein, homologous to bacterial porins and is localized in the OMM (Colombini 2012; Mertins et al. 2014; Neumann et al. 2010) as also observed in the mitochondria of rat cardiac myogenic cell line, H9C2 (Fig. 1). Geographical distribution of VDAC1 by stimulated emission depletion (STED) nanoscopy also indicates their unique clusters at nano-resolution in H9C2 cells (Fig. 1) as observed earlier in U2OS cells transfected with VDAC (Neumann et al. 2010). It is the principle mode of transport of ions and metabolites across the OMM, and also a probable contact point between the inner and outer membrane (Colombini 1979). VDAC had a serendipitous discovery; it occurred as a result of failed attempts in characterizing voltage-gated calcium sensitive channels from *Paramecium aurelia (Colombini and Mannella 1818)*. It is widely distributed in many species across the taxon, yeast have two (POR1 and POR2), and vertebrates have three isoforms (VDAC1, VDAC2, and VDAC3) (Szabo and Zoratti 2014). Humans, like all other vertebrates canonically encode three different isoforms. It is a well-characterized mitochondrial anion channel in terms of structure and function. Amongst the various isoforms, VDAC1 was the first one to be discovered. VDAC1 and VDAC2 have very strong pore-forming characteristics, while VDAC3 forms smaller conductance channels (Checchetto et al. 2014) and consequently modulates the physiological functions of other proteins.

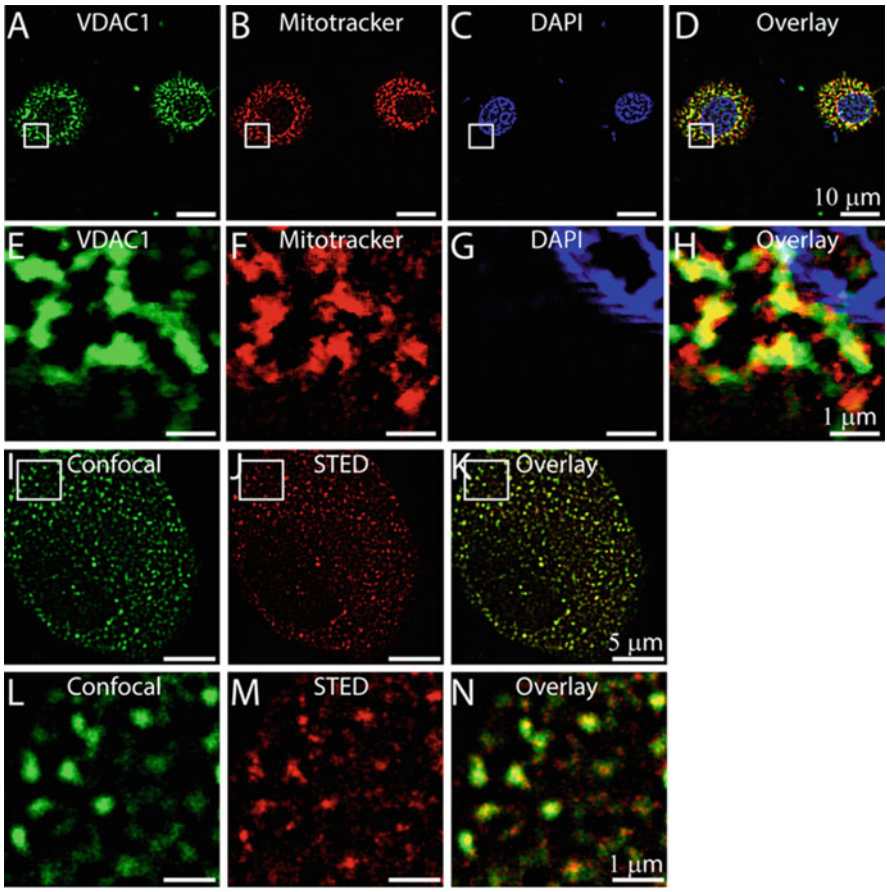

Fig. 1 Localization of VDAC1 in H9C2 cells. H9C2 cells loaded with mitotracker (**b**) were labeled with anti-VDAC1 antibody (**a**), and stained with a nuclear marker, DAPI (**c**). (**d**) is merge image of **a, b**, and **c** showing colocalization of VDAC1 to the mitochondria of H9C2 cells. **e**, **f**, **g**, and **h** are enlarged images of the squared regions **a**, **b**, **c**, and **d**, respectively. STED microscopy of H9C2 cells showing expression of VDAC1 (**j**, **k**). Confocal image of H9C2 cells labeled with anti-VDAC1 antibody (**i**) Corresponding STED image (**j**), overlay of **i** and **j** highlights increased resolution of VDAC1 localization to mitochondria with STED microscopy (**k**). **l**, **m**, and **n** are enlarged images of the squared region in **i**, **j**, and **k**, respectively

2.1.1 Structural Insights

VDAC, a small, >30 kDa protein consisting of 280 amino acid residues, shows high sequence homology (>80 %) within the same species. Using X-ray crystallography and nuclear magnetic resonance (NMR), three independent studies (Hiller et al. 2008; Ujwal et al. 2008; Bayrhuber et al. 2008) have revealed that VDAC forms β-barrel structures similar to bacterial porins, comprising of 19 unique β-strands connected with 18 loop-like connections, and a short N-terminal α-helix between residues 7 and 17. All the β-barrel strands are oriented in the anti-parallel

fashion except strands 1 and 19 which are parallel to each other and close the β-barrel structure (Ujwal et al. 2008). The height and width of the β-barrel were reported to be 35 Å and 40 Å, respectively (Ujwal et al. 2008), and the path for solute passage measures ~15 Å by 1 Å. The N-terminal β-barrel strands (1–4) contribute to conformational stability of VDAC whereas Glu73 is a key residue determining the structural stability of the protein. This residue is conserved in all the VDAC isoforms present in humans. The N-terminal helix folds up inside the β-barrel wall to reach the midpoint of the hydrophobic portion of the membrane. The positively charged residues of the helix face the interior of the β-barrel, while the negatively charged residues interact with the most conserved residues in the β-barrel wall of VDAC proteins (Bayrhuber et al. 2008). As a result the N-terminus is a site for many molecular interactions. The preceding N-terminal region is lightly attached to the β-barrel wall and orients towards the pore outlet (Bayrhuber et al. 2008). Each loop contributes a specific role in channel-gating, ion-selectivity, pore size, and structural stability of VDAC. The long loops fold into the pore lumen and determine the ion-selectivity and the gating of the channel. The short loops provide structural support by linking with the adjacent β-strands. The stability of β-strands is also important for the voltage-gating, and protein–protein interactions of VDAC. Similar to other porins, the C-terminal region extends to the intermembrane space (IMS) (Bayrhuber et al. 2008; Schulz 2002). Various studies indicate the ability of VDAC to form oligomers (Malia and Wagner 2007). Using crystallographic symmetry operators, a model for dimeric form of VDAC was constructed which indicated that the dimeric interface residues reside in β-strands, β1 (Ile-27, Leu-29), β2 (Glu-50, Thr-51), β18 (Leu-257, Leu-259), and β19 (Leu-277) (Bayrhuber et al. 2008).

2.1.2 Electrophysiological Properties

The channel properties of VDAC have been studied mainly by using planar bilayer systems. VDAC forms a large voltage dependent pore with maximum conductance of 4–5 nS in symmetrical 1 M KCl (Szabo and Zoratti 2014; Colombini 2004; Bera and Ghosh 2001). At lower holding potentials of 20–30 mV, VDAC exists in fully open state and exhibits weak anion-selectivity, whereas at higher holding potentials, it is cation selective, and the conductance observed is approximately half of the original conductance shown by the "closed" state of the channel (Szabo and Zoratti 2014). Exposed charge residues of the pore are the key determinants for anion selectivity. Amino-terminal sequence determines the voltage-induced gating of VDAC (Mannella 1997; Shoshan-Barmatz et al. 1797). Although channel activity is observed in bilayers, a consistent conductance is not yet recorded through patch clamping studies (Kinnally and Antonsson 2007). VDAC possesses a binding site for Ca^{2+} (Gincel et al. 2001) and also transports calcium (Ca^{2+}), ATP, and superoxides. The open state of the channel poorly conducts Ca^{2+} ($P_{Cl^-}/P_{Ca^{2+}}$ ~25), while partially closed state still prefers Cl^- over Ca^{2+}, with a $P_{Cl^-}/P_{Ca^{2+}}$ as 1–4.5 (Szabo and Zoratti 2014; Tan and Colombini 1768). Mitochondrial VDAC-like conductance was also observed in excised patches of plasma membrane

(Bahamonde et al. 2003) of neuroblastoma cells. Thus, suggesting VDAC as a molecular correlate of the plasma membrane maxi Cl^- channels (Bahamonde et al. 2003). In another study, electrophysiological and immunocytochemical evidences showed the activation of VDAC in plasma membrane of neurons during apoptosis (Elinder et al. 2005). These observations are supported by the identification of a VDAC isoform (pl-VDAC) containing a leader sequence for its trafficking to the plasma membrane (Buettner et al. 2000; De Pinto et al. 2010) and also the presence of VDAC in caveolae (Bahamonde et al. 2003).

2.1.3 Regulation of VDAC

VDAC is regulated by many cellular and extracellular factors. It gets phosphorylated by Ser-Thr kinases GSK3β (Das et al. 2008; Sheldon et al. 2011), tyrosine kinase (Salvi et al. 2005), protein kinase A (PKA) (Banerjee and Ghosh 2006), and nima-related kinases (NEK) (Chen et al. 2009; Chen et al. 2010). Phosphorylation regulates the apoptotic activity of VDAC via modulating its interaction with cytoskeletal components (Kerner et al. 1818). However, phosphorylation does not affect the current magnitude and the open probability in the positive clamping potentials, but lowers both in the negative clamping potentials (Bera and Ghosh 2001). Further, it has been shown that phosphorylation of VDAC by JNK3 leads to channel closure and thereby causes cell death (Gupta and Ghosh 2015). VDAC has been shown to interact with multiple factors ranging from pyridine dinucleotides (Lee et al. 1996; Zizi et al. 1994), lipids including cholesterol (Betaneli et al. 2012; Rostovtseva and Bezrukov 2008), Bcl-xL (Malia and Wagner 2007; Arbel et al. 2012), creatine kinases (Beutner et al. 1996; Brdiczka et al. 1994), Ca^{2+}, etc. Interaction of VDAC with tubulin (Sheldon et al. 2011) results in its increased sensitivity to voltages causing a channel closure at potentials as low as 10 mV. It also has been known to be inhibited by gelsolin, a Ca^{2+}-dependent actin-binding regulatory protein, thereby exerting anti-apoptotic effects (Kusano et al. 2000).

2.1.4 Functions

VDAC has been extensively studied with respect to its role in apoptosis. It is known to interact with Bax and Bak to form a large pore and stimulate efflux of cytochrome c (Shimizu et al. 2000; Shimizu et al. 1999; Banerjee and Ghosh 2004) which is a hallmark of apoptosis. Although, VDAC2 is shown to be pro-apoptotic, facilitating sensitivity to t-Bid (Yamagata et al. 2009), its deletion in lymphocytes was fatal (Cheng et al. 2003) but was rescued by deletion of Bak, reasserting VDAC2's role in apoptosis. There are contradictory reports arguing VDACs (VDAC1 and VDAC3) role in apoptosis; mouse embryonic fibroblasts (MEFs) lacking either one of these isoforms showed no response to their apoptotic response to Bax overexpression and also showed no role of VDAC in cytochrome c release (Baines et al. 2007). Bcl-2 family members like BclxL inhibit VDAC's activity and exhibit their anti-apoptotic activity (Arbel et al. 2012) via interaction with their N-terminal segment (Malia and Wagner 2007; Abu-Hamad et al. 2009). Overexpression of plant VDACs in jurkat cells (T cell line) induced apoptosis that can be blocked by Bcl-2 and the VDAC inhibitors (Godbole et al. 1642).

VDACs have also been widely studied in cancers due to their association with hexokinase 1 (HK1) and hexokinase 2 (HK2) which are over-expressed in glycolytic cancers (Wolf et al. 2011). HKs via binding to VDAC1 provide a metabolic benefit to cells, and suppress apoptosis, causing a cell proliferative advantage and increased resistance to chemotherapy (Shoshan-Barmatz et al. 1848). However, the association of VDAC and HK is also regulated by cyclophilin D (CypD) (Machida et al. 2006) and its acetylation status which is in turn modulated by deacetylases like sirtuin-3 (Shulga et al. 2010; Verma et al. 2013). VDAC is prone to oxidative damage when its tyrosine residues get converted to 3-nitrotyrosine by reactive nitrogen species (RNS) including NO and peroxynitrite, produced upon oxidative stress during aging. Therefore, increased VDAC nitration leads to VDAC-dependent, rapid, and massive cytochrome c release. These functions signify the very important physiological role of VDAC in cell life and death. VDAC has been arguably considered to be a component of mitochondrial permeability transition pore (mPTP) (Zoratti and Szabo 1995), as the electrophysiological property of VDAC resembled to that of mPTP (Szabo et al. 1993). It was also predicted to be in the dimeric state in mPTP complex (Szabo and Zoratti 1993). Molkentin and his group further showed that mitochondria from the VDAC null mutant mice were susceptible to Ca^{2+} and oxidative stress induced mPTP formation instigating a debatable role of VDACs in mPTP formation (Baines et al. 2007). A recent study showed the SGP7 (mitochondrial AAA protease) which is an essential component of mPTP complex interacts with VDAC (Shanmughapriya et al. 2015). These studies further suggest that VDACs role in forming mPTP complex is dispensable and probably regulates mPTP formation via interacting with modulators like Bax, Bak, and SGP7.

3 Anion Channels of Inner Mitochondrial Membranes

As stated above, it was widely believed that the inner membrane of the mitochondria is unlikely to contain ion channels, as any movement of ions across it would degenerate the proton motive force driving ATP synthesis (O'Rourke 2007). Although, later, multiple conductances were recorded in IMM, no specifics on molecular identity of these channels are known (Szabo and Zoratti 2014). The realization for the existence of ion channel in inner membrane came from early studies on mitochondrial swelling, which were induced by movement of cations and anions into the matrix compartments (O'Rourke 2007).

3.1 Inner Membrane Anion Channel: Historical Perspective and Ion Channel Properties

Around the 1960s, Azzi et al. promoted the idea of Mg^{2+} governing the permeability of the mitochondrial membrane to univalent cations, which switches to anion, upon increasing pH (Azzi and Azzone 1965; Azzi and Azzone 1967). However,

mechanism for this ion extrusion was uncertain. In 1969, Brierley observed that the permeability of the inner membrane to anions is very low at acidic pH in heart mitochondria, which further increased under alkaline conditions (Brierley 1969; Garlid and Beavis 1986). These findings supported the concept of Azzi et al., about the existence of an Mg^{2+} and pH dependent anion transport pathway in IMM (Garlid and Beavis 1986). Furthermore, the transport of several anions like Cl^{-}, Br^{-}, I^{-}, SCN^{-}, NO^{3-}, $PO_4{}^{-}$, HCO^{3-}, and $SO_4{}^{2-}$ across the mitochondrial membrane was demonstrated (Garlid and Beavis 1986; Stockdale et al. 1970), thus indicating the presence of an IMAC in the mitochondria (Garlid and Beavis 1986).

3.1.1 Electrophysiological Properties of IMAC

Initial patch clamp studies performed on IMM revealed a slightly anion-selective channel with a mean conductance of ~107 pS under symmetrical (*cis:trans*) 150 mM KCl condition (Sorgato et al. 1987). It was named as mitochondrial centum picosiemens (mCS) channel. This was followed by observations of multiple anion channel conductances, on mitoplasts or mitochondrial membrane vesicles (Ballarin and Sorgato 1995; Borecky et al. 1997; De Marchi et al. 1777; Hayman et al. 1993; Kinnally et al. 1992; Klitsch and Siemen 1991) isolated from different tissues (brown adipose tissue, heart, and liver). Patch clamp studies on mitoplasts isolated from brown fat adipose tissue showed a ~108 pS conductance in symmetrical 150 mM KCl solution (Borecky et al. 1997; Klitsch and Siemen 1991). This channel was a candidate for IMAC as the channel currents were inhibited by purine nucleotides, Mg^{2+} as well as variations in pH (Borecky et al. 1997). In both liver and cardiac tissues, Sorgato et al. found a ~100 pS voltage-sensitive anion channel (Sorgato et al. 1989). In yet another study, two distinct anion channel currents were observed in sheep mitoplasts with one showing conductance of 100 pS (150 mM KCl) and the other of 50 pS (Hayman et al. 1993). Remarkably, both species of channel activity lasted for 10–15 min and then exhibited irreversible inactivation which could be a result of desensitization of these channels (Sun et al. 2002). $P_{Cl}{}^{-}/P_K{}^{+}$ values for both the channels were also different. Small conductance channel was shown to be non-selective between anions. Although the channel conductances were similar to that observed by Sorgato et al.(Sorgato et al. 1987), neither of these channels were sensitive to ATP, Mg^{2+}, or pH changes, a property exhibited by IMAC.

Interestingly, three classes of IMM channel activities were recorded in rat cardiac mitochondria, using patch clamp studies (Kinnally et al. 1992; Kinnally et al. 1991). The "107 pS activity" was slightly anion-selective and voltage-dependent (opens with positive potentials towards matrix); a Ca^{2+} or voltage-dependent "multiple conductance channel" (MCC) activity exhibited a single-channel conductance ranging from ~40 to over 1,000 pS, and a "low-conductance channel" (LCC) showing conductance of ~15 pS that was receptive to pH and Mg^{2+} changes. Thus, MCC was considered responsible for the Ca^{2+}-induced permeability transition observed in mitochondrial suspensions. LCC was suggested to be similar to IMAC currents with anion selectivity in the order of $SCN^{-} > NO3^{-} > Cl^{-} > P_i$.

All of the IMM channels reported so far are highly regulated and have a very low open probability under physiological conditions.

IMAC currents were also observed even in yeast (both wild type and VDAC less strain), but the properties of these channels differed (Ballarin and Sorgato 1996). Here, two kinds of channel activity were recorded: a small conductance channel of ~45 pS, which decreased at negative holding potentials, and large conductance channel, whose conductance increased at positive holding potentials (~400 pS at −40 mV; ~800 pS at 40 mV). Unlike IMAC, these channels were unaffected by matrix Ca^{2+} and Mg^{2+}. However, both showed ATP-dependence, indicated by the complete inhibition of the activity of the small channel, and the loss of voltage-dependency for large conductance channel in the presence of nucleotides. Another study revealed the existence of phosphate carrier (PIC) protein, as mitochondria purified from *Saccharomyces cerevisiae* upon reconstitution in liposomes showed anion-selective single-channel currents in patch clamp experiments (Herick et al. 1997). It exhibited a frequent conductance of ~40 ± 10 pS, and was inhibited by higher concentration of phosphate. Although PIC had similar conductance to that of small conducting channel earlier isolated from yeast, its activity was inhibited by Mg^{2+} and Ca^{2+} unlike the later one. This further indicated the existence of multiple IMM anion channel conductances in yeast.

In addition, pH-regulated plant inner mitochondrial anion channel (PIMAC) mediated flux of Cl^-, NO_3^-, succinate, malate, and oxaloacetate was shown in potato tuber mitochondria (Beavis and Vercesi 1992), as well as in durum wheat seedlings and Jerusalem artichoke tubers (Laus et al. 2008). Like yeast IMACs, they exhibit different properties than the mammalian IMAC, because of the lack of inhibition by recognized inhibitors of mammalian IMACs. Maize IMAC shows temperature independent open probability in contrast to that of mammalian IMAC, but this level of activity varied with the cold tolerance levels of the maize population. Although these studies point out the presence of IMAC in mitochondria, more data is required to establish the molecular identity of these channels.

3.1.2 IMAC Modulators

Light scattering on swelling mitoplasts was used to describe IMAC in initial studies (Garlid and Beavis 1986; Beavis and Vercesi 1992; Halle-Smith et al. 1988; Powers and Beavis 1991; Zernig et al. 1990). IMAC has been shown to be modulated by palmitoyl-CoA ($IC_{50} \sim 2.5$ μM) (Halle-Smith et al. 1988), Mg^{2+} (Garlid and Beavis 1986; Beavis and Vercesi 1992), ($IC_{50} \sim 38$ μM), pH ($pIC_{50} \sim 7.7$) (Beavis and Vercesi 1992; Beavis 1992), *N,N′*-dicyclohexylcarbodiimide, mercurial (chloromercuribenzene sulfonate and mersalyl), and amphiphilic amines such as propranolol and triorganotins (Powers and Beavis 1991). Tributyltin (TBT), a potent triorganotin, inhibits malonate transport via IMAC at 0.9 nmol/mg and 95 % efficiency (Powers and Beavis 1991). IMAC like other chloride channels is reversibly inhibited by stilbene derivatives such as 4,4′-Diisothiocyanato-2,2′-stilbenedisulfonic acid disodium salt (DIDS) (Beavis and Davatol-Hag 1996). Other inhibitors include amiodarone, amitriptyline, dibucaine, Ro 5–4864, and thiol cross linkers, like N-ethylmaleamide. Even inhibitors of adenine

nucleotide translocase (ANT) block the anion transport by IMAC (Powers et al. 1994). The plant IMACs unlike that of mammalian are not inhibited by matrix Mg^{2+}, mercurials, or *N*,*N*′-dicyclohexylcarbodiimide but by other well-known IMAC inhibitors such as propranolol ($IC_{50} = 14$ μM), TBT ($IC_{50} = 4$ nmol/mg), and the ANT inhibitors like erythrosin B and Cibacron Blue 3GA. Ca^{2+} also modulated the activity of IMAC in mitochondrial swelling experiments (Zernig et al. 1990). In addition, benzodiazepines that inhibit mitochondrial benzodiazepine receptor (mBzR) present in the OMM also blocked IMAC activity but the mechanism of inhibition is unclear. Even though the functions of these channels are poorly understood, their tight regulation suggests their import role in maintaining physiology of the cell by possibly regulating mitochondrial function.

3.1.3 Functions

IMACs are mainly implicated in volume homeostasis. IMAC has been proposed to play a role in postischemic electrical and contractile dysfunction in the heart (O'Rourke 2007; Akar et al. 2005; Aon et al. 1762). In adult cardiomyocytes, it was observed that inhibitors of IMAC prevented reverse oscillatory mitochondrial depolarizations induced by substrate deprivation (O'Rourke 2000) and oxidative stress (Aon et al. 2003). In spite of multiple conductance observed in the IMM, it is challenging to assign specific physiological roles due to its ambiguous molecular identity.

3.2 Chloride Intracellular Channel Proteins

These are unique class of intracellular channel proteins consisting of single transmembrane domain, and exist in dimorphic state of both soluble and integral membrane form (Ashley 2003; Singh 2010). There are seven paralogs of CLIC identified in mammals to date, namely CLIC1–CLIC6 (Singh 2010) which includes CLIC5A and CLIC5B (isoforms of CLIC5). They are also conserved among prokaryotes and widely distributed across other eukaryotic species, including three isoforms, identified in invertebrates (*Dm*CLIC in *Drosophila melanogaster*, EXC4 and EXL1 in *Caenorhabditis elegans*) (Berry et al. 2003; Littler et al. 2008) and four in plants (*At*DHAR1-4 in *Arabidopsis thaliana*) (Singh 2010; Littler et al. 2010; Elter et al. 2007). CLICs, as the name specifies, are localized to intracellular organelles (Singh 2010) specifically nuclear membrane (Valenzuela et al. 1997; Ulmasov et al. 2007), secretory vesicles of hippocampal neurons (Chuang et al. 1999), caveolae (Edwards and Kahl 2010), trans-Golgi network (Edwards and Kahl 2010), endoplasmic reticulum, (Duncan et al. 1997; Ponnalagu et al. 2016b) and mitochondria (Ponnalagu et al. 2016a; Edwards and Kahl 2010; Ponnalagu et al. 2016b; Fernandez-Salas et al. 1999).

3.2.1 Structural Insights

CLIC proteins show high sequence and structural similarity to members of omega glutathione-S-transferase (GST) family of proteins (Singh 2010). They contain

~280 amino acid residues, with a conserved C-terminal domain. CLIC5B and CLIC6 contain an additional hydrophobic N-terminal domain (Singh 2010; Littler et al. 2010). Until now, crystal structures of only the soluble forms of CLIC are obtained. CLIC4 and CLIC1 show high structural similarity due to 67 % sequence homology. Mammalian CLICs also share structural similarity to invertebrates. All CLICs contain 10 α-helices (h1, h2, h3, h4a, h4b, h5, h6, h7, h8, and h9) and four β-strands (s1, s2, s3, and s4) except in EXC4, where helix h4 is unbroken but has an additional h10 helix (Littler et al. 2008). Soluble structures of EXC4 and *Dm*CLIC indicate that the putative transmembrane domain is formed by an α-helix (h1) and a β-strand (s2) (Littler et al. 2008). In mammalian CLICs the putative transmembrane domain (C24-46) comprises the pore, and plays a role in ion transport and targeting the protein to the membrane (Singh 2010; Singh and Ashley 2006; Singh and Ashley 2007). For CLIC1 it is shown that modification of Cys24 inhibits the channel activity (Singh and Ashley 2006). Cys24 and its equivalent conserved residues in other CLIC proteins play a key role in the redox-regulation of CLIC1, CLIC4, and CLIC5 (Singh 2010). Also, it is observed that CLIC proteins insert into planar bilayers with the N-terminus located in the luminal side and the C-terminus towards the cytosolic side (Singh and Ashley 2006; Singh and Ashley 2007). Auto insertion of CLIC1 to the bilayers increased with the formation of disulphide bond between the residues Cys24 and Cys59 upon oxidation, indicating the role of Cys24 in integration of soluble form of CLIC to membranes. However, the residue Cys59 is not conserved in all other CLICs, and also it is not very clear how these residues form disulphide bonds in membrane form as they are oriented at opposite directions. Therefore, the role of disulphide bonds in insertion of CLICs is still questionable. As CLICs have single transmembrane domain, it is predicted that at least four molecules of them will be necessary to form a functional ion channel in the membrane (Singh 2010). Conformation flexibility of putative transmembrane increased in an acidic pH of ~5.5, thereby increasing the auto insertion into the membrane and exhibiting enhanced single-channel conductance (Stoychev et al. 2009).

3.2.2 Mitochondrial CLIC Proteins

Even though, CLICs were named as intracellular ion channels, their cellular localization is not systematically explored chiefly due to lack of specific antibodies. However, information on localization of some of the CLIC proteins is available (Singh 2010). Out of six CLIC proteins, CLIC4 was characterized as a putative mitochondrial ion channel protein (Szabo and Zoratti 2014), and was demonstrated to reside in mitochondria of keratinocytes (Fernandez-Salas et al. 2002), where it is involved in Ca^{2+}-induced differentiation of keratinocytes (Suh et al. 2007). Hence, CLIC4 is also known as mitochondrial CLIC4 (mtCLIC4). Furthermore, R (+)-Methylindazone, R (+)-[(6, 7-Dichloro-2-cyclopentyl-2, 3-dihydro-2-methyl-1-oxo-1H-inden-5-yl)-oxy] acetic acid (IAA-94)-sensitive CLIC-like currents were also observed in cardiac mitochondria (Misak et al. 2013) suggesting their presence in cardiac mitochondria. We have recently showed that CLIC4 and CLIC5 are cardiac mitochondrial proteins, where CLIC4 is localized to the OMM and CLIC5

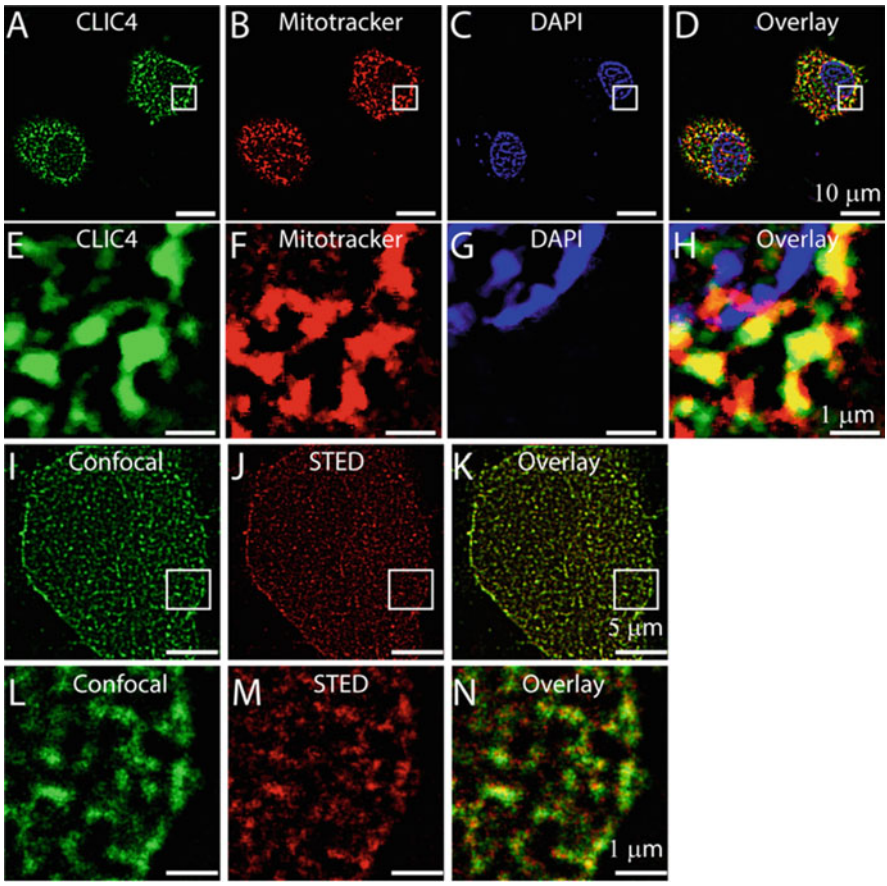

Fig. 2 Localization of CLIC4 in H9C2 cells. H9C2 cells loaded with mitotracker (**b**) were labeled with anti-CLIC4 antibody (**a**), and stained with DAPI (**c**). **d** is merge image of **a**, **b**, and **c** showing colocalization of CLIC4 to the mitochondria of H9C2 cells. **e**, **f**, **g**, and **h** are enlarged images of the squared regions in **a**, **b**, **c**, and **d**, respectively. STED microscopy of H9C2 cells showing expression of CLIC4 in the mitochondria (**j**, **k**). Confocal image of H9C2 cells labeled with anti-CLIC4 antibody (**i**). Corresponding STED image (**j**), overlay of **i** and **j** highlights increased resolution of CLIC4 localization to mitochondria with STED microscopy (**k**). **l**, **m**, and **n** are enlarged images of the squared region in **i**, **j**, and **k**, respectively

to the IMM of rat cardiac mitochondria (Ponnalagu et al. 2016a; Ponnalagu et al. 2016b). CLIC1 and CLIC2 are chiefly localized to the endoplasmic reticulum of adult cardiomyocytes (Ponnalagu et al. 2016a; Ponnalagu et al. 2016b).

In agreement to these studies here we further show mitochondrial localization of CLIC4 in H9C2 cardiac cells (Fig. 2a–h). We also investigated localization of CLIC5 and found it to be localized to the mitochondria of H9C2 cells (Fig. 3a–h). Interestingly, bioinformatic analysis of CLICs revealed the absence of any mitochondrial targeting sequence (MTS) (Ponnalagu et al. 2016a), further indicating a

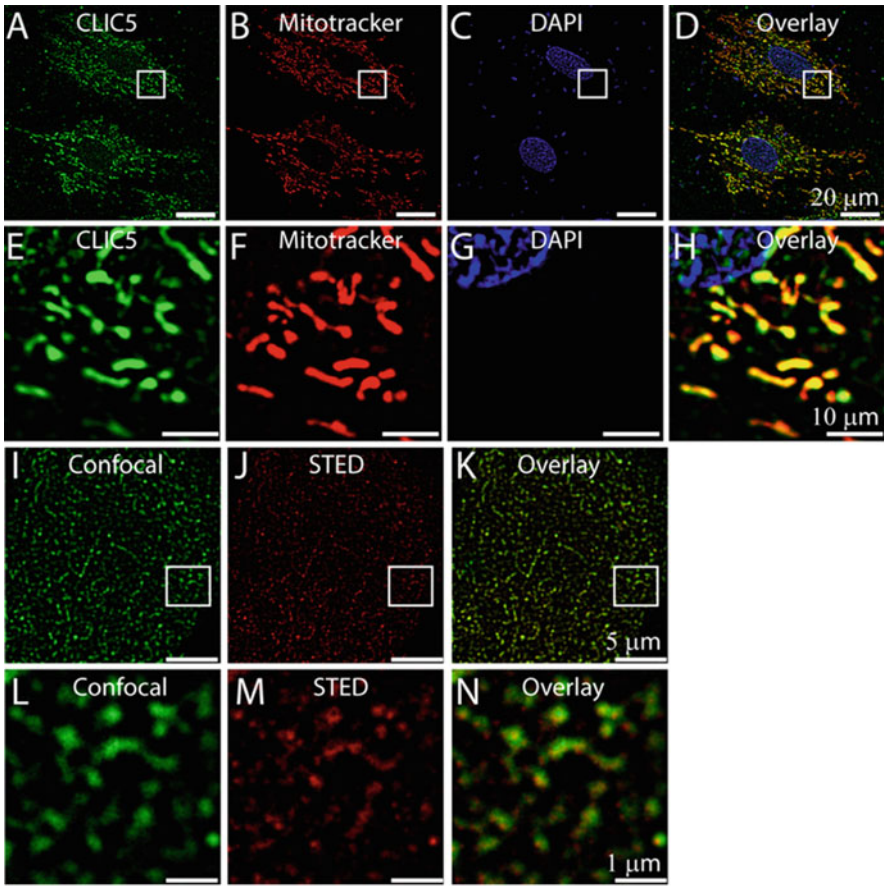

Fig. 3 Localization of CLIC5 in H9C2 cells. Mitotracker-labeled (**b**) H9C2 cells were incubated with anti-CLIC5 antibody (**a**), and stained with DAPI (**c**). **d** is merge image of **a**, **b**, and **c** showing colocalization of CLIC5 to the mitochondria of H9C2 cells. **e**, **f**, **g**, and **h** are enlarged images of the squared regions in **a**, **b**, **c**, and **d**, respectively. STED microscopy of H9C2 cells showing expression of CLIC5 in the mitochondria (**j**, **k**). Confocal image of H9C2 cells labeled with anti-CLIC5 antibody (**i**) Corresponding STED image (**j**), overlay of **i** and **j** highlights increased resolution of CLIC5 localization to mitochondria with STED microscopy (**k**). **l**, **m**, and **n** are enlarged images of the squared region in **i**, **j**, and **k**, respectively

unique unconventional mechanism for their mitochondrial localization in H9C2 cells. To understand geographical distribution of CLIC4 and CLIC5 in cardiac cells, we acquired super resolution images by using a custom-built STED microscope (Rodríguez et al. 2012). Figures 2i–n and 3i–n revealed a unique cluster distribution of CLIC4 (80 ± 10 nm, $n = 500$ clusters) and CLIC5 (98 ± 12 nm $n = 500$ clusters) in H9C2 cells. These results indicate that even though CLIC4 and CLIC5 localize to mitochondria of H9C2 cells, they follow a distinct distribution either due to differential localization within the mitochondria as observed in

adult cardiac mitochondria (Ponnalagu et al. 2016a; Singh et al. 2012b) or due to unique functional roles.

3.2.3 Electrophysiological Properties

Channel activity of CLIC proteins have been carried out in artificial bilayers (Singh and Ashley 2006; Tulk et al. 2002; Warton et al. 2002) using either recombinant proteins or ectopic expression in mammalian cells (Valenzuela et al. 1997; Tonini et al. 2000). CHO-K1 cells transfected with CLIC1 showed a single-channel conductance of 8 pS in cell and nuclear patch clamping studies (Valenzuela et al. 1997). Conductance of all CLICs varied depending on the lipid composition, recording conditions, and redox environment of the bilayers (Singh 2010; Singh and Ashley 2006). All the CLICs form NSAC as they do not differentiate between cations and anions; however, they turn into anion-selective in the presence of large cations like Tris (Singh 2010; Singh and Ashley 2006). Arg29 and Lys37 in the putative transmembrane domain have been shown to play a role in ion channel function. Lys37 altered the single-channel conductance whereas Arg29 affected the open probability of a single-channel in response to variation in membrane potential (Averaimo et al. 2013). Ionic conductance of CLIC1 and CLIC5 is regulated by actin (Singh et al. 2007), but not for CLIC4. Single-channel biophysics of CLICs in their native environment is not yet reported.

3.2.4 Functions

CLICs are multifunctional proteins. They involve in membrane trafficking, cytoskeletal function (Berryman et al. 2004), apoptosis (Fernandez-Salas et al. 2002; Suh et al. 2004), cell cycle control (Valenzuela et al. 2000), tubulogenesis (Berry et al. 2003), VEGF-mediated angiogenesis of endothelial cells (Tung et al. 2009), mitosis, and differentiation (Suh et al. 2007). CLIC2 was reported to modulate the function of ryanodine receptors (RyR1 and RyR2) (Board et al. 2004; Takano et al. 2012). In humans, the transcriptional analysis showed the downregulation of CLIC2 in dilated cardiomyopathy patients (Diaz et al. 2010) indicating its possible role in cardiac physiology. CLIC4 also showed a role in regulating the endothelial function, where CLIC4 deficient animals were shown to be resistant to pulmonary hypertension (Wojciak-Stothard et al. 2014). Diaz et al. showed blocking chloride channels using IAA-94, prevented the cardio protective effects of ischemic pre-conditioning (IPC) as there was increased myocardial infarction (MI) due to ischemic/reperfusion (IR) injury in both the heart as well as isolated adult cardiomyocytes (Diaz et al. 1999). It was also shown that other chloride channel blockers like DIDS, 5-Nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), and 4-acetamido-4′-isothiocyanato-stilbene-2,2′-disulfonic acid (SITS), protected hearts from MI further suggesting a probable cardio protective role of IAA-94 specific targets, CLICs (Zheng et al. 2013; Wang et al. 2015).

Overexpression of mtCLIC4 in keratinocytes induced its mitochondrial localization leading to reduced mitochondrial membrane potential and thereby triggering apoptosis (Fernandez-Salas et al. 2002). It was also demonstrated that RNAi suppression of CLIC4 enhanced H_2O_2-induced glioma cell apoptosis

(Xu et al. 2013). Recent study also showed that abrogation of CLIC5 increased the ROS generation of cardiac mitochondria in mice (Ponnalagu et al. 2016a). Thus, these studies provide evidence of CLIC in modulating mitochondrial function which is known to play a role in regulation of apoptosis, cardioprotection from IR injury, and their direct involvement in maintaining cellular physiology.

4 Other Chloride Transporters or Modulators in Mitochondria

Apart from the above mentioned anion channel conductances observed in both IMM and OMM, there are still other upcoming anion channels or modulators maintaining mitochondrial ion flux which needs to be further studied. We have discussed some of them in the following section.

4.1 Mitochondrial Permeability Transition Pore

mPTP is a non-selective channel present in the mitochondrial inner membrane which allows a solute of up to 1.5 kDa to pass through it resulting in dissipation of electrochemical proton gradient, inhibition of ATP synthesis further leading to cell death (Szabo and Zoratti 2014; Bernardi et al. 2006). The conductance of this channel varies from 0.9 to 1.3 pS, with mild anion-selectivity and can also switch to cation-selective channel (Szabo and Zoratti 2014), therefore they are considered as non-selective channels present in the mitochondrial membrane. Opening of the channel is reliant on mitochondrial Ca^{2+} concentrations and matrix alkalization. Earlier it was shown that PTP is inhibited by submicromolar concentration of an immunosuppressant drug, cyclosporine A (CsA) (Fournier et al. 1987; Crompton et al. 1988; Broekemeier et al. 1989; Davidson and Halestrap 1990) thus indicating the presence of CypD as the molecular component of the channel. Multiple studies have predicted that mPTP comprises of VDAC (Szabo et al. 1993; Szabo and Zoratti 1993), ANT (Kokoszka et al. 2004), CypD, and hexokinase. As discussed earlier in Sect. 2.1.4, interaction of SGP7 and VDAC was found to be essential for formation of mPTP (Shanmughapriya et al. 2015). A latest study has also suggested the likelihood of F_1F_O ATPase itself as forming a PTP (Halestrap 2014; Bonora et al. 2013). Further, studies on identifying the molecular correlate of mPTP are still underway, and usage of a multidisciplinary approach will certainly provide much desired information (Singh et al. 2012a; Singh 2010; Rodríguez et al. 2012; Singh et al. 2012b; Singh et al. 2009). Activity of the channel is modulated by CsA, matrix acidification, adenine nucleotides, and divalent cations apart from Ca^{2+}(Szabo and Zoratti 2014). In spite of its unclear molecular identity, multiple studies signify its key role in IR injury (Akar et al. 2005; Halestrap et al. 2004), neurodegeneration (Schinzel et al. 2005) in pathological disorders, apoptosis, and progression of cancer (Szabo and Zoratti 2014).

4.2 Maxi-Chloride Channel

This section summarizes the evidence of the presence of Maxi Cl^- channel in mitochondria (De Marchi et al. 1777). Patch clamp analysis of liver mitoplast revealed a channel activity similar to monomeric state of PTP. The occurrence of this activity is not found to be consistent in liver mitochondria but was observed more frequently in human colon tumor 116 (HCT116) cells. The conductance of the channel recorded in HCT116 cells was ~400 pS. This channel was considered to be anion-selective in fourfold KCl gradient and at reversal potentials in the range of -26 to -31 mV. The ratio of permeability coefficients reported was $P_{Cl}{}^-/P_K{}^+ \approx 7$–18. As the channel activity represented the monomeric form of PTP, VDAC was considered as a probable candidate for this channel activity. The channel activity did not differ upon VDAC deletion, thus, ruling out the possibility of VDAC as its molecular component. The channel occasionally switched to cation-selective state. Interestingly, the activity was inhibited by only DIDS and SITS but not by IAA-94, NPPB, or niflumic acid. Properties of this channel were similar to swelling-activated and voltage-inactivated maxi-chloride channels reported earlier (Sabirov et al. 2006). Although molecular structure of the channels is unknown but is considered to have a strong link with PTP.

4.3 Uncoupling Proteins

UCP is a ~33 kDa protein, primarily acting as an H^+ carrier in the inner membrane of mitochondria. There are five isoforms of UCP identified in mammals. Anion channel transport was associated with them as it was also demonstrated to play a role in mitochondrial swelling (Nicholls and Lindberg 1973), and Cl^- transport was confirmed in vesicles reconstituted with UCP (Huang and Klingenberg 1996; Jezek et al. 1990). There are six transmembrane domains and in case of UCP1 and UCP2, it is shown that transmembrane 2 (TM2) contributes to anion conductance (Yamaguchi et al. 2004). Also, arginine residues of TM2 were shown to be crucial for anion transport (Hoang et al. 2015). The channel exhibited an inward-rectification with a single-channel conductance of 75 ± 6 pS at positive holding potentials, and 84 ± 8 pS at negative holding potentials. Divalent cations like Mg^{2+} and Ca^{2+} aided in the occurrence of these channels. The permeability to ions decreased in the order of $Cl^- > Br^- > F^- > SCN^- > I^- > NO3^- >$ gluconate. Like all other uncharacterized mitochondrial anion channels, the channel activity was sensitive to nucleotides (Huang and Klingenberg 1996). Although, UCP is required to dissipate the proton gradient to provide energy for oxidative phosphorylation, these studies speculate the potential of UCP behaving as an anion channel.

4.4 Vacuolating Cytotoxin A

VacA, the vacuolating cytotoxin A, as the name implies forms large vacuoles in cells and is one of the major virulence factors released by *Helicobacter pylori*

(Rassow 2011). VacA toxin is the prominent mediator in *H. pylori*-induced apoptosis of cells (Cover and Blanke 2005; Boquet et al. 2003), and is also known to form anion channels (Iwamoto et al. 1999; Czajkowsky et al. 1999; Szabo et al. 1999) of low conductance of ~10 pS. This conductivity is completely inhibited by the chloride channel blocker NPPB (Szabo et al. 1999; Tombola et al. 2000). Previous studies also demonstrated that VacA intoxicates mammalian cells by reducing mitochondrial membrane potential and inducing apoptosis (Willhite and Blanke 2004). Based on these studies a model has been hypothesized to link the channel activity with its function: VacA after entering mammalian cells localizes to the mitochondria, and modulates mitochondrial membrane permeability by forming a channel resulting in cytochrome c release. Thus, VacA joins the ranks of anion channels playing a role in intrinsic pathway of apoptosis (Willhite and Blanke 2004).

5 Pharmacological Evidence of Cardiac Mitochondrial Anion Channels

Most of the chloride channels in mitochondria have been identified and characterized using pharmacological inhibitors. However the specificity of these pharmacological agents is not yet established, and is shown to be non-specific at different concentrations which is a limitation for their usage. Nevertheless, these pharmacological modulators have played a significant part in deciphering the molecular identity and physiological functions of these channels. Thus, in this section we investigated the presence of putative chloride channels in cardiac mitochondria of *Rattus norvegicus* and their respective roles in modulating mitochondrial ROS generation (Murphy 2009) using these pharmacological inhibitors of chloride channels.

Mitochondrial Ca^{2+} capacity and ROS generation play a key role in multiple pathologies, including cardiac IR injury, neurodegenerative diseases, diabetes, cancer, and premature aging (Sena and Chandel 2012). In a recent study, mitochondrial Ca^{2+} capacity was used to identify components of mPTP (Shanmughapriya et al. 2015). Here, we have incorporated widely used canonical anion channel blockers, and studied their impact on mitochondrial ROS generation to identify possible mitochondrial anion channels present in the heart. Cl^- channel inhibitors used are IAA-94 (Landry et al. 1989; Weber-Schurholz et al. 1993), Anthracene-9-carboxylic acid (A9C) (Al Khamici et al. 2015), NPPB (Malekova et al. 2007), DIDS (Malekova et al. 2007), glibenclamide (Sheppard and Welsh 1992), and 4-[(2-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1*H*-inden-5-yl)oxy]butanoic acid (DCPIB) (Bourke et al. 1981; Decher et al. 2001) that are known blockers of CLICs (IAA-94 and A9C both), calcium-activated chloride channel, CLC's, cystic fibrosis transmembrane conductance regulator (CFTR), and swelling-activated chloride channels, respectively. Further, earlier studies have shown the presence of these channels in the heart (Misak et al. 2013; Duan 2013; Baumgarten et al. 2005).

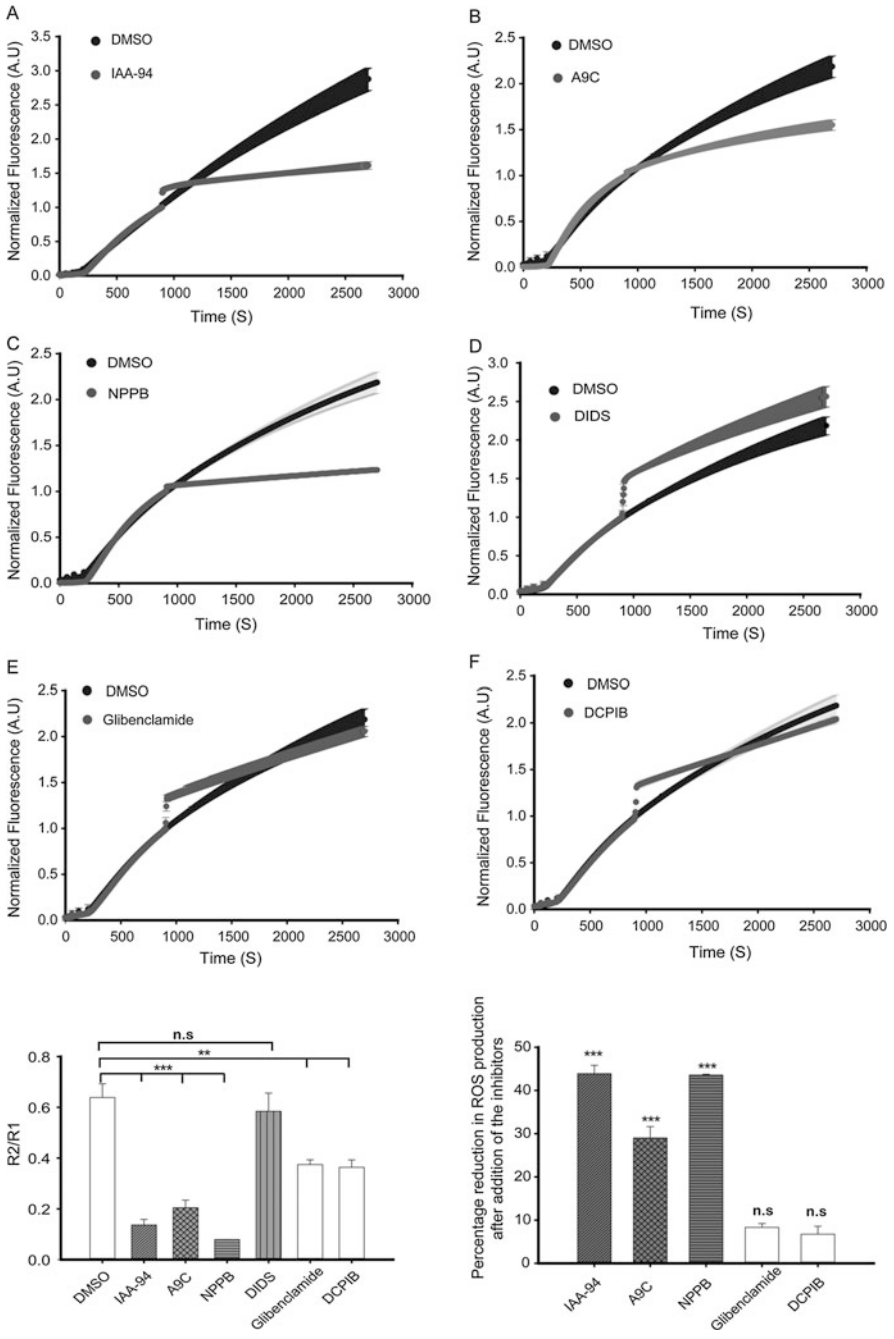

Fig. 4 Differential modulation of the rate of ROS generation in complex II/III of cardiac mitochondria by various chloride channel inhibitors. Cardiac mitochondria isolated from *R. norvegicus* and the ROS generated by complex II/III was measured as described earlier. Profile representing the rate of ROS production by cardiac mitochondria in the presence of DMSO (*black*) vs. 100 μM IAA-94 (*gray*, **a**), 100 μM A9C (*gray*, **b**), 50 μM NPPB (*gray*, **c**), 300 μM

Electron transport chain complex II/III were activated with its substrate, succinate and the ROS generated was measured using amplex red as an indicator of H_2O_2 produced, in fluorescence spectrophotometer F-2710 as described previously (Singh et al. 2012b). It was observed that there was significant reduction in the rate as well as total ROS generation (relative to DMSO control) after addition of inhibitors such as A9C (100 μM), IAA-94 (100 μM), and NPPB (50 μM) (Fig. 4a–c, g and h). This further indicated the possible presence of CLICs, and calcium-activated chloride channel in the cardiac mitochondria isolated from *R. norvegicus*. There was no change in the rate of ROS production in case of 300 μM DIDS (CLC inhibitor) (Fig. 4d, g). Also, there was a non-specific immediate spike in the rate of ROS production observed for most of the inhibitors. 100 μM of each glibenclamide, and DCPIB showed significant reduction in the rate of ROS production (Fig. 4e–g), but no significant reduction in the total ROS produced was observed relative to vehicle control (DMSO) at the end of 45 min (Fig. 4h). This could be either due to reversible inhibitory effect of these channel inhibitors or unhealthy condition of the mitochondria in the presence of a drug. These results further strengthen the existence of Cl^- channels in the mitochondria, and also provide evidence for their role in modulating mitochondrial function. Thus, molecular identity of other anion channels needs to be further elucidated, as they could be a probable candidate for developing new therapeutic tools.

6 Concluding Remarks

Chloride channels always remained in rear seat as they are considered as "unimportant leaks" associated with cation channels in excitable cells (Jentsch et al. 2004; Ashley 2003; Jentsch et al. 2002). This is mainly due to lack of specific tools/ligands/modulators to characterize them. In spite of lack of efficient tool to identify channel recordings in intracellular organelles, several mitochondrial anion channel conductances (summarized in Table 1) have been reported but the molecular correlate of these channels is unknown or debatable. So far, molecular identities

Fig. 4 (continued) DIDS (*gray*, **d**), 100 μM glibenclamide (*gray*, **e**), and 100 μM DCPIB (*gray*, **f**). R1: Initial rate of ROS production which is 15 min prior to addition of the inhibitors, R2: rate of ROS production calculated after 15 min of addition of inhibitors. R2/R1 significantly decreased in presence of the chloride channel inhibitors IAA-94, A9C, NPPB, glibenclamide, and DCPIB (**g**). IAA-94, A9C, and NPPB showed significant reduction in the total ROS produced (relative to DMSO) at the end of 45 min whereas negligible change in ROS production relative to DMSO control was observed in case of glibenclamide and DCPIB (**h**). One way ANOVA was used to calculate the statistical significance (***$p \leq 0.0005$, **$p \leq 0.005$). *IAA-94* R(+)-Methylindazone, R(+)-[(6,7-Dichloro-2-cyclopentyl-2,3-dihydro-2-methyl-1-oxo-1H-inden-5-yl)-oxy]acetic acid, *A9C* anthracene-9-carboxylic acid, *NPPB* (5-nitro-1-(3-phenylpropylamino)benzoic acid, *DIDS* 4,4′-Diisothiocyano-2,2′-stilbenedisulfonic acid, *DCPIB* 4-[(2-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1*H*-inden-5-yl)oxy]butanoic acid ($n \geq 4$, independent preparations with each pharmacological agent used)

Table 1 Mitochondria ion channels, their localization, selectivity, and conductance

Name	Location	Selectivity	References
VDAC1	OMM	Cl selective at the open state of channel [$P_{Ca} = 0.94 \pm 0.05$, $P_{Cl} = 22.6 \pm 0.2 (\times 10^{-16}$ L/s)], also permeable to calcium at the closed state [$P_{Ca} = 4 \pm 1$, $P_{Cl} = 5 \pm 1 (\times 10^{-16}$ L/s)], conductance of 2.4–4.3 nS at Vm = 10 mV	Szabo and Zoratti (2014), Tan and Colombini (1768), and De Pinto et al. (1987)
VDAC2	OMM	Cl selective, conductance varied as it showed lower conductance (1.55 ± 0.01 nS at 5 mV) and higher conductance (1.71 ± 0.01 nS at 12 mV). On an average conductance reported were 3.79 ± 0.10 nS.	Xu et al. (1999) and Menzel et al. (2009)
VDAC3	OMM	Cl selective at a holding potential of 12 mV, a small conductance of 0.68 nS−0.71 nS at tenfold KCl gradient	Checchetto et al. (2014), Colombini (2004), and Xu et al. (1999)
CLIC4	OMM	15 pS (500/50 mM KCl), 30 pS (140 mM KCl), 1 pS (300:140 mM choline Cl), 43 (50 mM choline chloride)	Ponnalagu et al. (2016a), Duncan et al. (1997), Ponnalagu et al. (2016b), Singh and Ashley (2007), and Proutski et al. (2002)
IMAC	IMM	P_{Cl}/P_K is 4.5, 107–110 pS in symmetrical 150 mM KCl.	Sorgato et al. (1987), Klitsch and Siemen (1991), and Sorgato et al. (1989)
CLIC5	IMM	Varied from ~3–116 pS (500/50 mM KCl) when reconstituted in planar bilayers, 26 pS, 100 pS and 400 pS (140 mM KCl), 100/280 pS (140 mM), 42 pS (140 mM KCl)	Ponnalagu et al. (2016a), Ponnalagu et al. (2016b), Singh et al. (2007), Landry et al. (1989), Weber-Schurholz et al. (1993), and Edwards et al. (1998)
mPTP	IMM	0.9–1.5 nS (150 mM KCl), average conductance of ~ 1.3 nS in patch clamp studies, low anion selectivity, and sometimes exhibit cation selectivity	Szabo and Zoratti (2014), De Marchi et al. (2006), and Petronilli et al. (1989)
Maxi Cl channel	IMM	400 pS (fourfold KCl gradient) in patch clamp studies under reversal potentials of −26 to −31, $P_{Cl}/P_K \approx 7$ to 18	De Marchi et al. (1777)
UCP	IMM	~75 pS at positive holding potential (symmetrical 100 mM KCl) in patch clamp studies. ~84 pS at negative holding potential, inward rectifying Cl^- currents were observed.	Huang and Klingenberg (1996)

of only VDAC, CLIC4, and CLIC5 have been well established in mitochondria and many mitochondrial anion channels are yet to be identified.

Moreover, ongoing and upcoming studies signify the potential role of mitochondrial ion channels as regulators of mitochondrial morphology as well as physiology, contributors of cancer and cardiac-related diseases, and key participants in cell life and death. Despite their significant functions in cell fate determination, little effort has been put in developing specifically targeted therapeutics agents to modulate their activity, mainly because of lack of molecular identity and structure of these channels. Therefore, efficient tools/modulators should be generated in identifying the molecular structure of these channels, as this will aid in providing specific targets for development of novel therapeutics.

Enormous evidence supports the existence of multiple physiologically relevant anion channel conductance in the mitochondria. Hence, these channels cannot be ignored as unimportant leaks and more efforts should be carried out towards understanding the molecular identity and physiological function of these channels.

Acknowledgements We would like to thank Dr. Olimpia Meucci (DUCoM) for providing reagents, Jason Farber, Ahmed T. Hussain, and Kajol Shah for assistance with data analysis, and Dr. Shubha Gururaja Rao (DUCoM) for helpful comments and discussions. HS is supported by CTRI, CURE, AHA SDG (11SDG7230059), NIH NHLBI (1R01HL133050-01) and startup funds from DUCoM.

References

Abu-Hamad S, Arbel N, Calo D, Arzoine L, Israelson A, Keinan N, Ben-Romano R, Friedman O, Shoshan-Barmatz V (2009) The VDAC1 n-terminus is essential both for apoptosis and the protective effect of anti-apoptotic proteins. J Cell Sci 122:1906–1916

Akar FG, Aon MA, Tomaselli GF, O'Rourke B (2005) The mitochondrial origin of postischemic arrhythmias. J Clin Invest 115:3527–3535

Al Khamici H, Brown LJ, Hossain KR, Hudson AL, Sinclair-Burton AA, Ng JP, Daniel EL, Hare JE, Cornell BA, Curmi PM, Davey MW, Valenzuela SM (2015) Members of the chloride intracellular ion channel protein family demonstrate glutaredoxin-like enzymatic activity. PLoS One 10, e115699

Aon MA, Cortassa S, Akar FG, O'Rourke B (1762) Mitochondrial criticality: a new concept at the turning point of life or death. Biochim Biophys Acta 2006:232–240

Aon MA, Cortassa S, Marban E, O'Rourke B (2003) Synchronized whole cell oscillations in mitochondrial metabolism triggered by a local release of reactive oxygen species in cardiac myocytes. J Biol Chem 278:44735–44744

Arbel N, Ben-Hail D, Shoshan-Barmatz V (2012) Mediation of the antiapoptotic activity of Bcl-xL protein upon interaction with VDAC1 protein. J Biol Chem 287:23152–23161

Ashley RH (2003) Challenging accepted ion channel biology: P64 and the CLIC family of putative intracellular anion channel proteins (review). Mol Membr Biol 20:1–11

Averaimo S, Abeti R, Savalli N, Brown LJ, Curmi PM, Breit SN, Mazzanti M (2013) Point mutations in the transmembrane region of the CLIC1 ion channel selectively modify its biophysical properties. PLoS One 8, e74523

Azzi A, Azzone GF (1965) Swelling and shrinkage phenomena in liver mitochondria. Ii. Low amplitude swelling-shrinkage cycles. Biochim Biophys Acta 105:265–278

Azzi A, Azzone GF (1967) Swelling and shrinkage phenomena in liver mitochondria. Vi. - Metabolism-independent swelling coupled to ion movement. Biochim Biophys Acta 131:468–478

Bahamonde MI, Fernandez-Fernandez JM, Guix FX, Vazquez E, Valverde MA (2003) Plasma membrane voltage-dependent anion channel mediates antiestrogen-activated maxi cl- currents in c1300 neuroblastoma cells. J Biol Chem 278:33284–33289

Baines CP, Kaiser RA, Sheiko T, Craigen WJ, Molkentin JD (2007) Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death. Nat Cell Biol 9:550–555

Ballarin C, Sorgato MC (1995) An electrophysiological study of yeast mitochondria. Evidence for two inner membrane anion channels sensitive to ATP. J Biol Chem 270:19262–19268

Ballarin C, Sorgato MC (1996) Anion channels of the inner membrane of mammalian and yeast mitochondria. J Bioenerg Biomembr 28:125–130

Banerjee J, Ghosh S (2004) Bax increases the pore size of rat brain mitochondrial voltage-dependent anion channel in the presence of tBid. Biochem Biophys Res Commun 323:310–314

Banerjee J, Ghosh S (2006) Phosphorylation of rat brain mitochondrial voltage-dependent anion as a potential tool to control leakage of cytochrome c. J Neurochem 98:670–676

Bates E (2015) Ion channels in development and cancer. Annu Rev Cell Dev Biol 31:231–247

Baumgarten CM, Browe DM, Ren Z (2005) Swelling- and stretch-activated chloride channels in the heart: regulation and function. In: Kamkin A, Kiseleva I (eds) Mechanosensitivity in cells and tissues. Academia, Moscow, pp 79–102

Bayrhuber M, Meins T, Habeck M, Becker S, Giller K, Villinger S, Vonrhein C, Griesinger C, Zweckstetter M, Zeth K (2008) Structure of the human voltage-dependent anion channel. Proc Natl Acad Sci U S A 105:15370–15375

Beavis AD (1992) Properties of the inner membrane anion channel in intact mitochondria. J Bioenerg Biomembr 24:77–90

Beavis AD, Davatol-Hag H (1996) The mitochondrial inner membrane anion channel is inhibited by dids. J Bioenerg Biomembr 28:207–214

Beavis AD, Vercesi AE (1992) Anion uniport in plant mitochondria is mediated by a mg(2+)-insensitive inner membrane anion channel. J Biol Chem 267:3079–3087

Bera AK, Ghosh S (2001) Dual mode of gating of voltage-dependent anion channel as revealed by phosphorylation. J Struct Biol 135:67–72

Bernardi P, Krauskopf A, Basso E, Petronilli V, Blachly-Dyson E, Di Lisa F, Forte MA (2006) The mitochondrial permeability transition from in vitro artifact to disease target. FEBS J 273:2077–2099

Berry KL, Bulow HE, Hall DH, Hobert O (2003) A C. Elegans CLIC-like protein required for intracellular tube formation and maintenance. Science 302:2134–2137

Berryman M, Bruno J, Price J, Edwards JC (2004) CLIC-5A functions as a chloride channel in vitro and associates with the cortical actin cytoskeleton in vitro and in vivo. J Biol Chem 279:34794–34801

Betaneli V, Petrov EP, Schwille P (2012) The role of lipids in VDAC oligomerization. Biophys J 102:523–531

Beutner G, Ruck A, Riede B, Welte W, Brdiczka D (1996) Complexes between kinases, mitochondrial porin and adenylate translocator in rat brain resemble the permeability transition pore. FEBS Lett 396:189–195

Board PG, Coggan M, Watson S, Gage PW, Dulhunty AF (2004) CLIC-2 modulates cardiac ryanodine receptor Ca2+ release channels. Int J Biochem Cell Biol 36:1599–1612

Bonora M, Bononi A, De Marchi E, Giorgi C, Lebiedzinska M, Marchi S, Patergnani S, Rimessi A, Suski JM, Wojtala A, Wieckowski MR, Kroemer G, Galluzzi L, Pinton P (2013) Role of the c subunit of the FO ATP synthase in mitochondrial permeability transition. Cell Cycle 12:674–683

Boquet P, Ricci V, Galmiche A, Gauthier NC (2003) Gastric cell apoptosis and h. Pylori: has the main function of VacA finally been identified? Trends Microbiol 11:410–413

Borecky J, Jezek P, Siemen D (1997) 108-ps channel in brown fat mitochondria might be identical to the inner membrane anion channel. J Biol Chem 272:19282–19289

Bose T, Cieslar-Pobuda A, Wiechec E (2015) Role of ion channels in regulating ca (2)(+) homeostasis during the interplay between immune and cancer cells. Cell Death Dis 6, e1648

Bourke RS, Waldman JB, Kimelberg HK, Barron KD, San Filippo BD, Popp AJ, Nelson LR (1981) Adenosine-stimulated astroglial swelling in cat cerebral cortex in vivo with total inhibition by a non-diuretic acylaryloxyacid derivative. J Neurosurg 55:364–370

Brdiczka D, Kaldis P, Wallimann T (1994) In vitro complex formation between the octamer of mitochondrial creatine kinase and porin. J Biol Chem 269:27640–27644

Brierley GP (1969) Energy-linked alteration of mitochondrial permeability to anions. Biochem Biophys Res Commun 35:396–402

Broekemeier KM, Dempsey ME, Pfeiffer DR (1989) Cyclosporin a is a potent inhibitor of the inner membrane permeability transition in liver mitochondria. J Biol Chem 264:7826–7830

Buettner R, Papoutsoglou G, Scemes E, Spray DC, Dermietzel R (2000) Evidence for secretory pathway localization of a voltage-dependent anion channel isoform. Proc Natl Acad Sci U S A 97:3201–3206

Checchetto V, Reina S, Magri A, Szabo I, De Pinto V (2014) Recombinant human voltage dependent anion selective channel isoform 3 (hVDAC3) forms pores with a very small conductance. Cell Physiol Biochem 34:842–853

Chen Y, Craigen WJ, Riley DJ (2009) Nek1 regulates cell death and mitochondrial membrane permeability through phosphorylation of VDAC1. Cell Cycle 8:257–267

Chen Y, Gaczynska M, Osmulski P, Polci R, Riley DJ (2010) Phosphorylation by nek1 regulates opening and closing of voltage dependent anion channel 1. Biochem Biophys Res Commun 394:798–803

Cheng EH, Sheiko TV, Fisher JK, Craigen WJ, Korsmeyer SJ (2003) VDAC2 inhibits BAK activation and mitochondrial apoptosis. Science 301:513–517

Chuang JZ, Milner TA, Zhu M, Sung CH (1999) A 29 kDa intracellular chloride channel p64H1 is associated with large dense-core vesicles in rat hippocampal neurons. J Neurosci 19:2919–2928

Colombini M (1979) A candidate for the permeability pathway of the outer mitochondrial membrane. Nature 279:643–645

Colombini M (2004) VDAC: the channel at the interface between mitochondria and the cytosol. Mol Cell Biochem 256–257:107–115

Colombini M (2012) Mitochondrial outer membrane channels. Chem Rev 112:6373–6387

Colombini M, Mannella CA (1818) VDAC, the early days. Biochim Biophys Acta 2012:1438–1443

Cover TL, Blanke SR (2005) Helicobacter pylori VacA, a paradigm for toxin multifunctionality. Nat Rev Microbiol 3:320–332

Crompton M, Ellinger H, Costi A (1988) Inhibition by cyclosporin a of a Ca2 + −dependent pore in heart mitochondria activated by inorganic phosphate and oxidative stress. Biochem J 255:357–360

Czajkowsky DM, Iwamoto H, Cover TL, Shao Z (1999) The vacuolating toxin from helicobacter pylori forms hexameric pores in lipid bilayers at low pH. Proc Natl Acad Sci U S A 96:2001–2006

Das S, Wong R, Rajapakse N, Murphy E, Steenbergen C (2008) Glycogen synthase kinase 3 inhibition slows mitochondrial adenine nucleotide transport and regulates voltage-dependent anion channel phosphorylation. Circ Res 103:983–991

Davidson AM, Halestrap AP (1990) Partial inhibition by cyclosporin a of the swelling of liver mitochondria in vivo and in vitro induced by sub-micromolar [Ca2+], but not by butyrate. Evidence for two distinct swelling mechanisms. Biochem J 268:147–152

De Marchi U, Szabo I, Cereghetti GM, Hoxha P, Craigen WJ, Zoratti M (1777) A maxi-chloride channel in the inner membrane of mammalian mitochondria. Biochim Biophys Acta 2008:1438–1448

De Marchi U, Basso E, Szabo I, Zoratti M (2006) Electrophysiological characterization of the cyclophilin D-deleted mitochondrial permeability transition pore. Mol Membr Biol 23:521–530

De Pinto V, Ludwig O, Krause J, Benz R, Palmieri F (1987) Porin pores of mitochondrial outer membranes from high and low eukaryotic cells: biochemical and biophysical characterization. Biochim Biophys Acta 894:109–119

De Pinto V, Messina A, Lane DJ, Lawen A (2010) Voltage-dependent anion-selective channel (VDAC) in the plasma membrane. FEBS Lett 584:1793–1799

Decher N, Lang HJ, Nilius B, Bruggemann A, Busch AE, Steinmeyer K (2001) DCPIB is a novel selective blocker of I(Cl, swell) and prevents swelling-induced shortening of guinea-pig atrial action potential duration. Br J Pharmacol 134:1467–1479

Diaz RJ, Losito VA, Mao GD, Ford MK, Backx PH, Wilson GJ (1999) Chloride channel inhibition blocks the protection of ischemic preconditioning and hypo-osmotic stress in rabbit ventricular myocardium. Circ Res 84:763–775

Diaz RJ, Hinek A, Wilson GJ (2010) Direct evidence of chloride ion efflux in ischaemic and pharmacological preconditioning of cultured cardiomyocytes. Cardiovasc Res 87:545–551

Duan DD (2013) Phenomics of cardiac chloride channels. Compr Physiol 3:667–692

Duncan RR, Westwood PK, Boyd A, Ashley RH (1997) Rat brain p64H1, expression of a new member of the p64 chloride channel protein family in endoplasmic reticulum. J Biol Chem 272:23880–23886

Edwards JC, Kahl CR (2010) Chloride channels of intracellular membranes. FEBS Lett 584:2102–2111

Edwards JC, Tulk B, Schlesinger PH (1998) Functional expression of p64, an intracellular chloride channel protein. J Membr Biol 163:119–127

Elinder F, Akanda N, Tofighi R, Shimizu S, Tsujimoto Y, Orrenius S, Ceccatelli S (2005) Opening of plasma membrane voltage-dependent anion channels (VDAC) precedes caspase activation in neuronal apoptosis induced by toxic stimuli. Cell Death Differ 12:1134–1140

Elter A, Hartel A, Sieben C, Hertel B, Fischer-Schliebs E, Luttge U, Moroni A, Thiel G (2007) A plant homolog of animal chloride intracellular channels (CLICs) generates an ion conductance in heterologous systems. J Biol Chem 282:8786–8792

Fernandez-Salas E, Sagar M, Cheng C, Yuspa SH, Weinberg WC (1999) P53 and tumor necrosis factor alpha regulate the expression of a mitochondrial chloride channel protein. J Biol Chem 274:36488–36497

Fernandez-Salas E, Suh KS, Speransky VV, Bowers WL, Levy JM, Adams T, Pathak KR, Edwards LE, Hayes DD, Cheng C, Steven AC, Weinberg WC, Yuspa SH (2002) MtCLIC/CLIC4, an organellular chloride channel protein, is increased by DNA damage and participates in the apoptotic response to p53. Mol Cell Biol 22:3610–3620

Feske S, Wulff H, Skolnik EY (2015) Ion channels in innate and adaptive immunity. Annu Rev Immunol 33:291–353

Fournier N, Ducet G, Crevat A (1987) Action of cyclosporine on mitochondrial calcium fluxes. J Bioenerg Biomembr 19:297–303

Garlid KD, Beavis AD (1986) Evidence for the existence of an inner membrane anion channel in mitochondria. Biochim Biophys Acta 853:187–204

Gincel D, Zaid H, Shoshan-Barmatz V (2001) Calcium binding and translocation by the voltage-dependent anion channel: a possible regulatory mechanism in mitochondrial function. Biochem J 358:147–155

Godbole A, Varghese J, Sarin A, Mathew MK (1642) VDAC is a conserved element of death pathways in plant and animal systems. Biochim Biophys Acta 2003:87–96

Gupta R, Ghosh S (2015) Phosphorylation of voltage-dependent anion channel by c-jun n-terminal kinase-3 leads to closure of the channel. Biochem Biophys Res Commun 459:100–106

Halestrap AP (2014) The C ring of the F1FO ATP synthase forms the mitochondrial permeability transition pore: a critical appraisal. Front Oncol 4:234

Halestrap AP, Clarke SJ, Javadov SA (2004) Mitochondrial permeability transition pore opening during myocardial reperfusion–a target for cardioprotection. Cardiovasc Res 61:372–385
Halle-Smith SC, Murray AG, Selwyn MJ (1988) Palmitoyl-coa inhibits the mitochondrial inner membrane anion-conducting channel. FEBS Lett 236:155–158
Hayman KA, Spurway TD, Ashley RH (1993) Single anion channels reconstituted from cardiac mitoplasts. J Membr Biol 136:181–190
Herick K, Kramer R, Luhring H (1997) Patch clamp investigation into the phosphate carrier from saccharomyces cerevisiae mitochondria. Biochim Biophys Acta 1321:207–220
Hiller S, Garces RG, Malia TJ, Orekhov VY, Colombini M, Wagner G (2008) Solution structure of the integral human membrane protein VDAC-1 in detergent micelles. Science 321:1206–1210
Hoang T, Matovic T, Parker J, Smith MD, Jelokhani-Niaraki M (2015) Role of positively charged residues of the second transmembrane domain in the ion transport activity and conformation of human uncoupling protein-2. Biochemistry 54:2303–2313
Huang SG, Klingenberg M (1996) Chloride channel properties of the uncoupling protein from brown adipose tissue mitochondria: a patch-clamp study. Biochemistry 35:16806–16814
Hunter DR, Haworth RA, Southard JH (1976) Relationship between configuration, function, and permeability in calcium-treated mitochondria. J Biol Chem 251:5069–5077
Iwamoto H, Czajkowsky DM, Cover TL, Szabo G, Shao Z (1999) VacA from helicobacter pylori: a hexameric chloride channel. FEBS Lett 450:101–104
Jentsch TJ, Stein V, Weinreich F, Zdebik AA (2002) Molecular structure and physiological function of chloride channels. Physiol Rev 82:503–568
Jentsch TJ, Hubner CA, Fuhrmann JC (2004) Ion channels: function unravelled by dysfunction. Nat Cell Biol 6:1039–1047
Jezek P, Orosz DE, Garlid KD (1990) Reconstitution of the uncoupling protein of brown adipose tissue mitochondria. Demonstration of GDP-sensitive halide anion uniport. J Biol Chem 265:19296–19302
Kerner J, Lee K, Tandler B, Hoppel CL (1818) VDAC proteomics: post-translation modifications. Biochim Biophys Acta 2012:1520–1525
Kinnally KW, Antonsson B (2007) A tale of two mitochondrial channels, MAC and PTP, in apoptosis. Apoptosis 12:857–868
Kinnally KW, Zorov D, Antonenko Y, Perini S (1991) Calcium modulation of mitochondrial inner membrane channel activity. Biochem Biophys Res Commun 176:1183–1188
Kinnally KW, Antonenko YN, Zorov DB (1992) Modulation of inner mitochondrial membrane channel activity. J Bioenerg Biomembr 24:99–110
Klitsch T, Siemen D (1991) Inner mitochondrial membrane anion channel is present in brown adipocytes but is not identical with the uncoupling protein. J Membr Biol 122:69–75
Kokoszka JE, Waymire KG, Levy SE, Sligh JE, Cai J, Jones DP, MacGregor GR, Wallace DC (2004) The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore. Nature 427:461–465
Kusano H, Shimizu S, Koya RC, Fujita H, Kamada S, Kuzumaki N, Tsujimoto Y (2000) Human gelsolin prevents apoptosis by inhibiting apoptotic mitochondrial changes via closing VDAC. Oncogene 19:4807–4814
Landry DW, Akabas MH, Redhead C, Edelman A, Cragoe EJ Jr, Al-Awqati Q (1989) Purification and reconstitution of chloride channels from kidney and trachea. Science 244:1469–1472
Laus MN, Soccio M, Trono D, Cattivelli L, Pastore D (2008) Plant inner membrane anion channel (PIMAC) function in plant mitochondria. Plant Cell Physiol 49:1039–1055
Leanza L, Biasutto L, Manago A, Gulbins E, Zoratti M, Szabo I (2013) Intracellular ion channels and cancer. Front Physiol 4:227
Lee AC, Xu X, Colombini M (1996) The role of pyridine dinucleotides in regulating the permeability of the mitochondrial outer membrane. J Biol Chem 271:26724–26731
Littler DR, Harrop SJ, Brown LJ, Pankhurst GJ, Mynott AV, Luciani P, Mandyam RA, Mazzanti M, Tanda S, Berryman MA, Breit SN, Curmi PM (2008) Comparison of vertebrate

and invertebrate CLIC proteins: the crystal structures of caenorhabditis elegans EXC-4 and drosophila melanogaster dmCLIC. Proteins 71:364–378
Littler DR, Harrop SJ, Goodchild SC, Phang JM, Mynott AV, Jiang L, Valenzuela SM, Mazzanti M, Brown LJ, Breit SN, Curmi PM (2010) The enigma of the CLIC proteins: ion channels, redox proteins, enzymes, scaffolding proteins? FEBS Lett 584:2093–2101
Lodish H, Berk A, Zipursky SL, Matsudaira P, Baltimore D, Darnell J (2000) Section 15.4 Intracellular ion environment and membrane electric potential. In: Molecular cell biology, 4th edn. Scientific American Books, New York
Machida K, Ohta Y, Osada H (2006) Suppression of apoptosis by cyclophilin D via stabilization of hexokinase ii mitochondrial binding in cancer cells. J Biol Chem 281:14314–14320
Malekova L, Tomaskova J, Novakova M, Stefanik P, Kopacek J, Lakatos B, Pastorekova S, Krizanova O, Breier A, Ondrias K (2007) Inhibitory effect of DIDS, NPPB, and phloretin on intracellular chloride channels. Pflugers Arch 455:349–357
Malia TJ, Wagner G (2007) Nmr structural investigation of the mitochondrial outer membrane protein VDAC and its interaction with antiapoptotic bcl-xl. Biochemistry 46:514–525
Mannella CA (1997) Minireview: on the structure and gating mechanism of the mitochondrial channel, VDAC. J Bioenerg Biomembr 29:525–531
Menzel VA, Cassara MC, Benz R, de Pinto V, Messina A, Cunsolo V, Saletti R, Hinsch KD, Hinsch E (2009) Molecular and functional characterization of VDAC2 purified from mammal spermatozoa. Biosci Rep 29:351–362
Mertins B, Psakis G, Essen LO (2014) Voltage-dependent anion channels: the wizard of the mitochondrial outer membrane. Biol Chem 395:1435–1442
Misak A, Grman M, Malekova L, Novotova M, Markova J, Krizanova O, Ondrias K, Tomaskova Z (2013) Mitochondrial chloride channels: electrophysiological characterization and ph induction of channel pore dilation. Eur Biophys J 42:709–720
Murphy MP (2009) How mitochondria produce reactive oxygen species. Biochem J 417:1–13
Neumann D, Buckers J, Kastrup L, Hell SW, Jakobs S (2010) Two-color sted microscopy reveals different degrees of colocalization between hexokinase-i and the three human VDAC isoforms. PMC Biophys 3:4
Nicholls DG, Lindberg O (1973) Brown-adipose-tissue mitochondria. The influence of albumin and nucleotides on passive ion permeabilities. Eur J Biochem 37:523–530
O'Rourke B (2000) Pathophysiological and protective roles of mitochondrial ion channels. J Physiol 529(Pt 1):23–36
O'Rourke B (2007) Mitochondrial ion channels. Annu Rev Physiol 69:19–49
O'Rourke B, Cortassa S, Aon MA (2005) Mitochondrial ion channels: gatekeepers of life and death. Physiology (Bethesda) 20:303–315
Perkins G, Renken C, Martone ME, Young SJ, Ellisman M, Frey T (1997) Electron tomography of neuronal mitochondria: three-dimensional structure and organization of cristae and membrane contacts. J Struct Biol 119:260–272
Petronilli V, Szabo I, Zoratti M (1989) The inner mitochondrial membrane contains ion-conducting channels similar to those found in bacteria. FEBS Lett 259:137–143
Ponnalagu D, Gururaja Rao S, Farber J, Xin W, Hussain AT, Shah K, Tanda S, Berryman M, Edwards JC, Singh H (2016a) Molecular identity of cardiac mitochondrial chloride intracellular channel proteins. Mitochondrion 27:6–14
Ponnalagu D, Rao SG, Farber J, Xin W, Hussain AT, Shah K, Tanda S, Berryman MA, Edwards JC, Singh H (2016b) Data supporting characterization of CLIC1, CLIC4, CLIC5 and dmCLIC antibodies and localization of CLICs in endoplasmic reticulum of cardiomyocytes. Data Brief 7:1038–1044
Powers MF, Beavis AD (1991) Triorganotins inhibit the mitochondrial inner membrane anion channel. J Biol Chem 266:17250–17256
Powers MF, Smith LL, Beavis AD (1994) On the relationship between the mitochondrial inner membrane anion channel and the adenine nucleotide translocase. J Biol Chem 269:10614–10620

Prindle A, Liu J, Asally M, Ly S, Garcia-Ojalvo J, Suel GM (2015) Ion channels enable electrical communication in bacterial communities. Nature 527:59–63

Proutski I, Karoulias N, Ashley RH (2002) Overexpressed chloride intracellular channel protein CLIC4 (p64H1) is an essential component of novel plasma membrane anion channels. Biochem Biophys Res Commun 297:317–322

Rassow J (2011) Helicobacter pylori vacuolating toxin a and apoptosis. Cell Commun Signal 9:26

Rodríguez PFG, Wu Y, Singh H, Toro L, Stefani E (2012) Building a fast scanning stimulated emission depletion microscope: a step by step guide. In: Méndez-Vilas A (ed) Current microscopy contributions to advances in science and technology. Formatex Research Center, pp 791–800

Roelfsema MR, Hedrich R, Geiger D (2012) Anion channels: master switches of stress responses. Trends Plant Sci 17:221–229

Rostovtseva TK, Bezrukov SM (2008) VDAC regulation: role of cytosolic proteins and mitochondrial lipids. J Bioenerg Biomembr 40:163–170

Sabirov RZ, Sheiko T, Liu H, Deng D, Okada Y, Craigen WJ (2006) Genetic demonstration that the plasma membrane maxianion channel and voltage-dependent anion channels are unrelated proteins. J Biol Chem 281:1897–1904

Salvi M, Brunati AM, Toninello A (2005) Tyrosine phosphorylation in mitochondria: a new frontier in mitochondrial signaling. Free Radic Biol Med 38:1267–1277

Schein SJ, Colombini M, Finkelstein A (1976) Reconstitution in planar lipid bilayers of a voltage-dependent anion-selective channel obtained from paramecium mitochondria. J Membr Biol 30:99–120

Schinzel AC, Takeuchi O, Huang Z, Fisher JK, Zhou Z, Rubens J, Hetz C, Danial NN, Moskowitz MA, Korsmeyer SJ (2005) Cyclophilin D is a component of mitochondrial permeability transition and mediates neuronal cell death after focal cerebral ischemia. Proc Natl Acad Sci U S A 102:12005–12010

Schulz GE (2002) The structure of bacterial outer membrane proteins. Biochim Biophys Acta 1565:308–317

Sena LA, Chandel NS (2012) Physiological roles of mitochondrial reactive oxygen species. Mol Cell 48:158–167

Shanmughapriya S, Rajan S, Hoffman NE, Higgins AM, Tomar D, Nemani N, Hines KJ, Smith DJ, Eguchi A, Vallem S, Shaikh F, Cheung M, Leonard NJ, Stolakis RS, Wolfers MP, Ibetti J, Chuprun JK, Jog NR, Houser SR, Koch WJ, Elrod JW, Madesh M (2015) Spg7 is an essential and conserved component of the mitochondrial permeability transition pore. Mol Cell 60:47–62

Sheldon KL, Maldonado EN, Lemasters JJ, Rostovtseva TK, Bezrukov SM (2011) Phosphorylation of voltage-dependent anion channel by serine/threonine kinases governs its interaction with tubulin. PLoS One 6, e25539

Sheppard DN, Welsh MJ (1992) Effect of ATP-sensitive K+ channel regulators on cystic fibrosis transmembrane conductance regulator chloride currents. J Gen Physiol 100:573–591

Shimizu S, Narita M, Tsujimoto Y (1999) Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature 399:483–487

Shimizu S, Ide T, Yanagida T, Tsujimoto Y (2000) Electrophysiological study of a novel large pore formed by bax and the voltage-dependent anion channel that is permeable to cytochrome c. J Biol Chem 275:12321–12325

Shoshan-Barmatz V, Keinan N, Abu-Hamad S, Tyomkin D, Aram L (1797) Apoptosis is regulated by the VDAC1 n-terminal region and by VDAC oligomerization: release of cytochrome c, aif and smac/diablo. Biochim Biophys Acta 2010:1281–1291

Shoshan-Barmatz V, Ben-Hail D, Admoni L, Krelin Y, Tripathi SS (1848) The mitochondrial voltage-dependent anion channel 1 in tumor cells. Biochim Biophys Acta 2015:2547–2575

Shulga N, Wilson-Smith R, Pastorino JG (2010) Sirtuin-3 deacetylation of cyclophilin D induces dissociation of hexokinase ii from the mitochondria. J Cell Sci 123:894–902

Singh H (2010) Two decades with dimorphic chloride intracellular channels (CLICs). FEBS Lett 584:2112–2121
Singh H, Ashley RH (2006) Redox regulation of CLIC1 by cysteine residues associated with the putative channel pore. Biophys J 90:1628–1638
Singh H, Ashley RH (2007) CLIC4 (p64H1) and its putative transmembrane domain form poorly selective, redox-regulated ion channels. Mol Membr Biol 24:41–52
Singh H, Cousin MA, Ashley RH (2007) Functional reconstitution of mammalian 'chloride intracellular channels' CLIC1, CLIC4 and CLIC5 reveals differential regulation by cytoskeletal actin. FEBS J 274:6306–6316
Singh H, Warburton S, Vondriska TM, Khakh BS (2009) Proteomics to identify proteins interacting with p2x2 ligand-gated cation channels. J Vis Exp
Singh H, Stefani E, Toro L (2012a) Intracellular bk(ca) (ibk(ca)) channels. J Physiol 590:5937–5947
Singh H, Lu R, Rodriguez PF, Wu Y, Bopassa JC, Stefani E, Toro L (2012b) Visualization and quantification of cardiac mitochondrial protein clusters with STED microscopy. Mitochondrion 12:230–236
Sollner K (1955) A physicochemical cell model which simultaneously accumulates anions and cations against concentration gradients. Arch Biochem Biophys 54:129–134
Sorgato MC, Keller BU, Stuhmer W (1987) Patch-clamping of the inner mitochondrial membrane reveals a voltage-dependent ion channel. Nature 330:498–500
Sorgato MC, Moran O, De Pinto V, Keller BU, Stuehmer W (1989) Further investigation on the high-conductance ion channel of the inner membrane of mitochondria. J Bioenerg Biomembr 21:485–496
Stockdale M, Dawson AP, Selwyn MJ (1970) Effects of trialkyltin and triphenyltin compounds on mitochondrial respiration. Eur J Biochem 15:342–351
Stoychev SH, Nathaniel C, Fanucchi S, Brock M, Li S, Asmus K, Woods VL Jr, Dirr HW (2009) Structural dynamics of soluble chloride intracellular channel protein CLIC1 examined by amide hydrogen-deuterium exchange mass spectrometry. Biochemistry 48:8413–8421
Suh KS, Mutoh M, Nagashima K, Fernandez-Salas E, Edwards LE, Hayes DD, Crutchley JM, Marin KG, Dumont RA, Levy JM, Cheng C, Garfield S, Yuspa SH (2004) The organellular chloride channel protein CLIC4/mtCLIC translocates to the nucleus in response to cellular stress and accelerates apoptosis. J Biol Chem 279:4632–4641
Suh KS, Mutoh M, Mutoh T, Li L, Ryscavage A, Crutchley JM, Dumont RA, Cheng C, Yuspa SH (2007) Clic4 mediates and is required for ca2 + −induced keratinocyte differentiation. J Cell Sci 120:2631–2640
Sun Y, Olson R, Horning M, Armstrong N, Mayer M, Gouaux E (2002) Mechanism of glutamate receptor desensitization. Nature 417:245–253
Szabo I, Zoratti M (1993) The mitochondrial permeability transition pore may comprise VDAC molecules. I. Binary structure and voltage dependence of the pore. FEBS Lett 330:201–205
Szabo I, Zoratti M (2014) Mitochondrial channels: ion fluxes and more. Physiol Rev 94:519–608
Szabo I, De Pinto V, Zoratti M (1993) The mitochondrial permeability transition pore may comprise VDAC molecules. Ii. The electrophysiological properties of VDAC are compatible with those of the mitochondrial megachannel. FEBS Lett 330:206–210
Szabo I, Brutsche S, Tombola F, Moschioni M, Satin B, Telford JL, Rappuoli R, Montecucco C, Papini E, Zoratti M (1999) Formation of anion-selective channels in the cell plasma membrane by the toxin VacA of helicobacter pylori is required for its biological activity. EMBO J 18:5517–5527
Takano K, Liu D, Tarpey P, Gallant E, Lam A, Witham S, Alexov E, Chaubey A, Stevenson RE, Schwartz CE, Board PG, Dulhunty AF (2012) An x-linked channelopathy with cardiomegaly due to a CLIC2 mutation enhancing ryanodine receptor channel activity. Hum Mol Genet 21:4497–4507
Tan W, Colombini M (1768) VDAC closure increases calcium ion flux. Biochim Biophys Acta 2007:2510–2515

Tombola F, Del Giudice G, Papini E, Zoratti M (2000) Blockers of VacA provide insights into the structure of the pore. Biophys J 79:863–873

Tonini R, Ferroni A, Valenzuela SM, Warton K, Campbell TJ, Breit SN, Mazzanti M (2000) Functional characterization of the NCC27 nuclear protein in stable transfected CHO-K1 cells. FASEB J 14:1171–1178

Toro L, Li M, Zhang Z, Singh H, Wu Y, Stefani E (2014) Maxik channel and cell signalling. Pflugers Arch 466:875–886

Tulk BM, Kapadia S, Edwards JC (2002) CLIC1 inserts from the aqueous phase into phospholipid membranes, where it functions as an anion channel. Am J Physiol Cell Physiol 282:C1103–C1112

Tung JJ, Hobert O, Berryman M, Kitajewski J (2009) Chloride intracellular channel 4 is involved in endothelial proliferation and morphogenesis in vitro. Angiogenesis 12:209–220

Ujwal R, Cascio D, Colletier JP, Faham S, Zhang J, Toro L, Ping P, Abramson J (2008) The crystal structure of mouse VDAC1 at 2.3 a resolution reveals mechanistic insights into metabolite gating. Proc Natl Acad Sci U S A 105:17742–17747

Ulmasov B, Bruno J, Woost PG, Edwards JC (2007) Tissue and subcellular distribution of CLIC1. BMC Cell Biol 8:8

Valenzuela SM, Martin DK, Por SB, Robbins JM, Warton K, Bootcov MR, Schofield PR, Campbell TJ, Breit SN (1997) Molecular cloning and expression of a chloride ion channel of cell nuclei. J Biol Chem 272:12575–12582

Valenzuela SM, Mazzanti M, Tonini R, Qiu MR, Warton K, Musgrove EA, Campbell TJ, Breit SN (2000) The nuclear chloride ion channel NCC27 is involved in regulation of the cell cycle. J Physiol 529(Pt 3):541–552

Verkman AS, Galietta LJ (2009) Chloride channels as drug targets. Nat Rev Drug Discov 8:153–171

Verma M, Shulga N, Pastorino JG (2013) Sirtuin-3 modulates Bak- and Bax-dependent apoptosis. J Cell Sci 126:274–288

Wang X, Cao Y, Shen M, Wang B, Zhang W, Liu Y, He X, Wang L, Xia Y, Ding M, Xu X, Ren J (2015) Dids reduces ischemia/reperfusion-induced myocardial injury in rats. Cell Physiol Biochem 35:676–688

Warton K, Tonini R, Fairlie WD, Matthews JM, Valenzuela SM, Qiu MR, Wu WM, Pankhurst S, Bauskin AR, Harrop SJ, Campbell TJ, Curmi PM, Breit SN, Mazzanti M (2002) Recombinant CLIC1 (NCC27) assembles in lipid bilayers via a ph-dependent two-state process to form chloride ion channels with identical characteristics to those observed in chinese hamster ovary cells expressing CLIC1. J Biol Chem 277:26003–26011

Weber-Schurholz S, Wischmeyer E, Laurien M, Jockusch H, Schurholz T, Landry DW, al-Awqati Q (1993) Indanyloxyacetic acid-sensitive chloride channels from outer membranes of skeletal muscle. J Biol Chem 268:547–551

Willhite DC, Blanke SR (2004) Helicobacter pylori vacuolating cytotoxin enters cells, localizes to the mitochondria, and induces mitochondrial membrane permeability changes correlated to toxin channel activity. Cell Microbiol 6:143–154

Wojciak-Stothard B, Abdul-Salam VB, Lao KH, Tsang H, Irwin DC, Lisk C, Loomis Z, Stenmark KR, Edwards JC, Yuspa SH, Howard LS, Edwards RJ, Rhodes CJ, Gibbs JS, Wharton J, Zhao L, Wilkins MR (2014) Aberrant chloride intracellular channel 4 expression contributes to endothelial dysfunction in pulmonary arterial hypertension. Circulation 129:1770–1780

Wolf A, Agnihotri S, Micallef J, Mukherjee J, Sabha N, Cairns R, Hawkins C, Guha A (2011) Hexokinase 2 is a key mediator of aerobic glycolysis and promotes tumor growth in human glioblastoma multiforme. J Exp Med 208:313–326

Xu X, Decker W, Sampson MJ, Craigen WJ, Colombini M (1999) Mouse VDAC isoforms expressed in yeast: channel properties and their roles in mitochondrial outer membrane permeability. J Membr Biol 170:89–102

Xu Y, Kang J, Yuan Z, Li H, Su J, Li Y, Kong X, Zhang H, Wang W, Sun L (2013) Suppression of CLIC4/mtCLIC enhances hydrogen peroxide-induced apoptosis in c6 glioma cells. Oncol Rep 29:1483–1491

Yamagata H, Shimizu S, Nishida Y, Watanabe Y, Craigen WJ, Tsujimoto Y (2009) Requirement of voltage-dependent anion channel 2 for pro-apoptotic activity of bax. Oncogene 28:3563–3572

Yamaguchi H, Jelokhani-Niaraki M, Kodama H (2004) Second transmembrane domain of human uncoupling protein 2 is essential for its anion channel formation. FEBS Lett 577:299–304

Zernig G, Graziadei I, Moshammer T, Zech C, Reider N, Glossmann H (1990) Mitochondrial ca2+ antagonist binding sites are associated with an inner mitochondrial membrane anion channel. Mol Pharmacol 38:362–369

Zheng XB, Wang R, Yang HL, Sun XL (2013) Effects of chloride ion channel and its blockers on myocardial ischemia reperfusion arrhythmias in rabbits. Zhonghua Yi Xue Za Zhi 93:1168–1173

Zizi M, Forte M, Blachly-Dyson E, Colombini M (1994) Nadh regulates the gating of VDAC, the mitochondrial outer membrane channel. J Biol Chem 269:1614–1616

Zoratti M, Szabo I (1995) The mitochondrial permeability transition. Biochim Biophys Acta 1241:139–176

Guide to the Pharmacology of Mitochondrial Potassium Channels

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Contents

Abstract

This chapter provides a critical overview of the available literature on the pharmacology of mitochondrial potassium channels. In the first part, the reader is introduced to the topic, and eight known protein contributors to the potassium permeability of the inner mitochondrial membrane are presented. The main part of this chapter describes the basic characteristics of each channel type mentioned in the introduction. However, the most important and valuable information included in this chapter concerns the pharmacology of mitochondrial potassium channels. Several available channel modulators are critically evaluated and rated by suitability for research use. The last figure of this chapter shows the results of this evaluation at a glance. Thus, this chapter can be very useful for beginners in

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H. Singh, S.-S. Sheu (eds.), *Pharmacology of Mitochondria*,
Handbook of Experimental Pharmacology 240, DOI 10.1007/164_2016_79

this field. It is intended to be a time- and resource-saving guide for those searching for proper modulators of mitochondrial potassium channels.

Keywords
Cytoprotection • Ischemic preconditioning • mitoBK$_{Ca}$ • Mitochondria • Mitochondrial potassium channels • mitoIK$_{Ca}$ • mitoK$_{ATP}$ • mitoKv1.3 • mitoKv7.4 • mitoSK$_{Ca}$ • mitoSLO2 • mitoTASK3 • Modulators of potassium channels • Potassium channel openers

1 Introduction

The pharmacology of mitochondrial potassium channels attracts the attention of many research groups due to the possibility that such channels may be involved in the regulation of cell physiology. Activation of mitochondrial potassium channels may induce protective cellular mechanisms, and mitochondrial channel inhibition may cause cell death (Szabo and Zoratti 2014).

Two contradictory statements can describe the pharmacology of mitochondrial potassium channels. First, the pharmacology of mitochondrial potassium channels can be described as simple and easy based on numerous experiments with plasmalemmal potassium channels. This is illustrated by charybdotoxin, a plasmalemmal potassium channel inhibitor that blocks large conductance calcium-regulated potassium channels both in the plasmalemma (BK$_{Ca}$ channels) and the inner mitochondrial membrane (IMM) (mitoBK$_{Ca}$ channels).

The second statement describing the pharmacology of mitochondrial potassium channels is that the pharmacology of mitochondrial potassium channels is complicated and difficult because mitochondria constitute a specific and unique environment for drugs. This environment is created by the high inner membrane potential (approximately 180 mV, with the mitochondrial matrix negatively charged) and the alkaline matrix. These properties promote the accumulation of both lipophilic and positively charged drugs. The alkaline matrix promotes accumulation of weak acids within mitochondria. This type of drug accumulation may lead to misinterpretation of changes in mitochondrial function as consequences of regulation of mitochondrial potassium channels, while they are just side effects. Additionally, mitochondrial potassium channels may have low affinity for drugs known to strongly interact with their plasmalemmal counterparts. This case is illustrated by the inhibitor of the ATP-regulated K^+ (K_{ATP}) channel glibenclamide, which has a high-affinity receptor in pancreatic β cell plasmalemma and a low-affinity receptor in mitochondrial membrane.

This chapter describes the pharmacology of mitochondrial potassium channels in the format of a guide intended for beginners. We hope that it will help the reader to rationally choose an optimal drug to study mitochondrial potassium channels.

2 Potassium Channels in the Inner Mitochondrial Membrane

The low permeability of the IMM to charged molecules is critical for maintaining the electrochemical proton gradient that drives ATP synthesis. However, the perpetual exchange of simple ions and complex molecules, such as pyruvate, fatty acids, ATP, or ADP, between the mitochondrial matrix and cytosol is essential for the proper function of mitochondria. Hence, the IMM harbors several types of transport proteins to facilitate this exchange. One of the most diverse groups of such proteins is mitochondrial potassium channels.

Potassium channels are notorious for their role in cytoprotection (Escande and Cavero 1992; Garlid 2000; Malinska et al. 2010). It is broadly accepted that preconditioning with potassium channel openers (KCOs) results in cytoprotection through activation of mitochondrial channels (Facundo et al. 2006). Several hypotheses link opening of mitochondrial potassium channels with cytoprotection (O'Rourke 2004; O'Rourke et al. 2005; Murphy and Steenbergen 2008; Chouchani et al. 2014). Although these hypotheses seem quite distinct, they do not necessarily need to be exclusive.

Mitochondrial potassium channels constitute the largest group of ion channels found in the IMM. Thus far, researchers have described the following eight channels [Fig. 1]: the ATP-regulated potassium channel (mitoK_{ATP}) (Inoue et al. 1991), the large conductance calcium-regulated potassium channel (mitoBK_{Ca}) (Siemen et al. 1999), the intermediate-conductance calcium-regulated potassium channel (mitoIK_{Ca}) (De Marchi et al. 2009), the small-conductance calcium-regulated potassium channel (mitoSK_{Ca}) (Stowe et al. 2013), the voltage-gated potassium channels mitoK_V1.3 (Szabò et al. 2005) and mitoK_V7.4 (Testai et al. 2015), the tandem pore domain acid-sensitive potassium channel type 3 (mitoTASK3) (Rusznák et al. 2008; Toczyłowska-Mamińska et al. 2013), and the SLO2 channel (mitoSLO2) (Wojtovich et al. 2011). It is important to acknowledge that these mitochondrial potassium channels were described in different cell types. Their expression pattern seems to be tissue-specific, and one should not expect that all of the presented proteins coexist in each mitochondrion. In fact, inner membranes of some mitochondria may not harbor any potassium channels.

Mitochondrial potassium channels have been best described in animals; however, they have also been found in the IMM of certain plants (Pastore et al. 1999, 2007; Koszela-Piotrowska et al. 2009; Matkovic et al. 2011) and unicellular protists (Kicinska et al. 2007; Costa and Krieger 2009; Laskowski et al. 2015) [Fig. 1].

Most mitochondrial potassium channels are believed to share structural and functional properties with their plasmalemmal counterparts. However, because the composition and role of the IMM are quite different than those of the plasmalemma, all properties of mitochondrial isoforms must be determined in experiments performed directly on mitochondria. Moreover, the concentrations of ions (especially K^+ and Ca^{2+}) in compartments separated by the plasmalemma and the IMM are different [Table 1]. According to the observations, the normal concentration of K^+ ions in the mitochondrial matrix is roughly the same as in the cytosol (140–150 mM), while it is significantly lower in the extracellular fluid (5 mM).

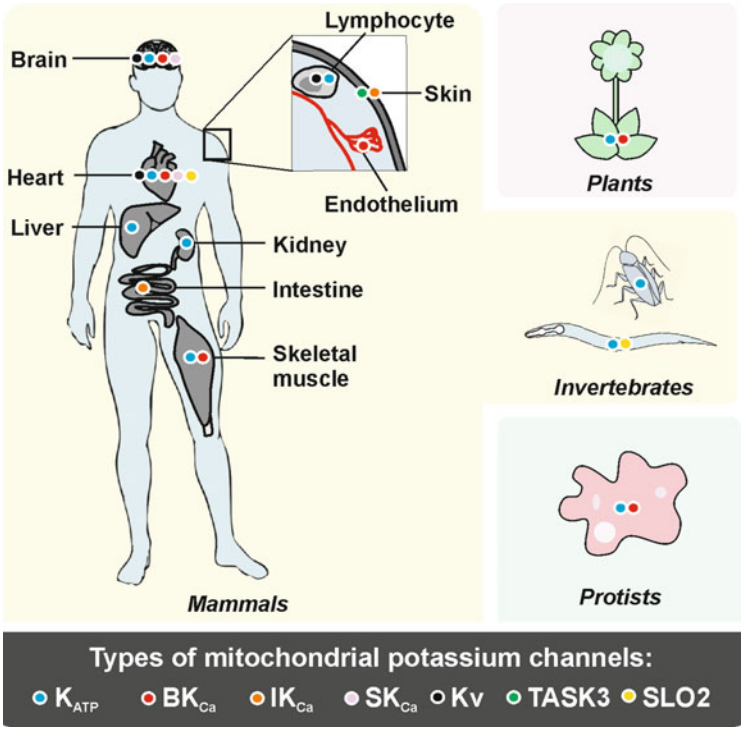

Fig. 1 Reported expression of mitochondrial potassium channels in eukaryotes

Table 1 Concentrations [mM] of potassium and calcium ions in the extracellular fluid, cytosol, and mitochondrial matrix

Ion	Extracellular fluid	Cytosol/Intermembrane space [mM]	Mitochondrial matrix
K^+	5	140–150	140–150
Ca^{2+}	1–2	0.0001	0.001–0.5

Hence, K^+ influx into the mitochondrial matrix upon the opening of mitochondrial potassium channels is driven by the electrical component of the electrochemical gradient at the cost of mitochondrial membrane potential. Thus, potassium channels found in mitochondria may play distinct roles and be regulated differently than their plasmalemmal counterparts.

Despite differences in function and mechanisms of regulation between plasmalemmal and mitochondrial potassium channels, most modulators interact with both types of channels. Notable exceptions are the inhibitor HMR1098, which is believed to block the plasmalemmal isoform of the K_{ATP} channel exclusively (Gögelein et al. 2001), and agitoxin-2, which inhibits the plasmalemmal but not the mitochondrial isoform of the Kv1.3 channel (Bednarczyk et al. 2010). Other

modulators of potassium channels are generally believed to interact with all isoforms of the target protein; however, their selectivity towards distinct isoforms may differ. In some tissues, certain drugs seem to target mitochondrial potassium channels with greater specificity than plasmalemmal isoforms. For example, the modulators of the K_{ATP} channel, diazoxide (channel opener), and 5-hydroxydecanoic acid (5-HD, channel inhibitor) are much more selective towards the mitoK_{ATP} channel. Hence, they are sometimes considered "mitochondrial potassium channel modulators" (Garlid et al. 1996). However, the side effects of these substances can be severe, as they often alter other mitochondrial functions. Consequently, we advise that the so-called mitochondrial potassium channel modulators should always be used with caution.

The number of substances that modulate the activity of potassium channels is enormous. Over 40 compounds modulate the BK_{Ca} channel alone. Therefore, in this chapter, we focus only on the substances whose effectiveness towards the mitochondrial isoforms of these proteins has already been evaluated. However, this does not mean that other modulators are ineffective. On the contrary, it is very likely that most may actually be quite effective but simply have not been proven yet.

2.1 Mitochondrial ATP-Regulated Potassium Channel

The plasmalemmal ATP-regulated potassium channel (K_{ATP}) is widely distributed within the human body. First described in cardiomyocytes (Noma 1983), it is also present in other tissues, such as skeletal muscles and the pancreas. In the latter, it plays a crucial role in the regulation of insulin secretion. K_{ATP} channels may have different structures and subunit compositions depending on their localization (Szabo and Zoratti 2014). In general, they consist of K^+-selective and pore-forming Kir6.X subunits and sulfonylurea receptor (SUR1/2) subunits. For example, the K_{ATP} channel found in sarcolemma consists of four Kir6.X subunits (either Kir6.1 or Kir6.2) and four SUR subunits (SUR1, SUR2, and SUR2B) (Inagaki et al. 1995). K_{ATP} channels are inhibited by ATP. Hence, by sensing the ATP/ADP ratio in the cytoplasm, they possess the unique ability to couple of cellular metabolism with membrane potential.

ATP-regulated potassium channels are also found in intracellular compartments. The mitochondrial (mitoK_{ATP}) isoform of this protein was, in fact, the first potassium channel described in the IMM (Inoue et al. 1991). Therefore, the body of literature concerning mitoK_{ATP} is relatively large, although virtually any aspect of studies on this protein generates controversy. Even its very existence in the IMM has sometimes been questioned. Still, most investigators agree that this protein, despite its low abundance, plays an important role in the physiology of mitochondria. It is well known for its putative involvement in ischemia/reperfusion preconditioning (Liu et al. 1999; Garlid et al. 1997; Murata et al. 2001). The conductance of mitoK_{ATP} calculated by electrophysiological experiments differs greatly among authors as follows: Inoue et al. reported 9–10 pS (Inoue et al. 1991), while Kicinska et al. put the value at 166 pS (Kicinska et al. 2007). This

inconsistency can be partially explained by the cooperative behavior of this channel; mitoK$_{ATP}$ tends to occur and function in clusters (Zoratti et al. 2009).

The molecular identity of the mitoK$_{ATP}$ channel has been subject to much speculation. First, canonical SUR1 and SUR2 proteins are not believed to be components of mitoK$_{ATP}$ channels. Second, genetic knockout of genes encoding Kir6.X proteins failed to suppress mitoK$_{ATP}$ activity (Miki et al. 2002; Wojtovich et al. 2013). These findings suggest that the mitoK$_{ATP}$ channel might be encoded by another gene(s). It was recently proposed that a certain splice variant of the renal outer medullary potassium channel (ROMK) may be the long-sought molecular constituent of the mitoK$_{ATP}$ channel (Foster et al. 2012).

The mitoK$_{ATP}$ channel is widely distributed among eukaryotic organisms. It was first described in rat liver mitochondria (Inoue et al. 1991) and then in several other mammalian cells and organs, including lymphocytes (Dahlem et al. 2004), heart (Garlid et al. 1996), brain (Bajgar et al. 2001; Kulawiak and Bednarczyk 2005), skeletal muscle (Debska et al. 2002), and kidney (Cancherini et al. 2003). It was also found in insects (Slocinska et al. 2013), *C. elegans* (Wojtovich et al. 2008), plants (Chiandussi et al. 2002; Pastore et al. 2007; Matkovic et al. 2011), and unicellular organisms (Kicinska et al. 2007) [Fig. 1].

More than 20 substances have proved to modulate the activity of the mitoK$_{ATP}$ channel (O'Rourke 2004). Unfortunately, most of them, including pinacidil, isoflurane, quinine, cromakalim, and sildenafil, modulate both plasmalemmal and mitochondrial isoforms of K$_{ATP}$ channels. Therefore, we decided to focus on modulators that are the most specific, and hence useful to research on the mitoK$_{ATP}$ channel.

In our opinion, three activators of the mitoK$_{ATP}$ channel deserve mention. First among them is diazoxide, which is generally considered to be a specific activator of the mitochondrial isoform of the K$_{ATP}$ channel (Garlid et al. 1996, 1997). The second is nicorandil (2-(Pyridine-3-carbonylamino)ethyl nitrate), a hybrid compound that activates mitoK$_{ATP}$ channels (Sato et al. 2000) and also possesses vasodilatory properties. Unfortunately, both diazoxide and nicorandil can also activate plasmalemmal K$_{ATP}$ channels at high concentrations and in certain cell types (Harvey and Ashford 1998; Sato et al. 2000). The third one BMS191095 also opens the mitoK$_{ATP}$ channel (Grover and Atwal 2002). It seems that this compound is highly selective towards the mitoK$_{ATP}$ channel and exerts a wide range of mitoK$_{ATP}$-mediated cytoprotective effects (Grover et al. 2001; Malinska et al. 2010). With regard to mitoK$_{ATP}$ channel inhibitors, again, three drugs deserve to be distinguished. First is 5-HD. The relatively simple structure of this compound compared with other modulators of potassium channels makes it unique. Furthermore, it is highly selective towards the mitoK$_{ATP}$ channel and generally does not inhibit the plasmalemmal isoform of this protein. Unfortunately, it was reported to inhibit the sarcK$_{ATP}$ channel at low pH (Notsu et al. 1992). Another inhibitor of the K$_{ATP}$ channel is the bee venom toxin tertiapin Q, which was investigated in recent reports on the molecular identity of mitoK$_{ATP}$ channel (as isoform of ROMK). The plasmalemmal ROMK channel proved to be effectively inhibited by tertiapin Q (Jin et al. 1998) as was the mitoK$_{ATP}$ channel (Foster et al. 2012). However, the obvious

problem with this toxin is the lack of selectivity towards the mitochondrial isoform of ROMK. The last mitoK$_{ATP}$ channel modulator that deserves mention is glibenclamide (O'Rourke 2004). It is a very well-studied compound from the class of antidiabetic drugs known as sulfonylureas. Its main role is to inhibit K$_{ATP}$ channels in the plasmalemma of pancreatic β cells, leading to the depolarization of the plasmalemma and eventually the release of stored insulin from β cells. Although glibenclamide acts through interaction with the SUR subunits of the channel, it can also inhibit the mitoK$_{ATP}$ channel. We mention this drug primarily to acknowledge its longtime use in research on both plasmalemmal and mitochondrial isoforms of K$_{ATP}$ channels. Yet, to inhibit the mitoK$_{ATP}$ channel we strongly advise using 5-HD or tertiapin Q instead. The structures of selected modulators of the mitoK$_{ATP}$ channel are presented in Fig. 2.

2.2 Mitochondrial Calcium-Regulated Potassium Channels

Three types of potassium channels are regulated by calcium ions in the IMM. They differ in terms of the potassium ion conductance. Hence, we distinguish channels as having large (BK$_{Ca}$), intermediate (IK$_{Ca}$), or small (SK$_{Ca}$) potassium conductance. The IK$_{Ca}$ channel was initially included in the SK$_{Ca}$ class (SK4). However, it was eventually upgraded to an independent class of potassium channels due to its significantly higher conductance. Nevertheless, both SK$_{Ca}$ and IK$_{Ca}$ channels share many structural and pharmacological features.

2.2.1 mitoBK$_{Ca}$ Channel

The BK$_{Ca}$ channel (large conductance calcium-regulated potassium channel: K$_{Ca}$1.1, SLO1) is ubiquitously expressed in both excitable and non-excitable cells. A functional BK$_{Ca}$ channel is composed of four identical α-subunits. Each spans the membrane seven times. The BK$_{Ca}$ channel represents a unique class of ion channels not only because of its high single channel conductance (~250–300 pS) but also because it can be activated by calcium ions alone, membrane depolarization alone, or synergistically by both (Magleby 2003). This dual regulation allows BK$_{Ca}$ channels to couple intracellular signaling to membrane potential and significantly modulate physiological responses, such as neuronal signaling and muscle contraction (Nardi and Olesen 2008). Additionally, there are four auxiliary regulatory β subunits (β1–β4) and four γ subunits (γ1–γ4) that can interact with the pore-forming α subunits and modulate the activity of assembled BK$_{Ca}$ channels (Contreras et al. 2013).

All isoforms of the BK$_{Ca}$ channel are products of alternative splicing of a single *Kcnma1* gene (Latorre and Brauchi 2006; Sakai et al. 2011). Unfortunately, the molecular identity of the mitoBK$_{Ca}$ isoform is not fully understood yet. However, the mitochondrial splice variant of the BK$_{Ca}$ channel is believed to have an extended C-terminal domain ending with the amino acid residues DEC (Singh et al. 2013). Expression of the mitoBK$_{Ca}$ channel was reported in several mammalian cell types, including heart (Xu et al. 2002), brain (Kulawiak and Bednarczyk

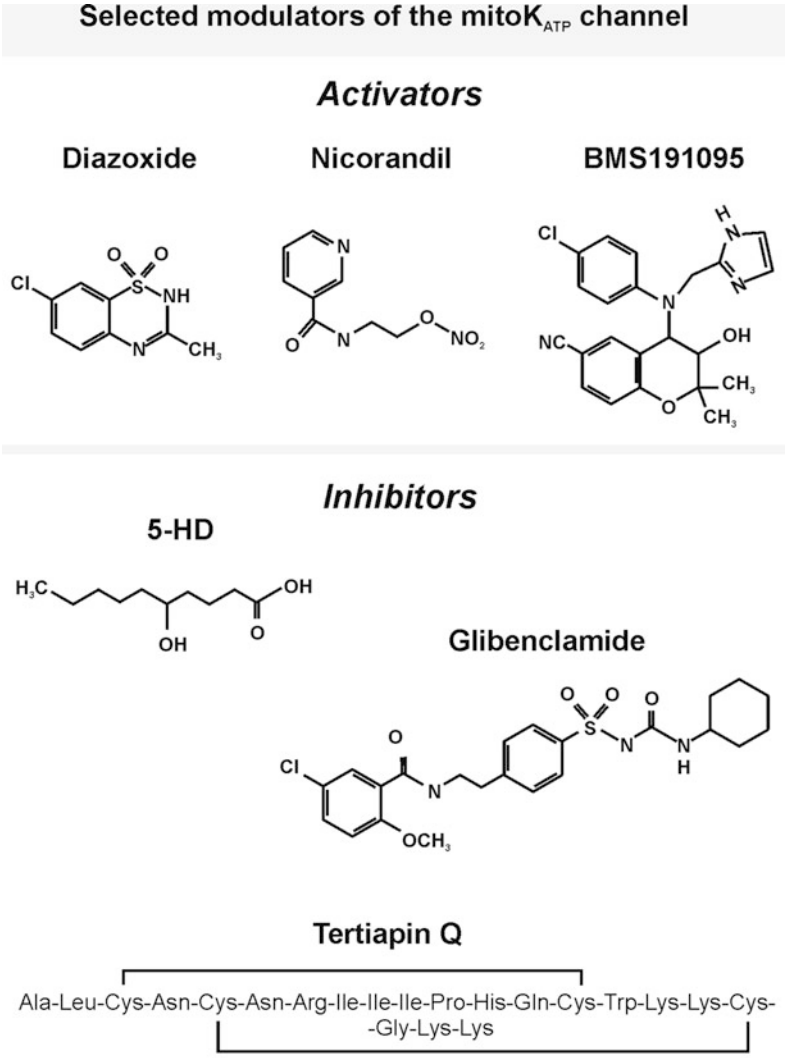

Fig. 2 Structural formulas of selected modulators of the mitoK$_{ATP}$ channel

2005; Piwonska et al. 2008; Skalska et al. 2009; Singh et al. 2016), skeletal muscle (Skalska et al. 2008), and endothelium (Bednarczyk et al. 2013a). It was also found in the mitochondria of certain plants (Koszela-Piotrowska et al. 2009) and members of the *Protista* kingdom (Laskowski et al. 2015) [Fig. 1].

A wide range of exogenous modulators are highly specific towards the BK$_{Ca}$ channel. Few are frequently used in experiments focused on mitoBK$_{Ca}$. By far, the most extensively used modulator is a compound developed by Neuro Search called

NS1619 (1,3-Dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one) (Siemen et al. 1999; Bednarczyk et al. 2013a, b). A second drug called NS11021 (N′-[3,5-Bis(trifluoromethyl)phenyl]-N-[4-bromo-2-(2H-tetrazol-5-yl)phenyl]-thiourea) was developed in the same laboratories and proved active towards mitoBK$_{Ca}$ (Bednarczyk et al. 2013a). Indole esters are another class of synthetic activators of the BK$_{Ca}$ channel disclosed by Ciba-Geigy (now Novartis). Two that are worth mentioning are CGS7184 (ethyl 1-[[(4-chlorophenyl) amino]oxo]-2-hydroxy-6-trifluoromethyl-1H-indole-3-carboxylate) (Kulawiak et al. 2008; Debska-Vielhaber et al. 2009) and CGS7181 (ethyl 2-hydroxy-1-[[(4-methylphenyl)amino]oxo]-6-trifluoromethyl-1H-indole-3-carboxylate) (Debska-Vielhaber et al. 2009). Both modulators proved to be effective in activating the mitoBK$_{Ca}$ channel.

Two major classes of mitoBK$_{Ca}$ channel inhibitors are commonly used. The first class contains the scorpion venom peptide toxins iberiotoxin and charybdotoxin (Galvez et al. 1990; Candia et al. 1992). The second class is represented by a series of indole-diterpenes belonging to a family of tremorgenic mycotoxins isolated from *Penicillium*, *Aspergillus*, and *Claviceps* fungi (Nardi and Olesen 2008). This group includes paxilline (Knaus et al. 1994), which is widely used as an inhibitor of the mitoBK$_{Ca}$ channel. However, in high concentrations, paxilline may affect other mitochondrial functions (Bednarczyk et al. 2008). The schematic structures of selected modulators of the mitoBK$_{Ca}$ channel are presented in Fig. 3.

2.2.2 mitoIK$_{Ca}$ Channel

The IK$_{Ca}$ channel (intermediate-conductance calcium-regulated potassium channel: K$_{Ca}$3.1, SK4, and Gardos channel) is expressed in the plasmalemma of blood cells and certain endothelia and epithelia in which it plays important physiological roles. This channel is thought to be expressed exclusively in the plasmalemma of non-excitable cells (Szabo and Zoratti 2014). Additionally, a wide body of evidence links expression of this protein with certain cancers. Its activity is regulated by calcium ions via tightly bound calmodulin, but, unlike the BK$_{Ca}$ channel, its probability of opening is not influenced by voltage. The IK$_{Ca}$ channel structurally resembles Kv channels and SK$_{Ca}$ channels. It consists of four pore-forming subunits. Each spans the plasmalemma six times. The conductance of this channel is significantly lower than that of the BK$_{Ca}$ channel but higher than that of the SK$_{Ca}$ channel and varies from 20 to 85 pS (Dale et al. 2016).

Plasmalemmal and mitochondrial isoforms of the IK$_{Ca}$ channel seem to be indistinguishable in terms of their molecular identity, pharmacology, and biophysical properties (Szabo and Zoratti 2014). The mitoIK$_{Ca}$ channel was found in cells derived from human colon cancer (De Marchi et al. 2009), in mouse embryonic fibroblasts and HeLa cells (Sassi et al. 2010) [Fig. 1].

Many agents are known to modulate the activity of the plasmalemmal IK$_{Ca}$ channel. However, in research concerning mitochondrial isoforms of this protein, three modulators have proved to be effective thus far, namely, the activator DCEBIO (5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazol-2-one) and the two inhibitors clotrimazole (De Marchi et al. 2009) and TRAM34 (1-[(2-Chlorophenyl)

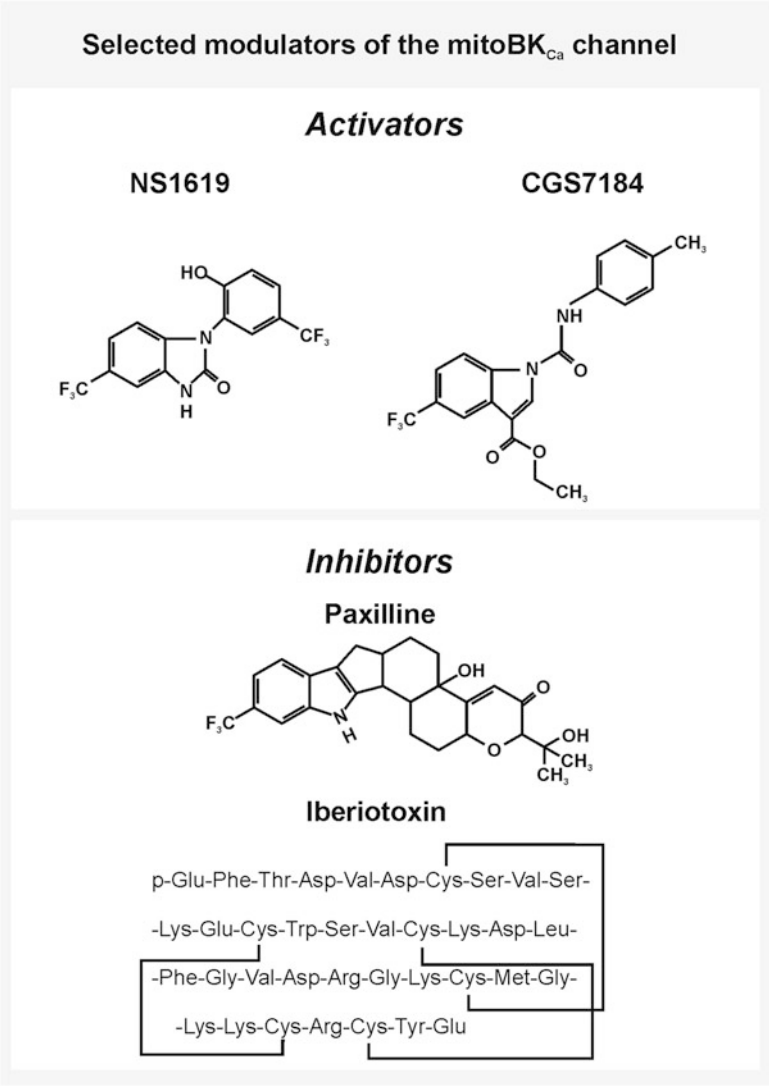

Fig. 3 Structural formulas of selected modulators of the mitoBK$_{Ca}$ channel

diphenylmethyl]-1H-pyrazole) (Sassi et al. 2010). Structural formulas of these compounds are presented in Fig. 4.

2.2.3 mitoSK$_{Ca}$ Channel

SK$_{Ca}$ channels (small-conductance calcium-regulated potassium channels: K$_{Ca}$2. X) constitute the last subfamily of calcium-regulated potassium channels. In contrast to BK$_{Ca}$ and IK$_{Ca}$ channels, their conductance oscillates approximately

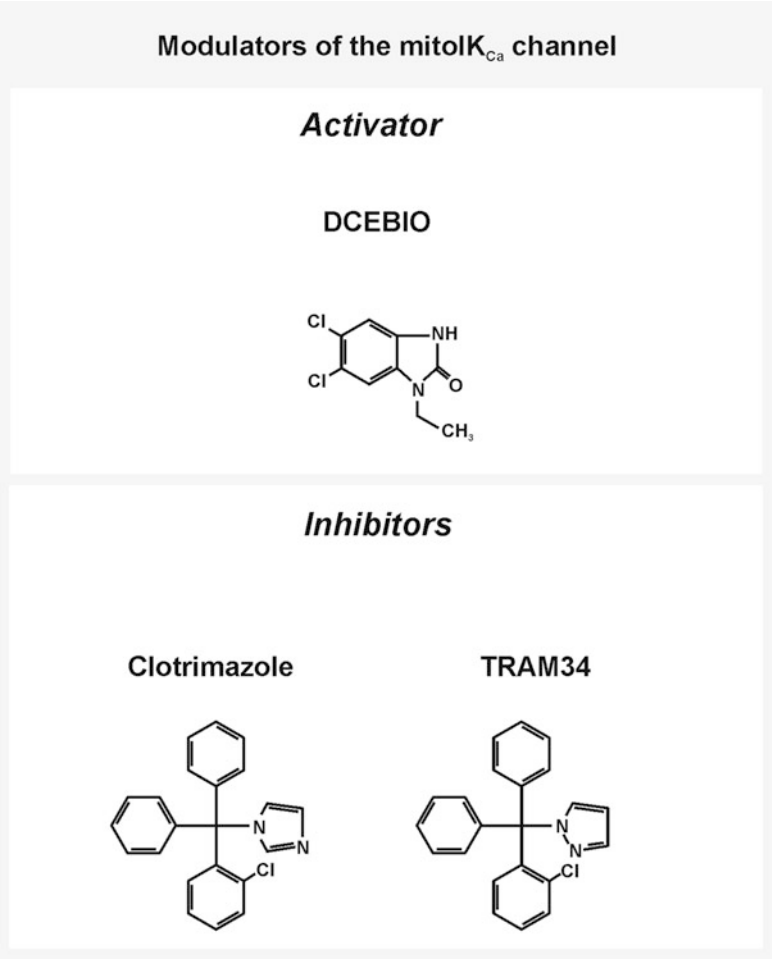

Fig. 4 Structural formulas of modulators of the mitoIK$_{Ca}$ channel

10 pS (hence the name). Similarly to IK$_{Ca}$ channels, they are insensitive to voltage and much more sensitive to Ca^{2+} ions than BK$_{Ca}$ channels due to calmodulin bound to their cytoplasmic tail. SK$_{Ca}$ channels are especially important for the proper function of neurons and other excitable cells. Upon an increase in intracellular Ca^{2+} concentration, SK$_{Ca}$ channels activate and allow K^+ ions to leave the cell according to the chemical gradient across the plasmalemma. This, in turn, leads to the hyperpolarization of the membrane, which limits the firing frequency of action potentials. SK$_{Ca}$ channels are homotetramers of pore-forming subunits that penetrate the plasmalemma six times. Structurally, they do not differ much from IK$_{Ca}$ channels.

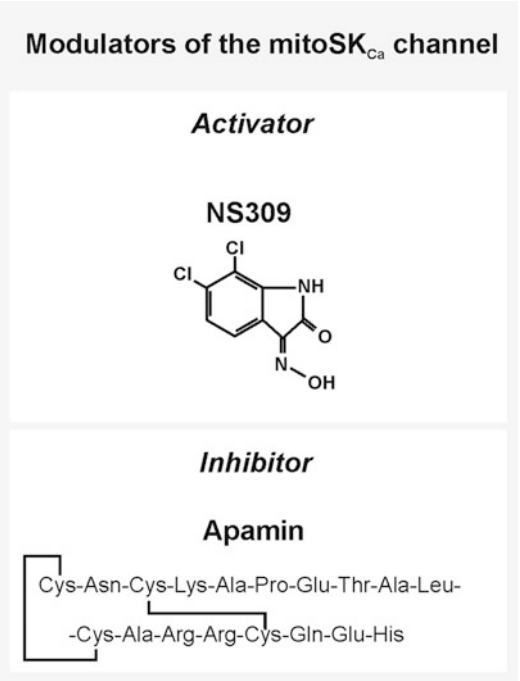

Fig. 5 Structural formulas of modulators of the mitoSK$_{Ca}$ channel (structures of DCEBIO and TRAM34 are found in Fig. 4)

Mitochondrial isoforms of SK$_{Ca}$ channels have been described thus far in cardiac muscle (Stowe et al. 2013) and brain (Dolga et al. 2014) [Fig. 1]. Unfortunately, our knowledge of the pharmacology of mitoSK$_{Ca}$ channels is still rather modest. Because IK$_{Ca}$ and SK$_{Ca}$ channels are very closely related, they partially share a common pharmacology. Thus, DCEBIO and TRAM34 modulate not only mitoIK$_{Ca}$ but also mitoSK$_{Ca}$ channels. Additionally, NS309 (6,7-Dichloro-1H-indole-2,3-dione 3-oxime) activates mitoSK$_{Ca}$ channels (Stowe et al. 2013) and the bee venom toxin apamin inhibits them (Dolga et al. 2013). Modulators of the mitoSK$_{Ca}$ channel are presented in Fig. 5.

2.3 Mitochondrial Voltage-Regulated Potassium Channels

Voltage-gated potassium channels (Kv) are by far the largest group of potassium channels found in animals. They are grouped into 12 classes that include more than 40 different proteins (Gutman et al. 2005). Therefore, it is not surprising that they were also found in the IMM. In fact, we expect that the number of voltage-gated potassium channels reported in the IMM will increase in the future.

2.3.1 mitoKv1.3 Channel

The Kv1.3 potassium channel is a member of the Shaker family of voltage-gated Kv channels. It is the main potassium channel in T lymphocytes but was also reported

in the plasmalemma of cells in the kidney, the central nervous system, brown and white fat, and epithelia. The functional channel is a homotetramer consisting of pore-forming subunits that span the plasmalemma six times. The channel is characterized by a conductance of approximately 100 pS and weak rectification at negative potentials.

Expression of the mitoKv1.3 channel has been reported in lymphocytes (Szabò et al. 2005) and brain (Bednarczyk et al. 2010) [Fig. 1]. One of the widely used mitoKv1.3 channel inhibitors is margatoxin, a peptide isolated from the venom of the scorpion *Centruroides margaritatus* (Szabò et al. 2005). However, mitoKv1.3 channels are insensitive to agitoxin-2, which inhibits the plasmalemmal counterpart of this channel (Bednarczyk et al. 2010). This may suggest that the pharmacology of mitochondrial and plasmalemmal isoforms of this channel differs. Another potent inhibitor of mitoKv1.3 channels is Shk (stichodactyla toxin), a peptide isolated from the sea anemone *Stichodactyla helianthus* (Gulbins et al. 2010). These two toxins may be useful in terms of single channel recordings of the mitoKv1.3 channel in patch-clamp experiments, but, due to their peptidyl nature, they are not suitable for blocking intracellular isoforms of the channels in intact cells. However, other non-peptidyl agents can. The most useful among them are clofazimine, 5-(4-phenylbutoxy)psoralen (Psora-4) and its derivative 5-(4-phenoxybutoxy)psoralen (PAP-1) (Schmitz and Sankaranarayanan 2005; Leanza et al. 2012). Unfortunately, no pharmacological activator of the mitoKv1.3 deserves recommendation. The structures of the described mitoKv1.3 channel inhibitors are presented in Fig. 6.

2.3.2 mitoKv7.4 Channel

Voltage-gated potassium channels type 7.X (7.1–7.5) are well-studied in the plasmalemma of many tissues and cells, including heart, neurons, epithelia, and smooth muscle. Kv7.4 is thought to play a vital role in the regulation of neuronal excitability, especially in sensory cells of the cochlea. The structure of this channel is not fully understood. The channel protein likely forms homomultimeric pores or heteromultimeric pores with Kv7.3 protein. The plasmalemmal Kv7.4 channel is bound to calmodulin, which acts as its sensor of the Ca^{2+} ion concentration. An increase in Ca^{2+} concentration results in channel inhibition (Sihn et al. 2016). The mitochondrial isoform of the Kv7.4 channel was discovered in cardiac mitochondria in 2015 (Testai et al. 2015); consequently, our knowledge of it is still limited. We do know, however, that its activity can be increased by the two aminopyridines, retigabine and flupirtine, and decreased by 0,10-bis (4-pyridinylmethyl)-9(10H)-anthracenone dihydrochloride (XE991) (Testai et al. 2015). The structures of these compounds are presented in Fig. 7.

2.4 Twin-Pore Domain mitoTASK3 Channel

The TASK3 channel ($K_{2P}9.1$) is structurally unique among mitochondrial potassium channels. It is a member of a large but poorly studied group of potassium

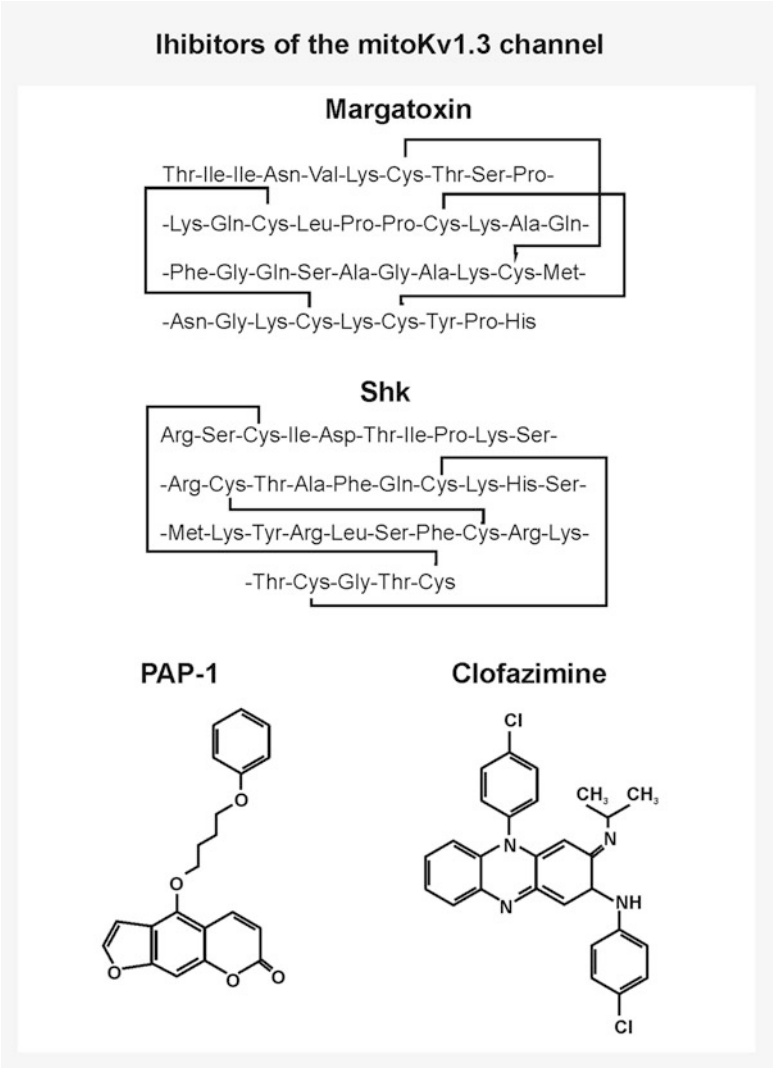

Fig. 6 Structural formulas of selected inhibitors of the mitoKv1.3 channel

channels discovered in the late 1990s. A pore-forming subunit of this channel consists of two pore domains and four transmembrane domains. The functional channel is assembled upon dimerization of pore-forming subunits. The TASK3 channel is expressed in plasmalemma predominantly in the nervous system, especially in the cerebellum. The channel is inhibited by extracellular acidification, arachidonic acid, and phorbol-12 myristate 13-acetate (TPA, PMA).

Expression of the mitoTASK3 channel was reported in cell lines derived from human skin in non-malignant keratinocytes (Rusznák et al. 2008; Toczyłowska-

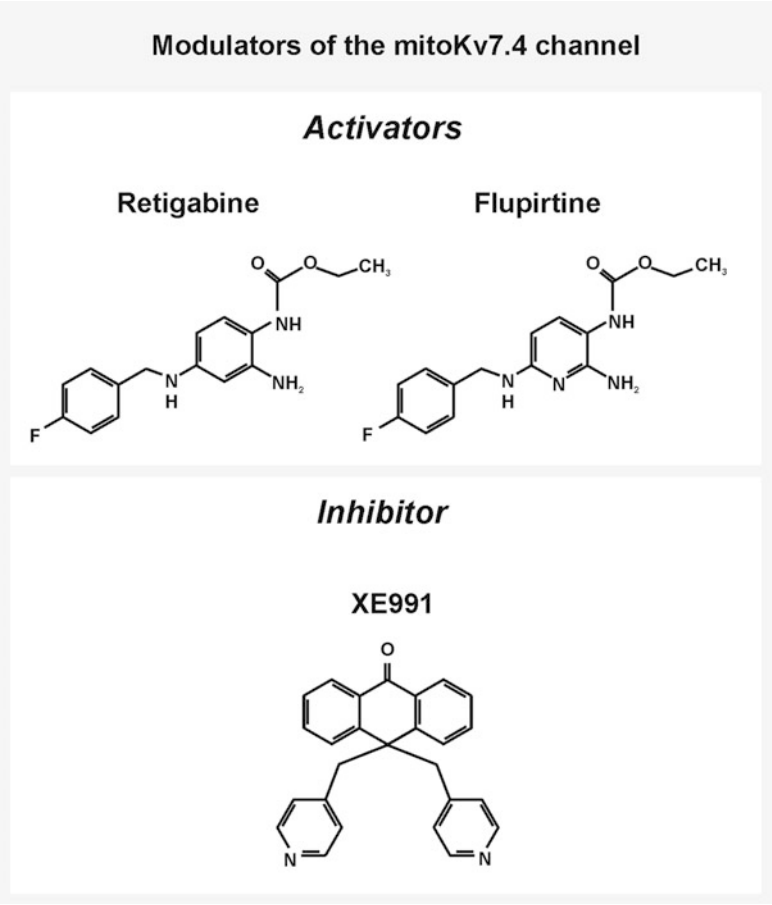

Fig. 7 Structural formulas of modulators of the mitoKv7.4 channel

Mamińska et al. 2013) and melanoma cells (Kosztka et al. 2011). Our knowledge of mitoTASK3 is very limited; however, we do know that it has rectifying properties and has a conductance of approximately 80 pS (Toczyłowska-Mamińska et al. 2013). Because there are only a handful of reports on the mitoTASK3 channel, only one modulator of this channel has been reported thus far; lidocaine was proven to inhibit mitoTASK3. The structure of lidocaine is presented in Fig. 8 (Toczyłowska-Mamińska et al. 2013).

2.5 MitoSLO2 Channel

Recent studies using BK_{Ca} channel knockout mice (*Kcnma1*$^{-/-}$) have challenged the molecular identity of the mitoBK_{Ca} channel in mouse heart mitochondria. It

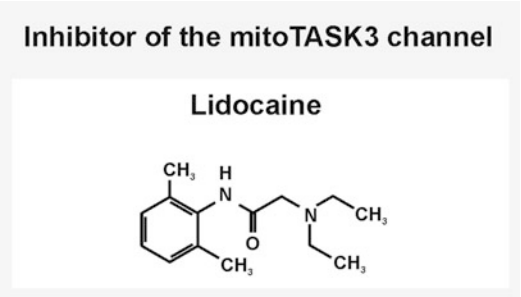

Fig. 8 Structural formula of the modulator of the mitoTASK3 channel

appeared that isoflurane-mediated cardioprotection was abolished by paxilline both in wild-type and *Kcnma1*$^{-/-}$ mice (Wojtovich et al. 2011). According to Wojtovich et al., SLO2, and not the SLO1 protein, is responsible for ischemia/reperfusion preconditioning in *C. elegans* and probably also in murine heart. There are two SLO2 proteins in mammals (SLO2.1 and SLO2.2) and one SLO2 protein in *C. elegans*. Mammalian SLO2 channels are encoded by the *Kcnt1* (SLO2.2/Slack protein) and *Kcnt2* (SLO2.1/Slick protein) genes. Unlike SLO1 (BK_{Ca}), these channels are activated by increases in intracellular sodium (K_{Na}) in mouse and by calcium ions in *C. elegans* (Wojtovich et al. 2011). Human mutations in plasmalemmal Slack channels lead to extremely severe learning impairments and interrupt development. This may suggest that K_{Na} channels play a vital role in neuronal plasticity and intellectual function (Kaczmarek 2013). Despite some differences, SLO2 paralogues share common structure with other potassium channels. A pore-forming subunit spans the membrane six times, and the transmembrane domains are virtually identical in Slick and Slack channels. Both N- and C-termini of the protein are located internally. The C-terminal domain of SLO2 proteins resembles that found in the BK_{Ca} channel (SLO1), with two RCK domains and phosphorylation sites. A functional channel is assembled from either a homo- or heterotetramer of pore-forming subunits.

The molecular identity of the proposed mitoSLO2 protein remains to be elucidated; we do not know which paralogue of the SLO2 channel, SLO2.1 or SLO2.2, constitutes the putative mitoSLO2 channel. Thus far, the mitoSLO2 channel has been found only in *C. elegans* and murine heart mitochondria [Fig. 1]. The mitoSLO2 channel, and not the mitoBK_{Ca} channel, was shown to be responsible for ischemic preconditioning (Wojtovich et al. 2011) in mice. However, the authors of this particular report do not preclude the presence of BK_{Ca} in the mitochondria or a role for BK_{Ca} channels in other protective paradigms.

The pharmacology of the mitoSLO2 channel, just as in the case of the mitoTASK3 channel, is not properly developed yet. Wojtovich et al. have exerted great effort to characterize the sensitivity of this channel towards several known modulators of other potassium channels. The mitoSLO2 channel appeared to be insensitive to glyburide and 5-HD, the inhibitors of the mitoK_{ATP} channel, and iberiotoxin, charybdotoxin, and apamin, the inhibitors of K_{Ca} channels. The channel was activated by bithionol (opener of the Slack channel) and isoflurane, and

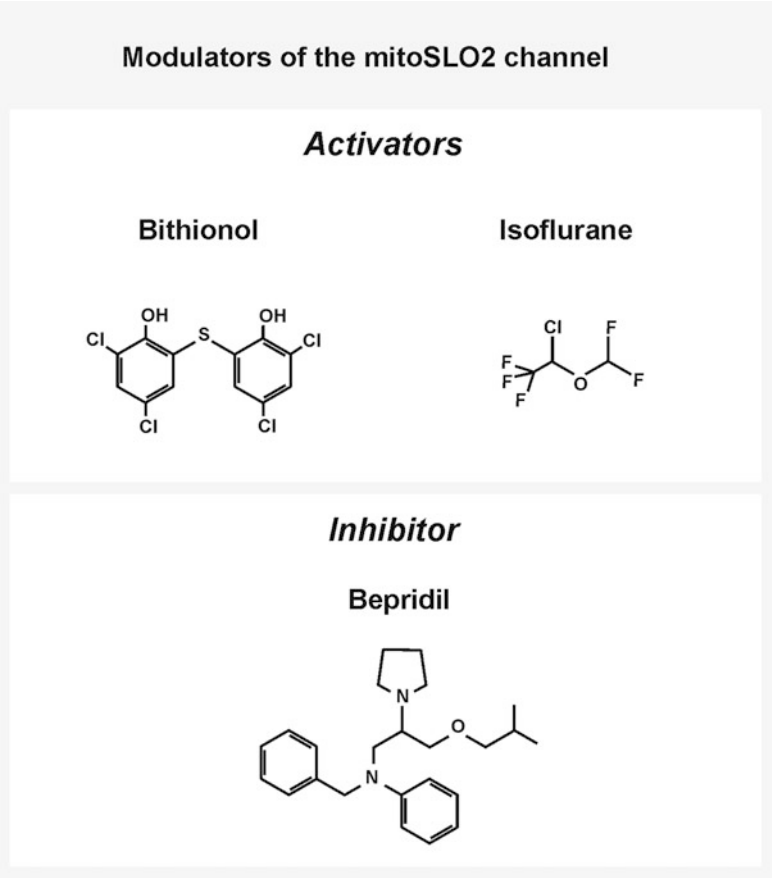

Fig. 9 Structural formulas of modulators of the mitoSLO2 channel

inhibited by bepridil and, surprisingly, paxilline. As for the usefulness of the modulators listed above, it seems that both bithionol and bepridil are more specific towards the mitoSLO2 channel. Isoflurane interacts with several other proteins, including GABA and glutamate receptors, many other potassium channels and calcium ATPase. Consequently, its impact on cells and mitochondria is multilevel and profound. Paxilline, which was already described as a useful inhibitor of the BK_{Ca} channel, is also not a good pharmacological candidate for the mitoSLO2 channel. The structures of selected modulators of mitoSLO2 are presented in Fig. 9.

3 Therapeutic Use and Toxicity of Mitochondrial Potassium Channels Modulators

Beginning with their discovery in 1991 (Inoue et al. 1991), mitochondrial potassium channels almost immediately were perceived as potential targets for therapies aimed at mitochondrial functions. Since then, a great number of substances have been proven to alter the activity of these channels. Most of them were described in the previous sections of this chapter. Yet, the real breakthrough was discovery of cardioprotective properties of KCOs made by Garlid's and Marban's groups. It appeared that preconditioning with the openers of the mitoK$_{ATP}$ channel can significantly decrease the size of ischemia/reperfusion-induced injury in cardiac muscle. Since then, the mitoK$_{ATP}$ channel has been found to take part in cytoprotective mechanisms in several tissues like liver, intestines, brain, and kidney (O'Rourke 2004). Later, it was shown that not only mitoK$_{ATP}$ but also mitoBK$_{Ca}$ channel can be responsible for ischemia/reperfusion-related cytoprotection (Xu et al. 2002). In fact, it became clear that the observed cytoprotective properties of KCOs rely on the influx of potassium ions into the mitochondrial matrix. We still do not know what is the exact mechanism of this phenomenon. A number of theories try to link influx of potassium ions into the mitochondrial matrix with cytoprotection (Laskowski et al. 2016). It seems that several interplaying mechanisms like decrease in reverse electron transfer and subsequent attenuation of ROS synthesis rate contribute to the potassium-related cytoprotection.

Even though the cytoprotective properties of several KCOs have been known for over 15 years, there are no approved therapies for humans yet that would take advantages of these effects. However, some drugs like diazoxide, glibenclamide, nicorandil, or pinacidil have been successfully administrated to humans for many years, but mainly due to their effect on plasmalemmal potassium channels or other, non-channel-related properties. Still, their safety and metabolism within the human body is well studied.

Considering the technical aspects of pharmacology of mitochondrial potassium channels one has to remember that it is associated with much more difficulties than pharmacology of their plasmalemmal counterparts. The most demanding challenge is delivery of the drug into mitochondria. First of all, drug that we chose should be small, lipophilic molecules that can cross intact plasmalemma of the living cell. This makes all peptide inhibitors of the channels (e.g., iberiotoxin, margatoxin, and apamin) virtually useless in research performed on the intact cells, tissues, or entire organisms. Second, an ideal situation would be accumulation of the drug within the mitochondrion. This can be achieved if the compound is positively charged because the matrix of energized mitochondria is charged negatively and more alkaline than cytoplasm. Moreover, it is crucial to remember that applied modulators of the mitochondrial potassium channels often also alter the activity of the plasmalemmal channels. Hence, it can be very difficult to distinguish between the effects caused by modulation of the mitochondrial and the plasmalemmal channels. In such case, one can think about performing experiments on isolated mitochondria instead of intact

cells, or about using modulators that are more specific towards mitochondrial potassium channels.

However, the major problem regarding modulators of potassium channels are their severe side effects. For instance, it was shown that on the top of its specific channel-activating properties, a mitoBK$_{Ca}$ channel opener NS1619 alters the function of other proteins like endoplasmic reticulum Ca^{2+}-ATPase, complex I, and ATP-synthase profoundly affecting the physiology of endothelial cells (Łukasiak et al. 2016). Similarly, paxilline which is an inhibitor of the same channel appeared to uncouple rather than counteract uncoupling of isolated, energized mitochondria (Bednarczyk et al. 2008). The issue of side effects of the modulators of mitochondrial potassium channels has been comprehensively reviewed in our previous work (Szewczyk et al. 2010). Fortunately, many of the side effects of potassium channel modulators can be avoided. The key factor that determines whether the drug can be relatively safely used is its concentration. There is one universal rule: use the lowest concentration of the drug possible. For electrophysiological experiments performed on individual single channels in the IMM or in planar lipid bilayers drugs can normally be used in smaller concentration than in experiments on either intact or perforated mitochondria. This is due to the fact that the access of the drug to the channel protein is somehow limited in the case of isolated mitochondria. We strongly advise to perform dose–response experiments for each used modulator in order to define its optimal concentration range so that the activity of the channel is affected, but low enough to minimize the side effects. Recommended concentrations of selected modulators of two best-studied mitochondrial potassium channels: mitoK$_{ATP}$ and mitoBK$_{Ca}$ are presented in Table 2.

As for the modulators of the six remaining types of mitochondrial potassium channels, we would prefer to refrain from giving any recommendations regarding their optimal concentrations in experiments. We believe that the body of literature regarding pharmacology of these channels is insufficient yet. At this point all we can advise is to perform dose–response experiments in order to choose the optimal range of concentration for each used modulator.

4 Final Remarks

The rational pharmacology of mitochondrial potassium channels should be preceded by the molecular identification of these proteins. Recent progress in this area may be a breakthrough for the development of "mitochondria targeted" drugs that

Table 2 Recommended concentration ranges of selected mitochondrial potassium channels modulators

Channel type	Modulator	Concentration range [μM]
mitoK$_{ATP}$	Diazoxide	10–100
	5-HD	150–500
	Glibenclamide	1–30
mitoBK$_{Ca}$	NS1619	1–10
	Iberiotoxin	0.1–2
	Paxilline	1–20

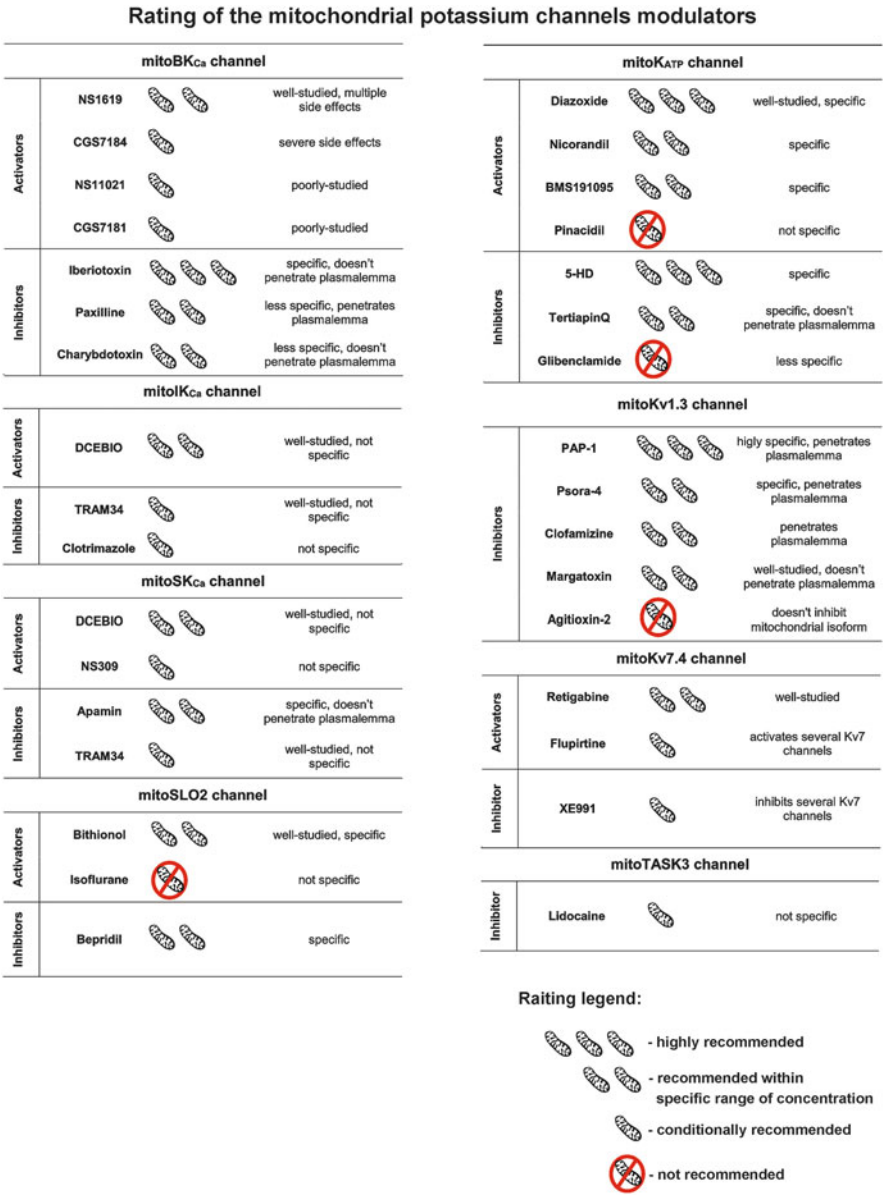

Fig. 10 Rating of selected modulators of mitochondrial potassium channels

regulate the activity of mitochondrial ion channels. In summary, modulators of greater specificity for these channels are required to understand their roles in mitochondria. Drugs specific to mitochondrial channels (i.e., lacking cross-

reactivity with plasmalemmal channels and other proteins present in mitochondria) need to be synthesized.

An evaluation of the utility of selected known modulators of mitochondrial potassium channels is presented at a glance in Fig. 10. This figure may be especially helpful for beginners in mitochondrial potassium channels pharmacology.

If the reader finds this guide useful, we would also recommend some additional readings. First, we strongly advise reading the review on mitochondrial ion channels written by Szabo and Zoratti (2014), which is the most current and comprehensive. This outstanding work provides deep insight into the issue of ion fluxes in mitochondria. The second reading is a special issue of FEBS Letters on itracellular ion channels (2010, volume 584, Issue 10). It contains several papers on the pharmacology of mitochondrial potassium channels and also addresses the problem of complex side effects of their modulators. Last, we recommend the review by Brian O'Rourke (2004), which concerns the issue of cardioprotective effects of certain openers of mitochondrial potassium channels.

Acknowledgements This work was supported by the Nencki Institute and partially by a grant from the National Centre of Research and Development, Poland No. MERIS PBS1/B8/1/2012 and by a grant from the Polish National Science Center No. 2015/17/B/NZ1/02496.

References

Bajgar R, Seetharaman S, Kowaltowski AJ, GArlid KD, Paucek P (2001) Identification and properties of a novel intracellular (mitochondrial) ATP-sensitive potassium channel in brain. J Biol Chem 276:33369–33374

Bednarczyk P, Barker GD, Halestrap AP (2008) Determination of the rate of K(+) movement through potassium channels in isolated rat heart and liver mitochondria. Biochim Biophys Acta 1777:540–548

Bednarczyk P, Kowalczyk JE, Beresewicz M et al (2010) Identification of a voltage-gated potassium channel in gerbil hippocampal mitochondria. Biochem Biophys Res Commun 397:614–620

Bednarczyk P, Koziel A, Jarmuszkiewicz W, Szewczyk A (2013a) Large-conductance Ca^{2+}-activated potassium channel in mitochondria of endothelial EA.hy926 cells. Am J Physiol Heart Circ Physiol 304:H1415–H1427

Bednarczyk P, Wieckowski MR, Broszkiewicz M et al (2013b) Putative structural and functional coupling of the mitochondrial BKCa channel to the respiratory chain. PLoS One 8:e68125

Cancherini DV, Trabuco LG, Rebouças NA, Kowaltowski AJ (2003) ATP-sensitive K^+ channels in renal mitochondria. Am J Physiol Renal Physiol 285:F1291–F1296

Candia S, Garcia ML, Latorre R (1992) Mode of action of iberiotoxin, a potent blocker of the large conductance Ca(2+)-activated K^+ channel. Biophys J 63:583–590

Chiandussi E, Petrussa E, Macrì F, Vianello A (2002) Modulation of a plant mitochondrial K^+ ATP channel and its involvement in cytochrome c release. J Bioenerg Biomembr 34:177–184

Chouchani ET, Pell VR, Gaude E et al (2014) Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. Nature 515(7527):431–435

Contreras GF, Castillo K, Enrique N et al (2013) A BK (Slo1) channel journey from molecule to physiology. Channels (Austin) 7:442–458

Costa ADT, Krieger MA (2009) Evidence for an ATP-sensitive K^+ channel in mitoplasts isolated from *Trypanosoma cruzi* and *Crithidia fasciculata*. Int J Parasitol 39:955–961

Dahlem YA, Horn TFW, Buntinas L et al (2004) The human mitochondrial KATP channel is modulated by calcium and nitric oxide: a patch-clamp approach. Biochim Biophys Acta 1656:46–56

Dale E, Staal RGW, Eder C, Möller T (2016) K_{Ca} 3.1-a microglial target ready for drug repurposing? Glia 64(10):1733–1741

De Marchi U, Sassi N, Fioretti B et al (2009) Intermediate conductance Ca^{2+}-activated potassium channel (KCa3.1) in the inner mitochondrial membrane of human colon cancer cells. Cell Calcium 45:509–516

Debska G, Kicinska A, Skalska J et al (2002) Opening of potassium channels modulates mitochondrial function in rat skeletal muscle. Biochim Biophys Acta 1556:97–105

Debska-Vielhaber G, Godlewski MM, Kicinska A et al (2009) Large-conductance K^+ channel openers induce death of human glioma cells. J Physiol Pharmacol 60:27–36

Dolga AM, Netter MF, Perocchi F et al (2013) Mitochondrial small conductance SK2 channels prevent glutamate-induced oxytosis and mitochondrial dysfunction. J Biol Chem 288:10792–10804

Dolga AM, de Andrade A, Meissner L et al (2014) Subcellular expression and neuroprotective effects of SK channels in human dopaminergic neurons. Cell Death Dis 5:e999

Escande D, Cavero I (1992) K^+ channel openers and "natural" cardioprotection. Trends Pharmacol Sci 13:269–272

Facundo HTF, Fornazari M, Kowaltowski AJ (2006) Tissue protection mediated by mitochondrial K^+ channels. Biochim Biophys Acta 1762:202–212

Foster DB, Ho AS, Rucker J et al (2012) Mitochondrial ROMK channel is a molecular component of mitoK(ATP). Circ Res 111:446–454

Galvez A, Gimenez-Gallego G, Reuben JP et al (1990) Purification and characterization of a unique, potent, peptidyl probe for the high conductance calcium-activated potassium channel from venom of the scorpion Buthus tamulus. J Biol Chem 265:11083–11090

Garlid KD (2000) Opening mitochondrial K(ATP) in the heart--what happens, and what does not happen. Basic Res Cardiol 95:275–279

Garlid KD, Paucek P, Yarov-Yarovoy V et al (1996) The mitochondrial KATP channel as a receptor for potassium channel openers. J Biol Chem 271:8796–8799

Garlid KD, Paucek P, Yarov-Yarovoy V et al (1997) Cardioprotective effect of diazoxide and its interaction with mitochondrial ATP-sensitive K^+ channels: possible mechanism of cardioprotection. Circ Res 81:1072–1082

Gögelein H, Ruetten H, Albus U et al (2001) Effects of the cardioselective KATP channel blocker HMR 1098 on cardiac function in isolated perfused working rat hearts and in anesthetized rats during ischemia and reperfusion. Naunyn Schmiedebergs Arch Pharmacol 364:33–41

Grover GJ, Atwal KS (2002) Pharmacologic profile of the selective mitochondrial-K(ATP) opener BMS-191095 for treatment of acute myocardial ischemia. Cardiovasc Drug Rev 20:121–136

Grover GJ, D'Alonzo AJ, Garlid KD et al (2001) Pharmacologic characterization of BMS-191095, a mitochondrial K(ATP) opener with no peripheral vasodilator or cardiac action potential shortening activity. J Pharmacol Exp Ther 297:1184–1192

Gulbins E, Sassi N, Grassmè H et al (2010) Role of Kv1.3 mitochondrial potassium channel in apoptotic signalling in lymphocytes. Biochim Biophys Acta 1797:1251–1259

Gutman GA, Chandy KG, Grissmer S et al (2005) International Union of Pharmacology. LIII. Nomenclature and molecular relationships of voltage-gated potassium channels. Pharmacol Rev 57:473–508

Harvey J, Ashford ML (1998) Diazoxide- and leptin-activated K(ATP) currents exhibit differential sensitivity to englitazone and ciclazindol in the rat CRI-G1 insulin- secreting cell line. Br J Pharmacol 124:1557–1565

Inagaki N, Gonoi T, Clement JP et al (1995) Reconstitution of IKATP: an inward rectifier subunit plus the sulfonylurea receptor. Science 270:1166–1170

Inoue I, Nagase H, Kishi K, Higuti T (1991) ATP-sensitive K^+ channel in the mitochondrial inner membrane. Nature 352:244–247

Jin W, Lu Z (1998) A novel high-affinity inhibitor for inward-rectifier K^+ channels. Biochemistry 37:13291–13299

Kaczmarek LK (2013) Slack, slick and sodium-activated potassium channels. ISRN Neurosci pii: 354262

Kicinska A, Swida A, Bednarczyk P et al (2007) ATP-sensitive potassium channel in mitochondria of the eukaryotic microorganism *Acanthamoeba castellanii*. J Biol Chem 282:17433–17441

Knaus HG, McManus OB, Lee SH et al (1994) Tremorgenic indole alkaloids potently inhibit smooth muscle high-conductance calcium-activated potassium channels. Biochemistry 33:5819–5828

Koszela-Piotrowska I, Matkovic K, Szewczyk A, Jarmuszkiewicz W (2009) A large-conductance calcium-activated potassium channel in potato (Solanum tuberosum) tuber mitochondria. Biochem J 424:307–316

Kosztka L, Rusznák Z, Nagy D et al (2011) Inhibition of TASK-3 (KCNK9) channel biosynthesis changes cell morphology and decreases both DNA content and mitochondrial function of melanoma cells maintained in cell culture. Melanoma Res 21:308–322

Kulawiak B, Bednarczyk P (2005) Reconstitution of brain mitochondria inner membrane into planar lipid bilayer. Acta Neurobiol Exp (Wars) 65(3):271–276

Kulawiak B, Kudin AP, Szewczyk A, Kunz WS (2008) BK channel openers inhibit ROS production of isolated rat brain mitochondria. Exp Neurol 212:543–547

Laskowski M, Kicinska A, Szewczyk A, Jarmuszkiewicz W (2015) Mitochondrial large-conductance potassium channel from *Dictyostelium discoideum*. Int J Biochem Cell Biol 60:167–175

Laskowski M, Augustynek B, Kulawiak B et al (2016) What do we not know about mitochondrial potassium channels? Biochim Biophys Acta 1857(8):1247–1257

Latorre R, Brauchi S (2006) Large conductance Ca^{2+}-activated K^+ (BK) channel: activation by Ca^{2+} and voltage. Biol Res 39:385–401

Leanza L, Henry B, Sassi N et al (2012) Inhibitors of mitochondrial Kv1.3 channels induce Bax/Bak-independent death of cancer cells. EMBO Mol Med 4:577–593

Liu Y, Sato T, Seharaseyon J, Szewczyk A, O'Rourke B, Marbán E (1999) Mitochondrial ATP-dependent potassium channels viable candidate effectors of ischemic preconditioning. Ann N Y Acad Sci 874:27–37

Łukasiak A, Skup A, Chlopicki S et al (2016) SERCA, complex I of the respiratory chain and ATP-synthase inhibition are involved in pleiotropic effects of NS1619 on endothelial cells. Eur J Pharmacol 786:137–147

Magleby KL (2003) Gating mechanism of BK (Slo1) channels: so near, yet so far. J Gen Physiol 121:81–96

Malinska D, Kulawiak B, Wrzosek A et al (2010) The cytoprotective action of the potassium channel opener BMS-191095 in C2C12 myoblasts is related to the modulation of calcium homeostasis. Cell Physiol Biochem 26:235–246

Matkovic K, Koszela-Piotrowska I, Jarmuszkiewicz W, Szewczyk A (2011) Ion conductance pathways in potato tuber (Solanum tuberosum) inner mitochondrial membrane. Biochim Biophys Acta 1807:275–285

Miki T, Suzuki M, Shibasaki T et al (2002) Mouse model of Prinzmetal angina by disruption of the inward rectifier Kir6.1. Nat Med 8:466–472

Murata M, Akao M, O'Rourke B, Marban E (2001) Mitochondrial ATP-sensitive potassium channels attenuate matrix Ca^{2+} overload during simulated ischemia and reperfusion: possible mechanism of cardioprotection. Circ Res 89:891–898

Murphy E, Steenbergen C (2008) Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury. Physiol Rev 88:581–609

Nardi A, Olesen S-P (2008) BK channel modulators: a comprehensive overview. Curr Med Chem 15:1126–1146

Noma A (1983) ATP-regulated K^+ channels in cardiac muscle. Nature 305:147–148

Notsu T, Tanaka I, Mizota M et al (1992) A cAMP-dependent protein kinase inhibitor modulates the blocking action of ATP and 5-hydroxydecanoate on the ATP-sensitive K^+ channel. Life Sci 51:1851–1856

O'Rourke B (2004) Evidence for mitochondrial K^+ channels and their role in cardioprotection. Circ Res 94:420–432

O'Rourke B, Cortassa S, Aon MA (2005) Mitochondrial ion channels: gatekeepers of life and death. Physiol (Bethesda) 20:303–315

Pastore D, Stoppelli MC, Di Fonzo N, Passarella S (1999) The existence of the K^+ channel in plant mitochondria. J Biol Chem 274:26683–26690

Pastore D, Trono D, Laus MN et al (2007) Possible plant mitochondria involvement in cell adaptation to drought stress. A case study: durum wheat mitochondria. J Exp Bot 58:195–210

Piwonska M, Wilczek E, Szewczyk A, Wilczynski GM (2008) Differential distribution of Ca^{2+}-activated potassium channel β4 subunit in rat brain: immunolocalization in neuronal mitochondria. Neuroscience 153:446–460

Rusznák Z, Bakondi G, Kosztka L et al (2008) Mitochondrial expression of the two-pore domain TASK-3 channels in malignantly transformed and non-malignant human cells. Virchows Arch 452:415–426

Sakai Y, Harvey M, Sokolowski B (2011) Identification and quantification of full-length BK channel variants in the developing mouse cochlea. J Neurosci Res 89:1747–1760

Sassi N, De Marchi U, Fioretti B et al (2010) An investigation of the occurrence and properties of the mitochondrial intermediate-conductance Ca^{2+}-activated K^+ channel mtKCa3.1. Biochim Biophys Acta 1797:1260–1267

Sato T, Sasaki N, O'Rourke B, Marbán E (2000) Nicorandil, a potent cardioprotective agent, acts by opening mitochondrial ATP-dependent potassium channels. J Am Coll Cardiol 35:514–518

Schmitz A, Sankaranarayanan A (2005) Design of PAP-1, a selective small molecule Kv1. 3 blocker, for the suppression of effector memory T cells in autoimmune diseases. Mol Pharmacol 68:1254–1270

Siemen D, Loupatatzis C, Borecky J et al (1999) Ca^{2+}-activated K channel of the BK-type in the inner mitochondrial membrane of a human glioma cell line. Biochem Biophys Res Commun 257:549–554

Sihn CR, Kim HJ, Woltz RL et al (2016) Mechanisms of calmodulin regulation of different isoforms of Kv7.4 K^+ channels. J Biol Chem 291:2499–2509

Singh H, Lu R, Bopassa JC et al (2013) mitoBKCa is encoded by the Kcnma1 gene, and a splicing sequence defines its mitochondrial location. Proc Natl Acad Sci 110(26):10836–10841

Singh H, Li M, Hall L et al (2016) MaxiK channel interactome reveals its interaction with GABA transporter 3 and heat shock protein 60 in the mammalian brain. Neuroscience 317:76–107

Skalska J, Piwońska M, Wyroba E et al (2008) A novel potassium channel in skeletal muscle mitochondria. Biochim Biophys Acta 1777:651–659

Skalska J, Bednarczyk P, Piwonska M et al (2009) Calcium ions regulate K^+ uptake into brain mitochondria: the evidence for a novel potassium channel. Int J Mol Sci 10(3):1104–1120

Slocinska M, Lubawy J, Jarmuszkiewicz W, Rosinski G (2013) Evidences for an ATP-sensitive potassium channel (KATP) in muscle and fat body mitochondria of insect. J Insect Physiol 59:1125–1132

Stowe DF, Gadicherla AK, Zhou Y et al (2013) Protection against cardiac injury by small Ca(2+)-sensitive K(+) channels identified in guinea pig cardiac inner mitochondrial membrane. Biochim Biophys Acta 1828:427–442

Szabo I, Zoratti M (2014) Mitochondrial channels: ion fluxes and more. Physiol Rev 94:519–608

Szabò I, Bock J, Jekle A et al (2005) A novel potassium channel in lymphocyte mitochondria. J Biol Chem 280:12790–12798

Szewczyk A, Kajma A, Malinska D et al (2010) Pharmacology of mitochondrial potassium channels: dark side of the field. FEBS Lett 584:2063–2069

Testai L, Barrese V, Soldovieri MV et al (2015) Expression and function of Kv7.4 channels in rat cardiac mitochondria: possible targets for cardioprotection. Cardiovasc Res 110(1):40–50

Toczyłowska-Mamińska R, Olszewska A, Laskowski M et al (2013) Potassium channel in the mitochondria of human keratinocytes. J Invest Dermatol 134(3):764–772

Wojtovich AP, Burwell LS, Sherman TA et al (2008) The C. elegans mitochondrial K+(ATP) channel: a potential target for preconditioning. Biochem Biophys Res Commun 376:625–628

Wojtovich AP, Sherman TA, Nadtochiy SM et al (2011) SLO-2 is cytoprotective and contributes to mitochondrial potassium transport. PLoS One 6:e28287

Wojtovich AP, Urciuoli WR, Chatterjee S et al (2013) Kir6.2 is not the mitochondrial KATP channel but is required for cardioprotection by ischemic preconditioning. Am J Physiol Heart Circ Physiol 304:H1439–H1445

Xu W, Liu Y, Wang S et al (2002) Cytoprotective role of Ca^{2+}- activated K^+ channels in the cardiac inner mitochondrial membrane. Science 298:1029–1033

Zoratti M, De Marchi U, Gulbins E, Szabò I (2009) Novel channels of the inner mitochondrial membrane. Biochim Biophys Acta 1787:351–363

The Mitochondrial Ca^{2+} Uniporter: Structure, Function, and Pharmacology

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H. Singh, S.-S. Sheu (eds.), *Pharmacology of Mitochondria*,
Handbook of Experimental Pharmacology 240, DOI 10.1007/164_2017_1

Abstract

Mitochondrial Ca^{2+} uptake is crucial for an array of cellular functions while an imbalance can elicit cell death. In this chapter, we briefly reviewed the various modes of mitochondrial Ca^{2+} uptake and our current understanding of mitochondrial Ca^{2+} homeostasis in regards to cell physiology and pathophysiology. Further, this chapter focuses on the molecular identities, intracellular regulators as well as the pharmacology of mitochondrial Ca^{2+} uniporter complex.

Keywords

Mitochondria • Mitochondrial Ca^{2+} uniporter • Mitochondrial Ca^{2+} uptake • Pharmacology

Abbreviations

$[Ca^{2+}]_c$	Cytosolic Ca^{2+} concentrations
$[Ca^{2+}]_m$	Mitochondrial Ca^{2+} concentrations
$[Ca^{2+}]_o$	Extramitochondrial free Ca^{2+} concentrations
ATP	Adenosine triphosphate
CaMK	Ca^{2+}/calmodulin kinase
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CoQ10	Coenzyme Q 10
CREB	Cyclic adenosine monophosphate response element–binding protein
EMRE	Essential MCU regulator
ER/SR	Endoplasmic reticulum/Sarcoplasmic reticulum
ICDH	Isocitrate dehydrogenase
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
IP_3R	Inositol 1,4,5-trisphosphate receptor
LETM1	Leucine zipper-EF-hand containing transmembrane protein 1
MAPK	Mitogen-activated protein kinase
MCU	Mitochondrial Ca^{2+} uniporter pore
MCUR1	Mitochondrial Ca^{2+} uniporter regulator 1
MICU1	Mitochondrial Ca^{2+} uptake 1
MICU2	Mitochondrial Ca^{2+} uptake 2
MICU3	Mitochondrial Ca^{2+} uptake 3
mPTP	Mitochondrial permeability transition pore
mRyR1	Mitochondrial ryanodine receptor 1
mtCU	Mitochondrial Ca^{2+} uniporter
NMR	Nuclear magnetic resonance
Npas4	Neuronal PAS Domain Protein 4

OGDH	α-Ketoglutarate/oxoglutarate dehydrogenase
OMM	Outer mitochondrial membrane
PDH	Pyruvate dehydrogenase
Pyk2	Proline-rich tyrosine kinase 2
RaM	Rapid mode of uptake
ROS	Reactive Oxygen Species
Ru360	Ruthenium 360
RyR	Ryanodine receptor
TASK-3	TWIK-related Acid-sensitive K^+ channel
TRPC3	Transient receptor potential channel 3
TRPV	Transient Receptor Potential Vanilloid
VDAC	Voltage dependent anion channel
α_1-AR	α1-Adrenoceptor

1 Introduction

Mitochondria play an important role in Ca^{2+} homeostasis, which is crucial for balancing cell survival and death (Giacomello et al. 2007; Duchen et al. 2008). During the 1950s it was observed that isolated mitochondria could accumulate Ca^{2+} (Carafoli 2010). Subsequently, an energy-driven accumulation of Ca^{2+} by isolated mitochondria was demonstrated (Vasington and Murphy 1962; Deluca and Engstrom 1961). It was initially thought that mitochondrial Ca^{2+} transport consists of an active uptake and passive release process (Chance 1965), but multiple groups [reviewed by Gunter et al. (1994)] showed that Ca^{2+} uptake across the inner mitochondrial membrane (IMM) is energetically favorable, while efflux requires electrogenic ion-exchange (antiport). This raised the possibility that mitochondria may play a significant role in the regulation or buffering of cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_c$) (Nicholls 1978). Though, mitochondria were one of the first organelle to be associated with intracellular Ca^{2+} handling, the relative low affinity of their Ca^{2+} transport systems led to the conclusion that they were physiologically irrelevant. It was demonstrated that in suspensions of respiring isolated rat liver mitochondria alone, the steady state extramitochondrial free Ca^{2+} concentrations ($[Ca^{2+}]_o$) of incubating solutions were about 0.5 μM (Becker et al. 1980). Addition of microsomes, which contain endoplasmic reticulum (ER) that has Ca^{2+} transport systems with a higher affinity for Ca^{2+} than that of mitochondria, was able to reduce $[Ca^{2+}]_o$ to 0.2 μM. Similar results were obtained in digitonin-permeabilized hepatocytes and thus brought forth the idea that the "set point" of $[Ca^{2+}]_c$ is established by the ER Ca^{2+} transport mechanisms and not the mitochondria (at ~0.2 μM) (Becker et al. 1980). However, interest revived in mitochondrial Ca^{2+} homeostasis in the 1990s when the development of Ca^{2+} sensors that can selectively measure the changes in the mitochondrial matrix Ca^{2+} concentrations ($[Ca^{2+}]_m$) allowed to demonstrate propagation of physiological Ca^{2+} signals from cytosol into the mitochondrial matrix. High Ca^{2+} microdomains at the ER/sarcoplasmic reticulum (SR) and mitochondria interface addressed the discrepancy between the relatively small (approximately 1 μM or less) global $[Ca^{2+}]_c$ peak levels and the much higher

in vitro activation range ($K_d \cong 50$ μM) for the mitochondrial Ca^{2+} uniporter (mtCU) in most tissues. The ER/SR, which possesses the Ca^{2+}-release channels, inositol 1,4,5-trisphosphate receptor (IP_3R), and/or ryanodine receptor (RyR), could release Ca^{2+} at the mitochondria/ER/SR junctions with concentrations sufficient to meet the threshold of the mtCU (Rizzuto and Pozzan 2006; O-Uchi et al. 2012). These groundbreaking studies repositioned mitochondria as key players in the dynamic regulation of cellular Ca^{2+} signaling under physiological conditions.

Ca^{2+} uptake into mitochondria was mostly considered to result from a single transport mechanism mediated by a Ca^{2+}-selective channel of the IMM, the mtCU (Gunter and Pfeiffer 1990). The electrophysiological characteristic of mtCU as a highly selective Ca^{2+} activated Ca^{2+} channel (I_{MiCa}) was confirmed by measuring total or single-channel ionic current from the IMM of mitoplasts (Kirichok et al. 2004). The discovery of the molecular identity of the mtCU protein complexes was tightly connected to the establishment of MitoCarta, a comprehensive mitochondrial protein compendium in 2008 (Pagliarini et al. 2008). Based on the establishment of this compendium, the Ca^{2+} sensing EF-hand regulator mitochondrial Ca^{2+} uptake 1 (MICU1) was identified first in 2010 as a regulator of the channel (Perocchi et al. 2010). With one or no predicted transmembrane domain, MICU1 has never been considered to form the mtCU pore. To that end, in 2011, a ~40 kDa protein with two transmembrane domains was discovered as the molecular identity of the mtCU pore termed MCU by the groups of Mootha and Rizzuto (De Stefani et al. 2011; Baughman et al. 2011). Following the identification of the MCU, other regulatory subunits were identified in the last 5 years. These findings open up exciting opportunities for using genetic approaches to elucidate molecular mechanisms that regulate mitochondrial Ca^{2+} uptake in a variety of cell types/tissues. Since the mechanisms for regulating mitochondrial Ca^{2+} concentrations ($[Ca^{2+}]_m$) are critical for fundamental cellular processes, the importance of understanding Ca^{2+} uptake mechanisms in physiology (Tarasov et al. 2012; Alam et al. 2012; Xu and Chisholm 2014) and pathophysiology (Mallilankaraman et al. 2012a; Huang et al. 2013; Csordas et al. 2013; Hall et al. 2014) has become increasingly relevant.

In this chapter, we review the current model of the mitochondrial Ca^{2+} influx mechanism, with special focus on the molecular identity of the mtCU complex.

Furthermore, the physiological, pathophysiological, and pharmacological implications of mitochondrial Ca^{2+} uptake and future directions of study are discussed.

2 Molecular Identities of Mitochondrial Ca^{2+} Channels/Transporters

2.1 Overview

Following the discovery of the pore, MCU, further regulatory subunits were identified, suggesting that the mtCU exists as a multi-protein complex capable of multiple states of MCU activity (De Stefani et al. 2011). Proteins in the mtCU

complex include transmembrane subunits [MCU, MCUb, and the essential MCU regulator (EMRE)], and membrane-associated regulatory subunits in the intermembrane space (IMS) (MICU1-3) (Fig. 1). Mitochondrial Ca^{2+} uniporter regulator 1 (MCUR1), another two transmembrane domain coiled-coil domain containing protein of the IMM was also proposed to interact with the MCU protein and to modulate the channel function (Mallilankaraman et al. 2012b); however, it was not present in the ~480 kDa uniporter holocomplex coined as the "uniplex" (Sancak et al. 2013). In addition to mtCU complex, we also briefly describe other mitochondrial Ca^{2+} channels/transporters that have been reported, which includes mitochondrial ryanodine receptor 1 (mRyR1), rapid mode of uptake (RaM), mCa1 and 2, Coenzyme Q 10 (CoQ10), the transient receptor potential channel 3 (TRPC3), and the Leucine zipper-EF-hand containing transmembrane protein 1 (LETM1).

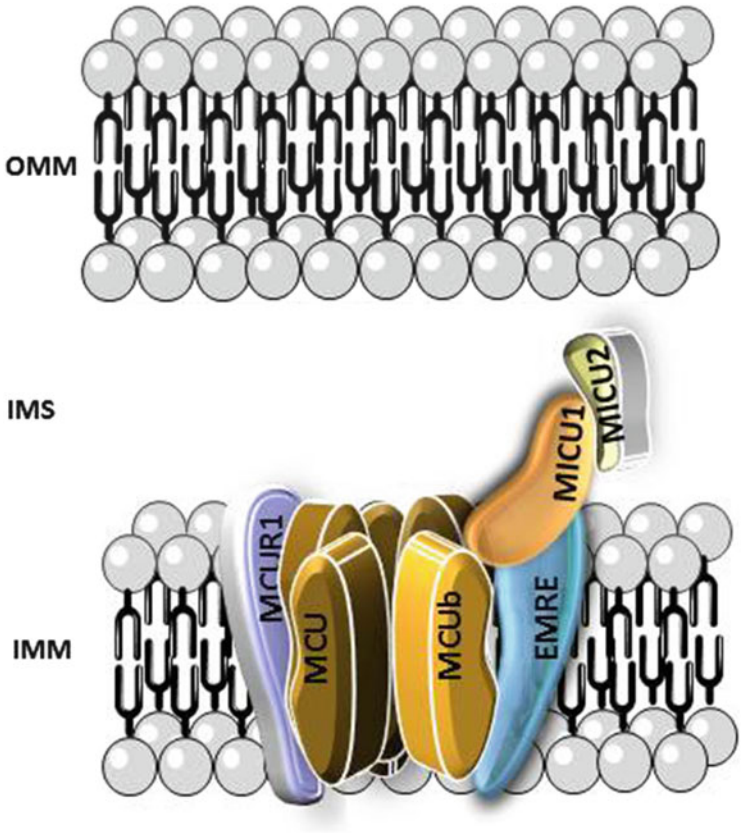

Fig. 1 The molecular structure of the mtCU complex. Composed of MCU and MCUb (the channel forming subunits) together with essential mtCU regulators, EMRE, MCUR1 and intermembrane space proteins, MICU1 and MICU2

2.2 mtCU Complex

2.2.1 MCU

The MCU gene (previously known as CCDC109A) is highly conserved across eukaryotes except yeast (De Stefani et al. 2011; Baughman et al. 2011). The MCU is a 40 kD protein that contains a proteolytically cleaved mitochondrial import sequence, two coiled-coil domains, two transmembrane domains, and a short motif of amino acids between the two transmembrane domains critical for Ca^{2+} transport (De Stefani et al. 2011; Baughman et al. 2011). MCU has been suggested to form the pore as a homo-oligomer and a recent study using nuclear magnetic resonance (NMR) demonstrated a pentameric stoichiometry (Oxenoid et al. 2016). Although there was originally some debate about the MCU topology, it is clear now that both the N- and C-termini face the mitochondrial matrix with a short motif of amino acids being exposed to the IMS (Martell et al. 2012). Overexpression of MCU increases the rate of mitochondrial Ca^{2+} influx in both intact and permeabilized cells, causing a significant decrease in $[Ca^{2+}]_c$ transients in intact cells (De Stefani et al. 2011). Further, the mutation of two negatively charged residues inside the highly conserved DIME motif (QxGxLAxLTWWxYSWDIMEPVTYF), in the IMS (D261Q/E264Q in human MCU) completely abolishes the MCU activity (De Stefani et al. 2011; Baughman et al. 2011). On the other hand, the partial knockdown of MCU greatly inhibits the rate and amplitude of mitochondrial Ca^{2+} entry (De Stefani et al. 2011; Baughman et al. 2011) whereas the knockout essentially eliminates rapid uptake of Ca^{2+} pulses (Sancak et al. 2013; Pan et al. 2013) and the expression of the wild-type MCU in MCU knockdown cells fully rescues Ca^{2+} uptake profile (Baughman et al. 2011). Thus, MCU is responsible for Ca^{2+} transport into the mitochondria. As of now, the essential role of MCU for mitochondrial Ca^{2+} uptake was validated in many cell types/tissues including the liver (Baughman et al. 2011), heart (Joiner et al. 2012), cardiomyocytes (Drago et al. 2012; O-Uchi et al. 2014), skeletal muscles (Pan et al. 2013), pancreatic β cells (Tarasov et al. 2012), neurons (Qiu et al. 2013), and mammary gland epithelial cells (Hall et al. 2014).

2.2.2 MCUb

MCUb, originally reported as CCDC109B, is a 33-kDa protein that shares 50% similarity to MCU with the key amino acid substitutions (R251W, E256V) in the DIME motif (Raffaello et al. 2013). Co-introduction of MCU and MCUb in a lipid bilayer dramatically decreases the open probability compared to only MCU incorporation. In addition, MCUb overexpression in intact cells decreases mitochondrial Ca^{2+} uptake in response to $[Ca^{2+}]_c$ increases, suggesting that MCUb interacts with MCU and acts as an endogenous dominant-negative subunit of the mtCU pore (Raffaello et al. 2013). Interestingly, the ratio of the amount of MCU and MCUb mRNA varies in different tissues (Sancak et al. 2013; Raffaello et al. 2013; Fieni et al. 2012). This raises the possibility that the ratio of MCU and MCUb expression may be one of the mechanisms that differentially regulate mitochondrial Ca^{2+} uptake in different tissues.

2.2.3 MICU1-3

MICU1 (previously known as CBARA1/EFHA3) is a 54-kDa protein with two highly conserved EF-hand Ca^{2+}-binding domains (Perocchi et al. 2010). The sub-mitochondrial localization of MICU1 has been a matter of debate (Perocchi et al. 2010; Mallilankaraman et al. 2012a; Hoffman et al. 2013) but recent proteomic mapping studies (Hung et al. 2014; Lam et al. 2015) as well as interactome analysis of the intermembrane space oxidoreductase MIA40 (Petrungaro et al. 2015) indicate that the MICU1 is a soluble (or membrane associated) protein in the IMS (Csordas et al. 2013; Patron et al. 2014; Wang et al. 2014), but not in the matrix. MICU1 is proposed to be pivotal in both the gatekeeping and cooperative activation of mtCU; keeping the channel closed at resting conditions, but promoting cooperative activation of the channel at high Ca^{2+} (Csordas et al. 2013; de la Fuente et al. 2014). Alternatively, MICU1 was also proposed to only convey either of these functions (gatekeeper (Mallilankaraman et al. 2012a; Hoffman et al. 2013), cooperative activator) (Patron et al. 2014).

Additionally, MICU isoforms, MICU2 (known as EFHA1) and MICU3 (known as EFHA2) are also identified (Plovanich et al. 2013). Both MICU2 and MICU3 possess the conserved EF-hand domains, but share only 25% sequence identity with MICU1 (Plovanich et al. 2013). Relative expression levels of these MICU isoforms vary across the different tissue types. MICU1 and MICU2 are ubiquitously expressed in mammalian tissues, whereas MICU3 is expressed only in the nervous system and skeletal muscle (Plovanich et al. 2013). Though the role of MICU1 and MICU2 have been extensively studied by several groups, but up to date there is no report attempted to characterize the MICU3 function. MICU2 forms a heterodimer with MICU1, thus indirectly associating with the MCU (Patron et al. 2014; Plovanich et al. 2013). Moreover, the stability of MICU2 is dependent on the level of MICU1 expression (Patron et al. 2014; Plovanich et al. 2013). Importantly, MICU2 inhibits the function of the MCU at lower $[Ca^{2+}]_c$ levels both in planar lipid bilayers and in intact cells (Patron et al. 2014; Matesanz-Isabel et al. 2016). These data lead to the suggestion that MICU2 would be the gatekeeper of MCU instead of MICU1, which would form a regulatory dimer with MICU2 to modulate MCU channel activity in opposite manner. On the other hand, a recent study by the Mootha group showed that upon disabling the Ca^{2+} sensing by their EF hands, MICU1 and MICU2 both would keep the channel closed and MICU1 would do this even if MICU2 was ablated (MICU2 KO) (Kamer and Mootha 2014). This would suggest that MICU1 alone can act as a gatekeeper but the gatekeeping activity would be lifted by lower $[Ca^{2+}]$ than that of MICU2 (Matesanz-Isabel et al. 2016). At low $[Ca^{2+}]_c$, the inhibitory effect of MICU2 is in dominance to safeguard minimal Ca^{2+} accumulation in the presence of a very large electromotive force for cation accumulation. At high $[Ca^{2+}]_c$, however, Ca^{2+}-dependent MICU2 inhibition and MICU1 activation warrant the mitochondria to respond rapidly for bringing adequate amount of Ca^{2+} into matrix during $[Ca^{2+}]_c$ oscillations so that Ca^{2+}-sensitive steps in ATP production can be stimulated efficiently. A very recent work by the Rizzuto/Raffaello group describes a splice variant of MICU1, termed MICU1.1 containing an insertion of 4 amino acids (EFWQ) at position

181 of MICU1, that is highly expressed in the skeletal muscle with increased Ca^{2+} binding affinity (Vecellio Reane et al. 2016). This splice variant seems to convey higher sensitivity (lower threshold) for the activation of mtCU further suggesting that MICU1 is instrumental in the gatekeeping of mtCU.

2.2.4 EMRE

EMRE (known as C22ORF32) is a 10-kDa protein that contains a single transmembrane domain and a highly conserved aspartate-rich C-terminal region (Sancak et al. 2013). While MCU and MICUs are well preserved across phylum, EMRE homologs are not present in plants, fungi, or protozoa, indicating that EMRE likely arose in the metazoan lineage (Sancak et al. 2013). However, within mammals, EMRE is ubiquitously expressed across tissues (Sancak et al. 2013). Importantly, it has been shown that knockdown or knockout of EMRE completely abolishes mitochondrial Ca^{2+} uptake, indicating that this protein is essential for the functional mtCU channel. EMRE interacts with MCU at the IMM and MICU1 at the IMS, acting as a retainer of MICU1/2 in the mtCU complex (Sancak et al. 2013; Kovacs-Bogdan et al. 2014; Tsai et al. 2016; Yamamoto et al. 2016; Vais et al. 2016). A majority of evidence suggest that the N-terminus of EMRE faces the matrix with the C-terminus facing the IMS (Tsai et al. 2016; Yamamoto et al. 2016; Tomar et al. 2016).

In addition to the $[Ca^{2+}]_c$ sensing via MICU, MCU may also be regulated by Ca^{2+} and Mg^{2+} from the matrix side. Recent work from the Foskett group has presented electrophysiological (mitoplast patch clamp) evidence for a biphasic (bell-shaped) Ca^{2+} regulation of mtCU from the matrix side with a matrix $[Ca^{2+}]$ activation window of ~0.01–2 μM. The acidic tail of EMRE was shown to be critical for this $[Ca^{2+}]$ regulation from the matrix side and, contrasting other works, was suggested that EMRE would rather have an N_{out}-C_{in} topology and its acidic tail would operate as the luminal Ca^{2+} sensor. Since MICU1/2 were also required for the matrix-side $[Ca^{2+}]$ regulation and considering the overwhelming evidence for EMRE's N_{in}-C_{out} topology, one could entertain an alternative mechanism for EMRE's contribution. EMRE may relay a signal from a distinct matrix Ca^{2+} sensor to the gatekeepers MICU1/2 via the interaction of its C-terminal acidic tail with a lysine-rich basic stretch of MICU1 (Tsai et al. 2016). As to the matrix Ca^{2+} sensor, very recently a comprehensive molecular structure (crystallography) study has identified a Ca^{2+}/Mg^{2+} binding acidic patch on the N-terminal matrix domain of MCU that conveys Mg^{2+} dependent inactivation of the channel (Lee et al. 2016). Further studies will be needed to clarify EMRE's role if any in this latter regulation.

2.2.5 MCUR1

MCUR1 (known as CCDC90A) is a 40-kDa protein that consists of two transmembrane domains and one coiled-coil region. The N- and C-termini of MICUR1 are proposed to face the IMS, thus the bulk of this protein exposed to the matrix (Mallilankaraman et al. 2012b). Knockdown of MCUR1 not only inhibits agonist-induced mitochondrial Ca^{2+} uptake, but also decreases basal $[Ca^{2+}]_m$. Overexpression of MCUR1 results in an increase of mitochondrial Ca^{2+} uptake,

but only when MCU exists, indicating that MCUR1 is required for Ca^{2+} uptake through the mtCU complex. MCUR1 interacts with MCU, but not with MICU1, suggesting that different compositions of the mtCU complex may exist. Shoubridge and colleagues raised a question about the direct involvement of MCUR1 in the regulation of the MCU complex (Paupe et al. 2015). They demonstrated that MCUR1 knockdown causes a drop of mitochondrial membrane potential ($\Delta\Psi_m$), proposed that the effect of MCUR1 on MCU activity may be indirect through changing the driving force of Ca^{2+} entry (Paupe et al. 2015). However, it was demonstrated that MCUR1 binds to the MCU-pore and EMRE through their coiled-coil domains which stabilizes the mtCU complex and loss of MCUR1 reduces the bioenergetics and promotes autophagy (Tomar et al. 2016). However, a recent study has shown that Drosophila cells lacking the MCUR1 homologue still exhibited typical mtCU Ca^{2+} uptake (Chaudhuri et al. 2016).

2.3 Other Channels

2.3.1 Transport Across the Outer Mitochondria Membrane

In order for Ca^{2+} to interact with the mtCU it must first travel across the outer mitochondrial membrane (OMM). Initially the OMM was considered to be freely permeable to Ca^{2+} mostly by way of the highly abundant voltage dependent anion channel (VDAC). Later, a pair of studies demonstrated that increasing the permeability of the OMM via overexpression of VDAC (Rapizzi et al. 2002) or via treatment with truncated Bid (tcBid) (Csordas et al. 2002) increased the rate of Ca^{2+} influx into the mitochondrial matrix from IP_3R-linked high [Ca^{2+}] microdomains. Moreover, it has been shown that physiological [Ca^{2+}] changes can enhance the cation (e.g., K^+) conductance of VDAC reconstituted in bilayer and also enhance the permeability of the OMM to H^+ and ATP in permeabilized cells (Bathori et al. 2006). Nevertheless, the cation permeability of VDAC reconstituted in a lipid bilayer has been reported higher in the closed state (Tan and Colombini 2007). Thus, VDAC expression levels as well as gating state can modulate mitochondrial Ca^{2+} entry.

2.3.2 mRyR1

Localized in the IMM, mRyR1 is an alternative mechanism for mitochondrial Ca^{2+} uptake in cardiac and neuronal cells (Jakob et al. 2014; Beutner et al. 2001, 2005). RyRs are the largest known ion channels of about >2 MDa. Three different subtypes of RyR isoforms (RyR1, RyR2, and RyR3) have been described and cloned, with different pharmacological properties and tissue-specific expression. RyR1, the primary isoform in the skeletal muscle, is considered to be the major Ca^{2+} release channel in SR (Marks et al. 1989); RyR2 is most abundant in cardiac muscle cells (Nakai et al. 1990) (and, in a lesser amount, the brain); RyR3 is widely expressed in the ER of different vertebrate tissues (Giannini et al. 1995) and may be coexpressed with RyR1 and RyR2. In cardiac muscle cells RyR2 is abundantly localized in the SR (Lanner et al. 2010), but RyR1 is also detectable both at the mRNA and protein levels (Munch et al. 2000; Jeyakumar et al. 2002).

Using immuno-gold particle and electron microscopy, we reported that a low level of RyR1 is expressed at the IMM in cardiomyocytes, and with higher Ca^{2+} conductance and higher K_m for Ca^{2+} binding as compared to mtCU, mitochondrial RyR (mRyR) channels serve as a fast and high affinity Ca^{2+} uptake pathway (Beutner et al. 2001, 2005). Owing to the remarkable biochemical, pharmacological, and functional similarity of RyR in cardiac mitochondria to those of RyR1 in skeletal muscle SR, we designated it as mRyR1 (Beutner et al. 2005). mRyR1 showed a bell-shaped Ca^{2+} dependence of [3H]ryanodine binding with maximal binding at approximately pCa of 4.4 and complete block at pCa2 suggestive of RyR1. Moreover, unlike the cardiac SR-RyR2, caffeine showed hardly any effect on ryanodine binding in mitochondria and binding was inhibited by 50% in the presence of 0.33 mmol L^{-1} Mg^{2+} (Zimanyi and Pessah 1991). In permeabilized cardiomyocytes, ruthenium red at a concentration of 1–5 μmol L^{-1} blocked mitochondrial Ca^{2+} uptake with no significant effect on SR Ca^{2+} release (Sharma et al. 2000). Single-channel characterization of the mRyR1 revealed a novel 225-pS cation-selective channel in heart mitoplasts, with 4 distinct channel conductance (100, 225, 700, and 1,000 pS in symmetrical 150 mmol L^{-1} CsCl), which was blocked by high concentrations of ruthenium red and ryanodine, known inhibitors of ryanodine receptors (Ryu et al. 2011). Ryanodine showed a concentration-dependent modulation of this channel, with low concentrations (10 μmol L^{-1}) stabilizing a subconductance state while high concentrations ($\geq$100 μmol L^{-1}) blocked the channel activity (Ryu et al. 2011).

Although both the mRyR1 and the MCU are inhibited by low concentrations of ruthenium red (1–5 μM) and Mg^{2+}, the unique single-channel characteristics of mRyR1 clearly differentiate it from previously identified mitochondrial ion channels. Further clarifications will be needed to distinct the roles of mRyR1 and mtCU in the physiological Ca^{2+} signaling activities of the cardiac muscle mitochondria. Interestingly, a recent paper shows that stimulation of IP_3R in adult cardiac myocytes with endothelin-1 causes Ca^{2+} release from the SR, which is uniquely tunneled to mitochondria via mRyR leading to stimulation of mitochondrial ATP production (Seidlmayer et al. 2016).

2.3.3 RaM

RaM, first studied in isolated liver mitochondria, is a kinetically distinct mode of mitochondrial Ca^{2+} uptake, capable of sequestering significant amounts of Ca^{2+} hundreds of times faster than the mtCU. RaM is activated only transiently, facilitates mitochondria to rapidly sequester Ca^{2+} at the beginning of each cytosolic Ca^{2+} pulse, and rapidly recovers between pulses, which allows mitochondria to respond to repetitive Ca^{2+} transients (Sparagna et al. 1995). Similar to mtCU and mRyR1, RaM was inhibited by ruthenium red, but required over an order of magnitude more than that required for the inhibition of mtCU (0.1 mmol L^{-1}). Likewise, RaM is also activated by polyamines, such as spermine, at a concentration of 0.1 mmol L^{-1} and displayed 3 times more of an increase in activity than mtCU (Gunter and Gunter 2001). In addition, a rapid mode of Ca^{2+} uptake was also proposed in isolated heart mitochondria but with some different transport features

from those of liver (Buntinas et al. 2001). The reset time was longer (>60 s) and with less sensitivity towards the inhibition by ruthenium red. Moreover, ATP and GTP activated RaM in liver but not in heart where RaM is activated by ADP and inhibited by AMP. Notably, RaM has always been considered to be potentially an "operating mode" of the uniporter instead of a distinct channel/transporter entity; however, there have been no studies to reconcile RaM with I_{MiCa} or with the thus far identified molecular components of the mtCU complex.

2.3.4 mCa 1 and 2

mCa1 and mCa2 are both voltage gated mitochondrial Ca^{2+} selective channels similar to mtCU with a maximal conductance of 10.9 and 6.56 pS, respectively, at 105 mmol L^{-1} [Ca^{2+}], and half saturating concentration (K_m) of 15.1 and 19.6 mmol L^{-1} [Ca^{2+}], respectively. They have unique single-channel characteristics and sensitivity to Ru360. mCa1 channels display higher single-channel amplitude, smaller opening time, a lower open probability ($P_O = 0.053$), and multiple sub-conductance states. While, mCa2 channels have a smaller single-channel amplitude with a lower conductance, longer openings, a higher open probability, and no sub-conductance states. Like MCU and RaM, both mCa1 and mCa2 were activated by spermine. However, mCa2 was only partially inhibited by μmol L^{-1} concentrations of Ru360 (Michels et al. 2009). Like RaM, mCa1/2 have not been studied further in the molecular era of mtCU to explore if it was indeed a distinct channel entity or rather the result of a particular (stoichiometric) permutation and/or post-translational modification of the mtCU complex constituents.

2.3.5 CoQ

CoQ10 is an essential component of the mitochondrial electron-transport chain (ETC) with the primary role as an electron and proton transporter. It was also reported that CoQ10 is a regulator of mitochondrial Ca^{2+} and redox homeostasis. Under physiological conditions, hydroxyl CoQs can bind and efficiently transport Ca^{2+}. Hydroxyl CoQs have a very high affinity for Ca^{2+} and therefore, can function at $[Ca^{2+}]_c$ lower than 0.5 μM and potentially even at resting $[Ca^{2+}]_c$ levels (Bogeski et al. 2011). This relatively slower Ca^{2+} transfer might be a component of the thus far unidentified source of small tonic Ca^{2+} accumulation observed in MCU knock-out cardiac mitochondria (Kwong et al. 2015; Luongo et al. 2015).

2.3.6 LETM1 and TRPC3

LETM1, initially identified as a K^+/H^+ exchanger, was recently reported as a Ca^{2+}/H^+ antiporter. Using an siRNA genome-wide screening in drosophila, it was reported to be localized at the IMM. It transports Ca^{2+} bidirectionally across the IMM in a pH gradient-dependent manner and is inhibited by ruthenium red (Jiang et al. 2009). However, a recent study with LETM1 protein reconstituted in liposomes demonstrated LETM1 as an electroneutral $1Ca^{2+}/2H^+$ antiporter, insensitive to ruthenium red (Tsai et al. 2014).

Lastly, TRPC3 was demonstrated as an alternative mitochondrial Ca^{2+} uptake pathway. It is permeable to Ca^{2+}, Na^+, and K^+ and can contribute to mitochondrial

Ca^{2+} uptake during conditions with a relatively high extramitochondrial [Ca^{2+}] (Feng et al. 2013).

3 Transcriptional/Post-transcriptional and Post-translational Regulation of the mtCU Complex

As described above (see Sect. 2), the mtCU is a multisubunit complex with many regulators. However, the expression patterns of each component are variable in a tissue-specific manner (Plovanich et al. 2013; Murgia and Rizzuto 2015) for adapting to the appropriate Ca^{2+} sensitivity by intracellular signals in each tissue. Therefore, it is of interest to elucidate how the mtCU complex is differentially regulated at the level of gene expression, which is linked to its modulation of mitochondrial Ca^{2+} uptake. Accordingly, it has been reported that transcriptional and post-transcriptional mechanisms can regulate MCU expression and activity to specific functional demands (Plovanich et al. 2013; Murgia and Rizzuto 2015; Marchi et al. 2013). For example, in neurons, synaptic activity suppresses MCU transcription through a nuclear Ca^{2+} signals, Ca^{2+}/calmodulin kinase (CaMK), and the transcription factor Npas4 dependent mechanism, preventing excitotoxic death (Qiu et al. 2013). In addition, the Ca^{2+}-regulated transcription factor cyclic adenosine monophosphate response element–binding protein (CREB) directly binds to the MCU promoter and stimulates MCU expression, regulating mitochondrial metabolism (Shanmughapriya et al. 2015). MCUb expression was also reported to be increased though independent of CREB activation (Shanmughapriya et al. 2015). It has been shown that MCU is also a target of microRNA-25 (miR-25), which can efficiently decrease MCU gene expression and activity (Marchi et al. 2013). Furthermore, analyses of post-translational modifications of the MCU components are ongoing. In 2012, Joiner et al. for the first time reported two Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) phosphorylation candidate motifs at the N-terminus of MCU. CaMKII resides endogenously in the mitochondrial matrix and is highly activated during pathophysiological conditions like ischemia reperfusion and myocardial infarction; promotes myocardial death via CaMKII-mediated increases in MCU current, by phosphorylation of MCU at serine 57 and 92. However, mitochondrial CaMKII inhibition reduced MCU current and was protective against ischemia/reperfusion injury, myocardial infarction, and neurohumoral injury (Joiner et al. 2012, 2014; Fieni et al. 2014). Recently, Lee et al. showed that MCU-S92A mutant expression failed to rescue the Ca^{2+} channel activity in an MCU knockdown cell line. In addition, they also presented the crystal structure of the N-terminal region of MCU including (S92) a potential CaMKII phosphorylation site and concluded them to be indispensable for modulation of channel activity (Lee et al. 2015). Additionally, our group demonstrated that α1-adrenoceptor (α1-AR) signaling activates Ca^{2+} and ROS dependent proline-rich tyrosine kinase 2 (Pyk2); translocates Pyk2 into the mitochondrial matrix. Activated Pyk2 interacts with MCU and directly phosphorylates MCU tyrosine residue(s) and enhances mitochondrial Ca^{2+} uptake by promoting MCU channel

oligomerization and formation of tetrameric channels (O-Uchi et al. 2014). However, persistent α1-AR stimulation increases ROS production, activates the mitochondrial permeability transition pore (mPTP) opening, and eventually leads to cell death via Pyk2 activation in cardiomyocytes (O-Uchi et al. 2014).

4 Physiological Roles of Mitochondrial Ca^{2+} Uptake

Mitochondrial Ca^{2+} has been implicated as an important regulator of fundamental cellular processes, which range from the regulation of cellular metabolism, buffering cytosolic Ca^{2+}, modulating cellular redox environments, to other cell-type specific functions. As described above, we have witnessed a rapid advance in our understanding of the role of mitochondrial Ca^{2+} uptake mechanisms in physiology and pathophysiology since the recent molecular discovery of the mtCU pore (i.e., MCU) and its regulators. Therefore, in the next sections, we summarize the role of mitochondrial Ca^{2+} uptake mechanisms highlighting the functions of the mtCU complex during physiological (Sect. 4) and pathological (Sect. 5) conditions.

4.1 Mitochondrial Ca^{2+} and Energy Metabolism

Mitochondrial Ca^{2+} uptake serves as one of the major factors for regulating cellular bioenergetics (Denton and McCormack 1980; Hajnoczky et al. 1995). Denton and McCormick in the 1980s demonstrated that mitochondrial Ca^{2+} plays an important role in regulating three Ca^{2+} dependent dehydrogenases: pyruvate dehydrogenase (PDH), α-ketoglutarate (also called oxoglutarate) dehydrogenase (OGDH), and NAD-isocitrate dehydrogenase (ICDH) (Denton 2009; McCormack et al. 1990) that are the rate-limiting enzymes in substrate supply for ATP synthesis (Jouaville et al. 1999). Of the three dehydrogenases, ICDH and OGDH are activated through the binding of Ca^{2+} (Rutter and Denton 1988) whereas, PDH activation depends on Ca^{2+}-dependent phosphatase mediated dephosphorylation step (Denton et al. 1972). Increase in mitochondrial Ca^{2+} uptake can activate oxidative metabolism via activated matrix dehydrogenases, resulting in an increased supply of reducing equivalents to drive respiratory chain activity and ATP synthesis (McCormack et al. 1990). Mitochondrial matrix Ca^{2+} also regulates bioenergetics by S100A1 mediated direct Ca^{2+}-dependent activation of F_0-F_1ATP synthase activity (Boerries et al. 2007; Glancy and Balaban 2012).

Surprisingly, mouse embryonic fibroblasts or isolated mitochondria from MCU-knockout mice have apparently well-maintained basal mitochondrial metabolic function and energetics, albeit with decreased Ca^{2+} uptake and lower resting Ca^{2+} levels (Perocchi et al. 2010; De Stefani et al. 2011; Baughman et al. 2011; Mallilankaraman et al. 2012b). Even more surprisingly, this lack of energetic phenotype extends to the beating heart in vivo under physiological conditions (approximately 500 beats/min), either in germline or inducible cardiac-specific

MCU knockout mice (Pan et al. 2013; Kwong et al. 2015; Luongo et al. 2015; Murphy et al. 2014). Likewise, though global MCU knockout displayed no evidence of Ca^{2+} uptake in mitochondria yet, basal ATP levels were not evidently altered, indicating that lack of MCU does not have marked impact on basal mitochondrial metabolism (Holmstrom et al. 2015). However, skeletal muscle showed a minor defect in muscle strength after endurance training (Pan et al. 2013). The mild phenotype of MCU knockout mice could be due to some kinds of adaptation in these animals (Murphy et al. 2014). Similarly, in a cardiac-specific MCU knockout mouse, there is no energetic phenotype in vivo under normal physiological conditions. However, these mice displayed a decreased β-adrenergic receptor-mediated fight or flight response for increased workload under stress and a decreased ischemia-reperfusion injury (Kwong et al. 2015; Luongo et al. 2015). Similar results have been obtained via cardiac-specific overexpression of a dominant-negative mutant MCU (Wu et al. 2015). These surprising findings have set a stage for seeking other compensatory or unknown mechanisms for the MCU-independent regulation of bioenergetics in beating heart (Harrington and Murphy 2015).

Knockdown of MCUR1 reduces mitochondrial Ca^{2+} uptake resulting in disruption of oxidative phosphorylation which activates AMP kinase-dependent pro-survival autophagy (Mallilankaraman et al. 2012b). However, in pancreatic β-cells, knockdown of MCU and MICU1 markedly reduced the mitochondrial Ca^{2+} uptake and showed that MCU- and MICU1-mediated Ca^{2+} uptake is critical for continual ATP synthesis, glucose metabolism, and insulin secretion (Tarasov et al. 2012; Alam et al. 2012). MCU silencing down-regulates the expression of respiratory chain complexes, mitochondrial metabolic activity, and oxygen consumption (Quan et al. 2015). In addition to MCU, absence of LETM1 decreased basal mitochondrial oxygen consumption, discernible inactivation of complex IV activity, and a drop in ATP production (Doonan et al. 2014). We recently reported that RyR1-overexpressing cardiac cells had higher mitochondrial ATP under basal conditions with augmented $[Ca^{2+}]_c$-dependent ATP production (O-Uchi et al. 2013), supporting our previous finding of a low respiratory control index in RyR1 knockout mice and insensitivity to $[Ca^{2+}]_c$ stimulation of O_2 consumption in mice.

4.2 Cytosolic Ca^{2+} Buffering

Apart from mitochondria's role as the main energy supplier, its implication in cytosolic Ca^{2+} buffering is becoming increasingly apparent. Mitochondria can directly influence the $[Ca^{2+}]_c$ by importing Ca^{2+} through the MCU and efflux through the Na^+/Ca^{2+} exchanger or H^+/Ca^{2+} exchangers (Gunter et al. 1994; Gunter and Pfeiffer 1990; Carafoli 1987; Thayer and Miller 1990; Cox and Matlib 1993). Since the resting $[Ca^{2+}]_c$ values are ~100 nM and the $\Delta\Psi_m$ is ~−180 mV, the prediction is that at electrochemical equilibrium, theoretical $[Ca^{2+}]_m$ values could be higher than 0.1 M (Pozzan et al. 2000). However, the low affinity of the MCU to Ca^{2+} (K_d around 10–50 μM), the presence of mitochondrial efflux mechanisms, and the decrease of $\Delta\Psi_m$ upon the cation influx would avert the attainment of

electrochemical equilibrium. Therefore, particularly under resting conditions, mitochondria may not uptake any Ca^{2+}. Based on these considerations, the evident discrepancy between the low affinity of MCU, the low concentration of global cytosolic Ca^{2+} signals, and the amplitude of $[Ca^{2+}]_m$ rises were resolved in the 1990s by the concept of a microdomain of high $[Ca^{2+}]_c$ between ER/SR and mitochondria contact areas (Rizzuto et al. 1993, 1998). According to which, mitochondria are strategically located in close proximity to ER/SR through tethering proteins (Csordas et al. 2006), and these close contact sites provide mitochondria preferential access to a much higher $[Ca^{2+}]$ than that measured in the bulk cytosol during Ca^{2+} release from ER/SR and able to activate the MCU. These local $[Ca^{2+}]$ exposures of the mitochondrial surface have been measured to be ~10 μM in average by means of "hotspot" mapping of OMM-targeted Ca^{2+} sensor proteins (Giacomello et al. 2010) or Ca^{2+} sensors directly targeted to the SR/ER-OMM focal contact areas utilizing a drug-inducible heterodimerization strategy (Csordas et al. 2010). In addition, there are reports that VDAC in the OMM and IP3 receptors in the ER are enriched at the mitochondria–ER interface, facilitating a Ca^{2+} transfer from the ER to the mitochondria (Szabadkai et al. 2006; Mendes et al. 2005; Malli et al. 2005). Several functional and morphological studies further suggested that mitochondria can form close contacts not only with ER/SR (Rizzuto et al. 1998; Csordas et al. 1999; Szalai et al. 2000) but also the Golgi apparatus (Dolman et al. 2005) and the plasma membrane (Malli et al. 2003; Park et al. 2001; Varadi et al. 2004). However, among these interactions, the ER/SR-mitochondria connections have gained much attention, and various proteins have been proposed to link mitochondria to the ER/SR such as MIRO, MFN2, and the Mmm1/Mdm10/Mdm12/Mdm34 complex (Rowland and Voeltz 2012; Grimm 2012). Therefore, ER/SR-mitochondria communication also serves as a highly localized Ca^{2+} buffering system. This in turn can modify the activity of any nearby Ca^{2+}-dependent proteins. Such regulation has been reported for IP_3R that display isoform-specific biphasic dependence on $[Ca^{2+}]_c$. Depending on the dominating IP_3R isoform, local Ca^{2+} clearance by mitochondria can either suppress IP_3R activation (and Ca^{2+} release from the ER) via reducing the local $[Ca^{2+}]$ (and so IP_3 sensitivity) over IP_3R clusters (Marchant et al. 2002; Hajnoczky et al. 1999); or do the opposite by decreasing $[Ca^{2+}]$ from high inhibitory to stimulatory range (Olson et al. 2010). By similar principles, local mitochondrial Ca^{2+} clearance has also been implicated in sustaining the activation of I_{CRAC}/Orai channels during store operated Ca^{2+} entry by relieving local feedback inhibition of the channels by Ca^{2+} (Hoth et al. 1997, 2000; Quintana et al. 2006).

4.3 Reactive Oxygen Species Generation

Mitochondria are a major source of ROS in the cell. It has been well recognized that $[Ca^{2+}]_m$ enhance ROS generation by stimulating the TCA cycle and oxidative phosphorylation (Perez-Campo et al. 1998; Sohal and Allen 1985) and/or triggering opening of mPTP (Brookes et al. 2004; Rasola and Bernardi 2011), which plays an

important role in the regulation of cellular function. For example, a recent study identified that mtCU-mediated mitochondrial Ca^{2+} uptake triggers mitochondrial ROS production and transient opening of the mPTP, which promotes wound repair and organismal survival (Xu and Chisholm 2014). In addition, it has been shown that mitochondrial Ca^{2+}-mediated ROS production modulates neural differentiation through activation of the Wnt/β-catenin pathway (Rharass et al. 2014). However, excess Ca^{2+} uptake by the mtCU can be detrimental for cells, triggering excessive ROS generation and initiating cell death pathways such as apoptosis (Mallilankaraman et al. 2012a; Huang et al. 2013; Csordas et al. 2013; Hall et al. 2014). Therefore, mitochondrial Ca^{2+} uptake can be either beneficial or detrimental depending on the amount of Ca^{2+} uptake and cellular conditions. We will discuss the pathological role of mitochondrial Ca^{2+} uptake in Sect. 5.

5 Pathological Roles of Mitochondrial Ca^{2+} Uptake

As shown in Sect. 4.2, mitochondrial Ca^{2+} uptake significantly contributes to buffering cytosolic Ca^{2+} under physiological Ca^{2+} release from ER/SR. However, intensive long-lasting pathophysiological release of Ca^{2+} from ER/SR causes persistent mitochondrial Ca^{2+} accumulation, which consequently triggers excessive ROS generation followed by ATP depletion, the long-lasting opening of the mPTP (Bernardi 2013; Rizzuto et al. 2012), and apoptotic/necrotic cascade (Rizzuto et al. 2012). Accordingly, MCU-overexpressing and MICU1-knockdown human cell lines lead to increased sensitivity to apoptosis (De Stefani et al. 2011; Mallilankaraman et al. 2012a). Moreover, human genetic disease associated with MICU1 null mutations exhibiting central nervous system (extrapyramidal symptoms, learning difficulties) and skeletal muscle (fatigue) phenotypes have been recently identified (Logan et al. 2014; Lewis-Smith et al. 2016). Liver-specific knockout of MICU1 has been recently shown to severely impair liver regeneration after partial hepatectomy, which phenotype could be almost completely rescued by administration of NIM811, a non-immunosuppressant mPTP inhibitor (Antony et al. 2016). In addition, MCU overexpression in *T. brucei* is also sensitized to apoptotic stress (Huang et al. 2013). However, MCU overexpression in a human breast adenocarcinoma cell line (Hall et al. 2014) and MCU-knockout mouse embryonic fibroblasts (Pan et al. 2013) show no difference in sensitivity to apoptosis.

As discussed above, although, mitochondrial Ca^{2+} increase has been associated with apoptosis in many pathological conditions (Giorgi et al. 2012); however, very little is known about the roles of mitochondrial Ca^{2+} signaling in cancer. Marchi et al. (2013) showed that microRNA-25 (miR-25) expression can decrease in MCU gene expression and activity. Specifically, miR-25 is up-regulated in human colon and prostate cancers, which leads to decreased MCU levels followed by reduced mitochondrial Ca^{2+} uptake and resistance to Ca^{2+}-dependent apoptotic challenges (Marchi et al. 2013). Consistent with these results, overexpression of MCU or knockdown of MICU1 in HeLa cervical cancer cells results in constitutive mitochondrial Ca^{2+} influx and increases HeLa cell sensitivity to hydrogen peroxide and

ceramide toxicity (De Stefani et al. 2011; Mallilankaraman et al. 2012a). In other cancer paradigms like in triple-negative breast cancer MCU has been identified as a promoter of progression/invasiveness by supporting the mitochondrial Ca^{2+}-ROS-HIF-1α signaling axis (Tosatto et al. 2016). Thus, the suppression of the MCU expression by miRNA provides initial clues to the relevance of this pathway in human cancers.

Recent studies show that genetic and molecular manipulation of the mtCU complex can also affect cell-type specific functions such as neurotransmission, growth, and development. MCU overexpression increases NMDA receptor-dependent excitotoxicity in mouse neurons via enhanced mitochondrial calcium uptake resulting in aggravated mitochondrial depolarization and neuronal injury. However, MCU knockdown protects neurons against NMDA receptor-mediated excitotoxic cell death (Qiu et al. 2013).

6 Pharmacological Modulators of the MCU

Despite the well-known role of the MCU as a key controller of Ca^{2+} homoeostasis, there is little information about its pharmacological regulation. Although, several pharmacological inhibitors have been described to modify the activity of the MCU, their lack of specificity and cellular permeability has limited their application (Table 1). One of the most widely studied and effective inhibitors is the hexavalent polysaccharide stain, ruthenium red, or its derivate Ru360 (Kirichok et al. 2004; Matlib et al. 1998). In 2011, De Stefani et al. demonstrated the MCU role as the channel-forming subunit, permeable to Ca^{2+} and inhibited by ruthenium red, in an isolated mitochondria. They reconstituted MCU in lipid bilayers and recorded

Table 1 Pharmacological modulators of MCU

Compound	Effect(s)	References
Ruthenium compound: ruthenium red, Ru360	Inhibitor	Kirichok et al. (2004) and Matlib et al. (1998)
Lanthanides: La^{3+}, Gd^{3+}, and Pr^{3+}	Inhibitor	Crompton et al. (1979)
Cardioactive drugs: quinidine, alprenolol, propranolol, oxyfedrine, and tetracaine	Inhibitor	Noack and Greeff (1971)
Amiloride analogs and derivatives	Inhibitor	Schellenberg et al. (1985)
Mg^{2+}	Inhibitor	Szanda et al. (2009)
KBR7943	Inhibitor	Santo-Domingo et al. (2007)
Minocycline	Inhibitor	Schwartz et al. (2013) and Csordas et al. (2012)
Polyamines: spermine and spermidine	Activator	Salvi and Toninello (2004)
Estrogen receptor agonists: 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT)	Activator	Lobaton et al. (2005)
The p38 MAP kinase inhibitor: SB202190	Activator	Montero et al. (2002)
Flavonoids	Activator	Montero et al. (2004)

ruthenium red-sensitive Ca^{2+} current with 6–7-pS single-channel activity (De Stefani et al. 2011). These findings were very recently supported by another patch-clamp experiment by Chaudhuri et al. (2013). They showed parallel changes in the mitochondrial Ca^{2+} current in an MCU knockdown and overexpression system. In addition, by exploiting the inhibitory characteristic of ruthenium red they further confirmed MCU as a pore-forming subunit of the channel complex. They demonstrated that a single point mutation (S259A) in the putative pore domain conferred resistance to ruthenium red (Baughman et al. 2011; Chaudhuri et al. 2013) without changing current magnitude indicating that ruthenium red directly targets the channel.

However, ruthenium red binds to and inhibits a wide variety of plasma membrane and intracellular Ca^{2+} and K^+ channels like Transient Receptor Potential Vanilloid (TRPV) (Amann and Maggi 1991; Hymel et al. 1988), TWIK-related Acid-sensitive K^+ channel (TASK-3) (Czirjak and Enyedi 2002), and RyR (MacQuaide et al. 2010). Ru360, a purified form of ruthenium red, is more effective than ruthenium red with an IC_{50} 5 nM vs 1 μM, respectively (Ying et al. 1991). Ru360 also demonstrates better specificity for the MCU over other Ca^{2+} channels in cardiac muscles (De Stefani et al. 2011; Baughman et al. 2011; Matlib et al. 1998). Earlier studies have reported a number of drugs exhibiting MCU inhibition such as the cardioactive drugs quinidine, alprenolol, propranolol, oxyfedrine, tetracaine (Noack and Greeff 1971), the diuretic, ethacrynic acid, amiloride analogs and derivatives (Schellenberg et al. 1985), and the antibiotic gentamicin (Sastrasinh et al. 1982). Minocycline, a tetracycline-derived antibiotic that has been used clinically to treat bacterial infections, is also a potent inhibitor for MCU (Schwartz et al. 2013). Mg^{2+}, an antagonist of mitochondrial Ca^{2+} uptake also inhibits the MCU at physiological concentrations (Szanda et al. 2009). Lanthanides such as La^{3+}, Gd^{3+}, and Pr^{3+} are also well-known competitive inhibitors and at low concentrations they may activate the uniporter's activation site and facilitate the transport of other ions (Mela 1969). However, they inhibit a variety of other Ca^{2+} channels and pumps too. Thiourea derivate KBR7943, originally an inhibitor of the plasma membrane Na^+/Ca^{2+} exchanger is also reported to have an inhibitory effect on the MCU (Santo-Domingo et al. 2007). In addition, MCU activity is also inhibited by adenine nucleotides; ATP being the most potent inhibitor (EC_{50} 0.6 mM) followed by ADP > AMP. Interestingly, AMPPNP, a non-hydrolysable analog of ATP was also found to be as efficient as ATP, suggesting that inhibitory action does not require ATP hydrolysis (Litsky and Pfeiffer 1997). On the other hand, uniporter activity is known to be activated by inorganic phosphate (Pi), which can accelerate the Ca^{2+} uptake rate by precipitating with Ca^{2+} in the mitochondrial matrix, and thereby lowering the $[Ca^{2+}]_m$ (Crompton et al. 1983). The Ca^{2+} influx rate and affinity for Ca^{2+} are modulated by protein kinases. Specifically, the ζ isoform of protein kinase C will activate, whereas the β/δ isoforms inactivate MCU (Pinton et al. 2004). Knockdown studies of p38 mitogen-activated protein kinase (MAPK) have resulted in an increase of mitochondrial Ca^{2+} uptake suggesting either itself or its downstream targets can inhibit MCU (Koncz et al. 2009; Szanda et al. 2008). Likewise, SB202190, an inhibitor of p38 MAPK, significantly activates mitochondrial Ca^{2+} uptake, both in

intact and in permeabilized cells (Montero et al. 2002). Other pharmacological activators include natural plant flavonoids (e.g., genistein, quercetin, kaempferol) (Montero et al. 2004), polyamines such as spermine and spermidine (Nicchitta and Williamson 1984; Salvi and Toninello 2004), and estrogens receptor agonists [4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT)] (Lobaton et al. 2005). Lastly, MCU mediated Ca^{2+} uptake also displays allosteric positive regulation by cytosolic Ca^{2+} in a calmodulin-dependent manner (Moreau et al. 2006; Putney and Thomas 2006) which was shown to be inhibited by calmodulin inhibitors (Csordas and Hajnoczky 2003).

7 Conclusions

Ca^{2+} uptake into the mitochondrial matrix plays a vital role in the regulation of multiple physiological and pathological processes, ranging from cytoplasmic Ca^{2+} signaling to bioenergetics and cell death. Mitochondria can uptake Ca^{2+} via multiple channels and pathways, however, the mtCU complex is the most prominent and well-characterized pathway. In this chapter, we have focused on the recent identification of the components of the mtCU complex as well as the other mitochondrial ion channels. Our understanding about the molecular complexity of mtCU gradually evolved from the concept of a single protein to macromolecular signaling complexes, which includes a Ca^{2+} pore-forming component and regulatory components controlling channel activity. We discussed the means by which multiple cell types and tissues regulate and use these channels to best-function for their physiological role in an organism, as well as how the dysfunction of this system can lead to pathophysiological conditions.

The recent characterization of the mtCU complex has opened up the possibility for precise crystal and cryo-electronmicroscopic structural information of the individual proteins as well as the complete complex. Finally, future insight into the transcriptional, post-transcriptional, and post-translational modifications of the multi-protein mtCU complex as well as other mitochondrial Ca^{2+} transport mechanisms will contribute to the development of more specific pharmacological tools and potentially therapeutic drugs.

Acknowledgments This work was partly supported by National Heart, Lung, and Blood Institute Grants 2R01 HL-093671 and HL-114760 (to S. S. Sheu), 1R01 HL-122124 (to G. Csordas and S. S. Sheu). American Heart Association Grant 16SDG27260248 (to J. O-Uchi).

References

Alam MR, Groschner LN, Parichatikanond W, Kuo L, Bondarenko AI, Rost R, Waldeck-Weiermair M, Malli R, Graier WF (2012) Mitochondrial Ca2+ uptake 1 (MICU1) and mitochondrial Ca2+ uniporter (MCU) contribute to metabolism-secretion coupling in clonal pancreatic beta-cells. J Biol Chem 287:34445–34454

Amann R, Maggi CA (1991) Ruthenium red as a capsaicin antagonist. Life Sci 49:849–856

Antony AN, Paillard M, Moffat C, Juskeviciute E, Correnti J, Bolon B, Rubin E, Csordas G, Seifert EL, Hoek JB, Hajnoczky G (2016) MICU1 regulation of mitochondrial Ca(2+) uptake dictates survival and tissue regeneration. Nat Commun 7:10955

Bathori G, Csordas G, Garcia-Perez C, Davies E, Hajnoczky G (2006) Ca2+-dependent control of the permeability properties of the mitochondrial outer membrane and voltage-dependent anion-selective channel (VDAC). J Biol Chem 281:17347–17358

Baughman JM, Perocchi F, Girgis HS, Plovanich M, Belcher-Timme CA, Sancak Y, Bao XR, Strittmatter L, Goldberger O, Bogorad RL, Koteliansky V, Mootha VK (2011) Integrative genomics identifies mcu as an essential component of the mitochondrial calcium uniporter. Nature 476:341–345

Becker GL, Fiskum G, Lehninger AL (1980) Regulation of free Ca2+ by liver mitochondria and endoplasmic reticulum. J Biol Chem 255:9009–9012

Bernardi P (2013) The mitochondrial permeability transition pore: a mystery solved? Front Physiol 4:95

Beutner G, Sharma VK, Giovannucci DR, Yule DI, Sheu SS (2001) Identification of a ryanodine receptor in rat heart mitochondria. J Biol Chem 276:21482–21488

Beutner G, Sharma VK, Lin L, Ryu SY, Dirksen RT, Sheu SS (2005) Type 1 ryanodine receptor in cardiac mitochondria: transducer of excitation-metabolism coupling. Biochim Biophys Acta 1717:1–10

Boerries M, Most P, Gledhill JR, Walker JE, Katus HA, Koch WJ, Aebi U, Schoenenberger CA (2007) Ca2+-dependent interaction of s100a1 with f1-atpase leads to an increased atp content in cardiomyocytes. Mol Cell Biol 27:4365–4373

Bogeski I, Gulaboski R, Kappl R, Mirceski V, Stefova M, Petreska J, Hoth M (2011) Calcium binding and transport by coenzyme Q. J Am Chem Soc 133:9293–9303

Brookes PS, Yoon Y, Robotham JL, Anders MW, Sheu SS (2004) Calcium, ATP, and ROS: a mitochondrial love-hate triangle. Am J Physiol Cell Physiol 287:C817–C833

Buntinas L, Gunter KK, Sparagna GC, Gunter TE (2001) The rapid mode of calcium uptake into heart mitochondria (RaM): comparison to RaM in liver mitochondria. Biochim Biophys Acta 1504:248–261

Carafoli E (1987) Intracellular calcium homeostasis. Annu Rev Biochem 56:395–433

Carafoli E (2010) The fateful encounter of mitochondria with calcium: how did it happen? Biochim Biophys Acta 1797:595–606

Chance B (1965) The energy-linked reaction of calcium with mitochondria. J Biol Chem 240:2729–2748

Chaudhuri D, Sancak Y, Mootha VK, Clapham DE (2013) MCU encodes the pore conducting mitochondrial calcium currents. Elife 2:e00704

Chaudhuri D, Artiga DJ, Abiria SA, Clapham DE (2016) Mitochondrial calcium uniporter regulator 1 (MCUR1) regulates the calcium threshold for the mitochondrial permeability transition. Proc Natl Acad Sci U S A 113:E1872–E1880

Cox DA, Matlib MA (1993) Modulation of intramitochondrial free Ca2+ concentration by antagonists of Na(+)-Ca2+ exchange. Trends Pharmacol Sci 14:408–413

Crompton M, Heid I, Baschera C, Carafoli E (1979) The resolution of calcium fluxes in heart and liver mitochondria using the lanthanide series. FEBS Lett 104:352–354

Crompton M, Kessar P, Al-Nasser I (1983) The alpha-adrenergic-mediated activation of the cardiac mitochondrial Ca2+ uniporter and its role in the control of intramitochondrial Ca2+ in vivo. Biochem J 216:333–342

Csordas G, Hajnoczky G (2003) Plasticity of mitochondrial calcium signaling. J Biol Chem 278:42273–42282

Csordas G, Thomas AP, Hajnoczky G (1999) Quasi-synaptic calcium signal transmission between endoplasmic reticulum and mitochondria. EMBO J 18:96–108

Csordas G, Madesh M, Antonsson B, Hajnoczky G (2002) Tcbid promotes Ca(2+) signal propagation to the mitochondria: control of Ca(2+) permeation through the outer mitochondrial membrane. EMBO J 21:2198–2206

Csordas G, Renken C, Varnai P, Walter L, Weaver D, Buttle KF, Balla T, Mannella CA, Hajnoczky G (2006) Structural and functional features and significance of the physical linkage between ER and mitochondria. J Cell Biol 174:915–921

Csordas G, Varnai P, Golenar T, Roy S, Purkins G, Schneider TG, Balla T, Hajnoczky G (2010) Imaging interorganelle contacts and local calcium dynamics at the ER-mitochondrial interface. Mol Cell 39:121–132

Csordas G, Varnai P, Golenar T, Sheu SS, Hajnoczky G (2012) Calcium transport across the inner mitochondrial membrane: molecular mechanisms and pharmacology. Mol Cell Endocrinol 353:109–113

Csordas G, Golenar T, Seifert EL, Kamer KJ, Sancak Y, Perocchi F, Moffat C, Weaver D, de la Fuente Perez S, Bogorad R, Koteliansky V, Adijanto J, Mootha VK, Hajnoczky G (2013) MICU1 controls both the threshold and cooperative activation of the mitochondrial Ca(2)(+) uniporter. Cell Metab 17:976–987

Czirjak G, Enyedi P (2002) Formation of functional heterodimers between the task-1 and task-3 two-pore domain potassium channel subunits. J Biol Chem 277:5426–5432

de la Fuente S, Matesanz-Isabel J, Fonteriz RI, Montero M, Alvarez J (2014) Dynamics of mitochondrial Ca2+ uptake in MICU1-knockdown cells. Biochem J 458:33–40

De Stefani D, Raffaello A, Teardo E, Szabo I, Rizzuto R (2011) A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter. Nature 476:336–340

Deluca HF, Engstrom GW (1961) Calcium uptake by rat kidney mitochondria. Proc Natl Acad Sci U S A 47:1744–1750

Denton RM (2009) Regulation of mitochondrial dehydrogenases by calcium ions. Biochim Biophys Acta 1787:1309–1316

Denton RM, McCormack JG (1980) The role of calcium in the regulation of mitochondrial metabolism. Biochem Soc Trans 8:266–268

Denton RM, Randle PJ, Martin BR (1972) Stimulation by calcium ions of pyruvate dehydrogenase phosphate phosphatase. Biochem J 128:161–163

Dolman NJ, Gerasimenko JV, Gerasimenko OV, Voronina SG, Petersen OH, Tepikin AV (2005) Stable golgi-mitochondria complexes and formation of golgi Ca(2+) gradients in pancreatic acinar cells. J Biol Chem 280:15794–15799

Doonan PJ, Chandramoorthy HC, Hoffman NE, Zhang X, Cardenas C, Shanmughapriya S, Rajan S, Vallem S, Chen X, Foskett JK, Cheung JY, Houser SR, Madesh M (2014) Letm1-dependent mitochondrial Ca2+ flux modulates cellular bioenergetics and proliferation. FASEB J 28:4936–4949

Drago I, De Stefani D, Rizzuto R, Pozzan T (2012) Mitochondrial Ca2+ uptake contributes to buffering cytoplasmic Ca2+ peaks in cardiomyocytes. Proc Natl Acad Sci U S A 109:12986–12991

Duchen MR, Verkhratsky A, Muallem S (2008) Mitochondria and calcium in health and disease. Cell Calcium 44:1–5

Feng S, Li H, Tai Y, Huang J, Su Y, Abramowitz J, Zhu MX, Birnbaumer L, Wang Y (2013) Canonical transient receptor potential 3 channels regulate mitochondrial calcium uptake. Proc Natl Acad Sci U S A 110:11011–11016

Fieni F, Lee SB, Jan YN, Kirichok Y (2012) Activity of the mitochondrial calcium uniporter varies greatly between tissues. Nat Commun 3:1317

Fieni F, Johnson DE, Hudmon A, Kirichok Y (2014) Mitochondrial Ca2+ uniporter and camkii in heart. Nature 513:E1–E2

Giacomello M, Drago I, Pizzo P, Pozzan T (2007) Mitochondrial Ca2+ as a key regulator of cell life and death. Cell Death Differ 14:1267–1274

Giacomello M, Drago I, Bortolozzi M, Scorzeto M, Gianelle A, Pizzo P, Pozzan T (2010) Ca2+ hot spots on the mitochondrial surface are generated by Ca2+ mobilization from stores, but not by activation of store-operated Ca2+ channels. Mol Cell 38:280–290

Giannini G, Conti A, Mammarella S, Scrobogna M, Sorrentino V (1995) The ryanodine receptor/calcium channel genes are widely and differentially expressed in murine brain and peripheral tissues. J Cell Biol 128:893–904

Giorgi C, Baldassari F, Bononi A, Bonora M, De Marchi E, Marchi S, Missiroli S, Patergnani S, Rimessi A, Suski JM, Wieckowski MR, Pinton P (2012) Mitochondrial Ca(2+) and apoptosis. Cell Calcium 52:36–43

Glancy B, Balaban RS (2012) Role of mitochondrial ca2+ in the regulation of cellular energetics. Biochemistry 51:2959–2973

Grimm S (2012) The ER-mitochondria interface: the social network of cell death. Biochim Biophys Acta 1823:327–334

Gunter TE, Gunter KK (2001) Uptake of calcium by mitochondria: transport and possible function. IUBMB Life 52:197–204

Gunter TE, Pfeiffer DR (1990) Mechanisms by which mitochondria transport calcium. Am J Physiol 258:C755–C786

Gunter TE, Gunter KK, Sheu SS, Gavin CE (1994) Mitochondrial calcium transport: physiological and pathological relevance. Am J Physiol 267:C313–C339

Hajnoczky G, Robb-Gaspers LD, Seitz MB, Thomas AP (1995) Decoding of cytosolic calcium oscillations in the mitochondria. Cell 82:415–424

Hajnoczky G, Hager R, Thomas AP (1999) Mitochondria suppress local feedback activation of inositol 1,4, 5-trisphosphate receptors by Ca2+. J Biol Chem 274:14157–14162

Hall DD, Wu Y, Domann FE, Spitz DR, Anderson ME (2014) Mitochondrial calcium uniporter activity is dispensable for MDA-MB-231 breast carcinoma cell survival. PLoS One 9:e96866

Harrington JL, Murphy E (2015) The mitochondrial calcium uniporter: mice can live and die without it. J Mol Cell Cardiol 78:46–53

Hoffman NE, Chandramoorthy HC, Shamugapriya S, Zhang X, Rajan S, Mallilankaraman K, Gandhirajan RK, Vagnozzi RJ, Ferrer LM, Sreekrishnanilayam K, Natarajaseenivasan K, Vallem S, Force T, Choi ET, Cheung JY, Madesh M (2013) MICU1 motifs define mitochondrial calcium uniporter binding and activity. Cell Rep 5:1576–1588

Holmstrom KM, Pan X, Liu JC, Menazza S, Liu J, Nguyen TT, Pan H, Parks RJ, Anderson S, Noguchi A, Springer D, Murphy E, Finkel T (2015) Assessment of cardiac function in mice lacking the mitochondrial calcium uniporter. J Mol Cell Cardiol 85:178–182

Hoth M, Fanger CM, Lewis RS (1997) Mitochondrial regulation of store-operated calcium signaling in T lymphocytes. J Cell Biol 137:633–648

Hoth M, Button DC, Lewis RS (2000) Mitochondrial control of calcium-channel gating: a mechanism for sustained signaling and transcriptional activation in T lymphocytes. Proc Natl Acad Sci U S A 97:10607–10612

Huang G, Vercesi AE, Docampo R (2013) Essential regulation of cell bioenergetics in trypanosoma brucei by the mitochondrial calcium uniporter. Nat Commun 4:2865

Hung V, Zou P, Rhee HW, Udeshi ND, Cracan V, Svinkina T, Carr SA, Mootha VK, Ting AY (2014) Proteomic mapping of the human mitochondrial intermembrane space in live cells via ratiometric apex tagging. Mol Cell 55:332–341

Hymel L, Schindler H, Inui M, Fleischer S (1988) Reconstitution of purified cardiac muscle calcium release channel (ryanodine receptor) in planar bilayers. Biochem Biophys Res Commun 152:308–314

Jakob R, Beutner G, Sharma VK, Duan Y, Gross RA, Hurst S, Jhun BS, O-Uchi J, Sheu SS (2014) Molecular and functional identification of a mitochondrial ryanodine receptor in neurons. Neurosci Lett 575:7–12

Jeyakumar LH, Gleaves LA, Ridley BD, Chang P, Atkinson J, Barnett JV, Fleischer S (2002) The skeletal muscle ryanodine receptor isoform 1 is found at the intercalated discs in human and mouse hearts. J Muscle Res Cell Motil 23:285–292

Jiang D, Zhao L, Clapham DE (2009) Genome-wide RNAi screen identifies Letm1 as a mitochondrial Ca2+/H+ antiporter. Science 326:144–147

Joiner ML, Koval OM, Li J, He BJ, Allamargot C, Gao Z, Luczak ED, Hall DD, Fink BD, Chen B, Yang J, Moore SA, Scholz TD, Strack S, Mohler PJ, Sivitz WI, Song LS, Anderson ME (2012) Camkii determines mitochondrial stress responses in heart. Nature 491:269–273

Joiner ML, Koval OM, Li J, He BJ, Allamargot C, Gao Z, Luczak ED, Hall DD, Fink BD, Chen B, Yang J, Moore SA, Scholz TD, Strack S, Mohler PJ, Sivitz WI, Song LS, Anderson ME, Joiner et al (2014) Reply. Nature 513:E3

Jouaville LS, Pinton P, Bastianutto C, Rutter GA, Rizzuto R (1999) Regulation of mitochondrial atp synthesis by calcium: evidence for a long-term metabolic priming. Proc Natl Acad Sci U S A 96:13807–13812

Kamer KJ, Mootha VK (2014) MICU1 and MICU2 play nonredundant roles in the regulation of the mitochondrial calcium uniporter. EMBO Rep 15:299–307

Kirichok Y, Krapivinsky G, Clapham DE (2004) The mitochondrial calcium uniporter is a highly selective ion channel. Nature 427:360–364

Koncz P, Szanda G, Fulop L, Rajki A, Spat A (2009) Mitochondrial Ca2+ uptake is inhibited by a concerted action of p38 mapk and protein kinase D. Cell Calcium 46:122–129

Kovacs-Bogdan E, Sancak Y, Kamer KJ, Plovanich M, Jambhekar A, Huber RJ, Myre MA, Blower MD, Mootha VK (2014) Reconstitution of the mitochondrial calcium uniporter in yeast. Proc Natl Acad Sci U S A 111:8985–8990

Kwong JQ, Lu X, Correll RN, Schwanekamp JA, Vagnozzi RJ, Sargent MA, York AJ, Zhang J, Bers DM, Molkentin JD (2015) The mitochondrial calcium uniporter selectively matches metabolic output to acute contractile stress in the heart. Cell Rep 12:15–22

Lam SS, Martell JD, Kamer KJ, Deerinck TJ, Ellisman MH, Mootha VK, Ting AY (2015) Directed evolution of apex2 for electron microscopy and proximity labeling. Nat Methods 12:51–54

Lanner JT, Georgiou DK, Joshi AD, Hamilton SL (2010) Ryanodine receptors: structure, expression, molecular details, and function in calcium release. Cold Spring Harb Perspect Biol 2: a003996

Lee Y, Min CK, Kim TG, Song HK, Lim Y, Kim D, Shin K, Kang M, Kang JY, Youn HS, Lee JG, An JY, Park KR, Lim JJ, Kim JH, Kim JH, Park ZY, Kim YS, Wang J, do Kim H, Eom SH (2015) Structure and function of the n-terminal domain of the human mitochondrial calcium uniporter. EMBO Rep 16:1318–1333

Lee SK, Shanmughapriya S, Mok MC, Dong Z, Tomar D, Carvalho E, Rajan S, Junop MS, Madesh M, Stathopulos PB (2016) Structural insights into mitochondrial calcium uniporter regulation by divalent cations. Cell Chem Biol 23:1157–1169

Lewis-Smith D, Kamer KJ, Griffin H, Childs AM, Pysden K, Titov D, Duff J, Pyle A, Taylor RW, Yu-Wai-Man P, Ramesh V, Horvath R, Mootha VK, Chinnery PF (2016) Homozygous deletion in MICU1 presenting with fatigue and lethargy in childhood. Neurol Genet 2:e59

Litsky ML, Pfeiffer DR (1997) Regulation of the mitochondrial Ca2+ uniporter by external adenine nucleotides: the uniporter behaves like a gated channel which is regulated by nucleotides and divalent cations. Biochemistry 36:7071–7080

Lobaton CD, Vay L, Hernandez-Sanmiguel E, Santodomingo J, Moreno A, Montero M, Alvarez J (2005) Modulation of mitochondrial Ca(2+) uptake by estrogen receptor agonists and antagonists. Br J Pharmacol 145:862–871

Logan CV, Szabadkai G, Sharpe JA, Parry DA, Torelli S, Childs AM, Kriek M, Phadke R, Johnson CA, Roberts NY, Bonthron DT, Pysden KA, Whyte T, Munteanu I, Foley AR, Wheway G, Szymanska K, Natarajan S, Abdelhamed ZA, Morgan JE, Roper H, Santen GW, Niks EH, van der Pol WL, Lindhout D, Raffaello A, De Stefani D, den Dunnen JT, Sun Y, Ginjaar I, Sewry CA, Hurles M, Rizzuto R, Consortium UK, Duchen MR, Muntoni F, Sheridan E (2014) Loss-of-function mutations in MICU1 cause a brain and muscle disorder linked to primary alterations in mitochondrial calcium signaling. Nat Genet 46:188–193

Luongo TS, Lambert JP, Yuan A, Zhang X, Gross P, Song J, Shanmughapriya S, Gao E, Jain M, Houser SR, Koch WJ, Cheung JY, Madesh M, Elrod JW (2015) The mitochondrial calcium

uniporter matches energetic supply with cardiac workload during stress and modulates permeability transition. Cell Rep 12:23–34
MacQuaide N, Ramay HR, Sobie EA, Smith GL (2010) Differential sensitivity of Ca(2)+ wave and Ca(2)+ spark events to ruthenium red in isolated permeabilised rabbit cardiomyocytes. J Physiol 588:4731–4742
Malli R, Frieden M, Osibow K, Zoratti C, Mayer M, Demaurex N, Graier WF (2003) Sustained Ca2+ transfer across mitochondria is essential for mitochondrial Ca2+ buffering, sore-operated Ca2+ entry, and Ca2+ store refilling. J Biol Chem 278:44769–44779
Malli R, Frieden M, Trenker M, Graier WF (2005) The role of mitochondria for Ca2+ refilling of the endoplasmic reticulum. J Biol Chem 280:12114–12122
Mallilankaraman K, Doonan P, Cardenas C, Chandramoorthy HC, Muller M, Miller R, Hoffman NE, Gandhirajan RK, Molgo J, Birnbaum MJ, Rothberg BS, Mak DO, Foskett JK, Madesh M (2012a) MICU1 is an essential gatekeeper for MCU-mediated mitochondrial Ca(2+) uptake that regulates cell survival. Cell 151:630–644
Mallilankaraman K, Cardenas C, Doonan PJ, Chandramoorthy HC, Irrinki KM, Golenar T, Csordas G, Madireddi P, Yang J, Muller M, Miller R, Kolesar JE, Molgo J, Kaufman B, Hajnoczky G, Foskett JK, Madesh M (2012b) MCUR1 is an essential component of mitochondrial Ca2+ uptake that regulates cellular metabolism. Nat Cell Biol 14:1336–1343
Marchant JS, Ramos V, Parker I (2002) Structural and functional relationships between Ca2+ puffs and mitochondria in xenopus oocytes. Am J Physiol Cell Physiol 282:C1374–C1386
Marchi S, Lupini L, Patergnani S, Rimessi A, Missiroli S, Bonora M, Bononi A, Corra F, Giorgi C, De Marchi E, Poletti F, Gafa R, Lanza G, Negrini M, Rizzuto R, Pinton P (2013) Downregulation of the mitochondrial calcium uniporter by cancer-related miR-25. Curr Biol 23:58–63
Marks AR, Tempst P, Hwang KS, Taubman MB, Inui M, Chadwick C, Fleischer S, Nadal-Ginard B (1989) Molecular cloning and characterization of the ryanodine receptor/junctional channel complex cDNA from skeletal muscle sarcoplasmic reticulum. Proc Natl Acad Sci U S A 86:8683–8687
Martell JD, Deerinck TJ, Sancak Y, Poulos TL, Mootha VK, Sosinsky GE, Ellisman MH, Ting AY (2012) Engineered ascorbate peroxidase as a genetically encoded reporter for electron microscopy. Nat Biotechnol 30:1143–1148
Matesanz-Isabel J, Arias-del-Val J, Alvarez-Illera P, Fonteriz RI, Montero M, Alvarez J (2016) Functional roles of MICU1 and MICU2 in mitochondrial Ca(2+) uptake. Biochim Biophys Acta 1858:1110–1117
Matlib MA, Zhou Z, Knight S, Ahmed S, Choi KM, Krause-Bauer J, Phillips R, Altschuld R, Katsube Y, Sperelakis N, Bers DM (1998) Oxygen-bridged dinuclear ruthenium amine complex specifically inhibits Ca2+ uptake into mitochondria in vitro and in situ in single cardiac myocytes. J Biol Chem 273:10223–10231
McCormack JG, Halestrap AP, Denton RM (1990) Role of calcium ions in regulation of mammalian intramitochondrial metabolism. Physiol Rev 70:391–425
Mela L (1969) Inhibition and activation of calcium transport in mitochondria. Effect of lanthanides and local anesthetic drugs. Biochemistry 8:2481–2486
Mendes CC, Gomes DA, Thompson M, Souto NC, Goes TS, Goes AM, Rodrigues MA, Gomez MV, Nathanson MH, Leite MF (2005) The type III inositol 1,4,5-trisphosphate receptor preferentially transmits apoptotic Ca2+ signals into mitochondria. J Biol Chem 280:40892–40900
Michels G, Khan IF, Endres-Becker J, Rottlaender D, Herzig S, Ruhparwar A, Wahlers T, Hoppe UC (2009) Regulation of the human cardiac mitochondrial Ca2+ uptake by 2 different voltage-gated Ca2+ channels. Circulation 119:2435–2443
Montero M, Lobaton CD, Moreno A, Alvarez J (2002) A novel regulatory mechanism of the mitochondrial Ca2+ uniporter revealed by the p38 mitogen-activated protein kinase inhibitor sb202190. FASEB J 16:1955–1957

Montero M, Lobaton CD, Hernandez-Sanmiguel E, Santodomingo J, Vay L, Moreno A, Alvarez J (2004) Direct activation of the mitochondrial calcium uniporter by natural plant flavonoids. Biochem J 384:19–24

Moreau B, Nelson C, Parekh AB (2006) Biphasic regulation of mitochondrial Ca2+ uptake by cytosolic Ca2+ concentration. Curr Biol 16:1672–1677

Munch G, Bolck B, Sugaru A, Schwinger RH (2000) Isoform expression of the sarcoplasmic reticulum Ca2+ release channel (ryanodine channel) in human myocardium. J Mol Med 78:352–360

Murgia M, Rizzuto R (2015) Molecular diversity and pleiotropic role of the mitochondrial calcium uniporter. Cell Calcium 58:11–17

Murphy E, Pan X, Nguyen T, Liu J, Holmstrom KM, Finkel T (2014) Unresolved questions from the analysis of mice lacking MCU expression. Biochem Biophys Res Commun 449:384–385

Nakai J, Imagawa T, Hakamat Y, Shigekawa M, Takeshima H, Numa S (1990) Primary structure and functional expression from cdna of the cardiac ryanodine receptor/calcium release channel. FEBS Lett 271:169–177

Nicchitta CV, Williamson JR (1984) Spermine. A regulator of mitochondrial calcium cycling. J Biol Chem 259:12978–12983

Nicholls DG (1978) The regulation of extramitochondrial free calcium ion concentration by rat liver mitochondria. Biochem J 176:463–474

Noack E, Greeff K (1971) Inhibition of calcium transport in mitochondria by -receptor blocking substances and its reactivation by phospholipids. Experientia 27:810–811

Olson ML, Chalmers S, McCarron JG (2010) Mitochondrial Ca2+ uptake increases Ca2+ release from inositol 1,4,5-trisphosphate receptor clusters in smooth muscle cells. J Biol Chem 285:2040–2050

O-Uchi J, Pan S, Sheu SS (2012) Perspectives on: SGP Symposium on Mitochondrial Physiology and Medicine. Molecular identities of mitochondrial Ca^{2+} influx mechanism: updated passwords for accessing mitochondrial Ca^{2+}-linked health and disease. J Gen Physiol 139:435–443

O-Uchi J, Jhun BS, Hurst S, Bisetto S, Gross P, Chen M, Kettlewell S, Park J, Oyamada H, Smith GL, Murayama T, Sheu SS (2013) Overexpression of ryanodine receptor type 1 enhances mitochondrial fragmentation and Ca^{2+}-induced ATP production in cardiac H9c2 myoblasts. Am J Physiol Heart Circ Physiol 305:H1736–H1751

O-Uchi J, Jhun BS, Xu S, Hurst S, Raffaello A, Liu X, Yi B, Zhang H, Gross P, Mishra J, Ainbinder A, Kettlewell S, Smith GL, Dirksen RT, Wang W, Rizzuto R, Sheu SS (2014) Adrenergic signaling regulates mitochondrial Ca2+ uptake through Pyk2-dependent tyrosine phosphorylation of the mitochondrial Ca2+ uniporter. Antioxid Redox Signal 21:863–879

Oxenoid K, Dong Y, Cao C, Cui T, Sancak Y, Markhard AL, Grabarek Z, Kong L, Liu Z, Ouyang B, Cong Y, Mootha VK, Chou JJ (2016) Architecture of the mitochondrial calcium uniporter. Nature 533:269–273

Pagliarini DJ, Calvo SE, Chang B, Sheth SA, Vafai SB, Ong SE, Walford GA, Sugiana C, Boneh A, Chen WK, Hill DE, Vidal M, Evans JG, Thorburn DR, Carr SA, Mootha VK (2008) A mitochondrial protein compendium elucidates complex i disease biology. Cell 134:112–123

Pan X, Liu J, Nguyen T, Liu C, Sun J, Teng Y, Fergusson MM, Rovira II, Allen M, Springer DA, Aponte AM, Gucek M, Balaban RS, Murphy E, Finkel T (2013) The physiological role of mitochondrial calcium revealed by mice lacking the mitochondrial calcium uniporter. Nat Cell Biol 15:1464–1472

Park MK, Ashby MC, Erdemli G, Petersen OH, Tepikin AV (2001) Perinuclear, perigranular and sub-plasmalemmal mitochondria have distinct functions in the regulation of cellular calcium transport. EMBO J 20:1863–1874

Patron M, Checchetto V, Raffaello A, Teardo E, Vecellio Reane D, Mantoan M, Granatiero V, Szabo I, De Stefani D, Rizzuto R (2014) MICU1 and MICU2 finely tune the mitochondrial Ca2+ uniporter by exerting opposite effects on mcu activity. Mol Cell 53:726–737

Paupe V, Prudent J, Dassa EP, Rendon OZ, Shoubridge EA (2015) CCDC90A (MCUR1) is a cytochrome c oxidase assembly factor and not a regulator of the mitochondrial calcium uniporter. Cell Metab 21:109–116

Perez-Campo R, Lopez-Torres M, Cadenas S, Rojas C, Barja G (1998) The rate of free radical production as a determinant of the rate of aging: evidence from the comparative approach. J Comp Physiol B 168:149–158

Perocchi F, Gohil VM, Girgis HS, Bao XR, McCombs JE, Palmer AE, Mootha VK (2010) Micu1 encodes a mitochondrial ef hand protein required for ca(2+) uptake. Nature 467:291–296

Petrungaro C, Zimmermann KM, Kuttner V, Fischer M, Dengjel J, Bogeski I, Riemer J (2015) The Ca(2+)-dependent release of the Mia40-induced MICU1-MICU2 dimer from MCU regulates mitochondrial Ca(2+) uptake. Cell Metab 22:721–733

Pinton P, Leo S, Wieckowski MR, Di Benedetto G, Rizzuto R (2004) Long-term modulation of mitochondrial Ca^{2+} signals by protein kinase C isozymes. J Cell Biol 165:223–232

Plovanich M, Bogorad RL, Sancak Y, Kamer KJ, Strittmatter L, Li AA, Girgis HS, Kuchimanchi S, De Groot J, Speciner L, Taneja N, Oshea J, Koteliansky V, Mootha VK (2013) MICU2, a paralog of MICU1, resides within the mitochondrial uniporter complex to regulate calcium handling. PLoS One 8:e55785

Pozzan T, Magalhaes P, Rizzuto R (2000) The comeback of mitochondria to calcium signalling. Cell Calcium 28:279–283

Putney JW Jr, Thomas AP (2006) Calcium signaling: double duty for calcium at the mitochondrial uniporter. Curr Biol 16:R812–R815

Qiu J, Tan YW, Hagenston AM, Martel MA, Kneisel N, Skehel PA, Wyllie DJ, Bading H, Hardingham GE (2013) Mitochondrial calcium uniporter mcu controls excitotoxicity and is transcriptionally repressed by neuroprotective nuclear calcium signals. Nat Commun 4:2034

Quan X, Nguyen TT, Choi SK, Xu S, Das R, Cha SK, Kim N, Han J, Wiederkehr A, Wollheim CB, Park KS (2015) Essential role of mitochondrial Ca^{2+} uniporter in the generation of mitochondrial pH gradient and metabolism-secretion coupling in insulin-releasing cells. J Biol Chem 290:4086–4096

Quintana A, Schwarz EC, Schwindling C, Lipp P, Kaestner L, Hoth M (2006) Sustained activity of calcium release-activated calcium channels requires translocation of mitochondria to the plasma membrane. J Biol Chem 281:40302–40309

Raffaello A, De Stefani D, Sabbadin D, Teardo E, Merli G, Picard A, Checchetto V, Moro S, Szabo I, Rizzuto R (2013) The mitochondrial calcium uniporter is a multimer that can include a dominant-negative pore-forming subunit. EMBO J 32:2362–2376

Rapizzi E, Pinton P, Szabadkai G, Wieckowski MR, Vandecasteele G, Baird G, Tuft RA, Fogarty KE, Rizzuto R (2002) Recombinant expression of the voltage-dependent anion channel enhances the transfer of Ca2+ microdomains to mitochondria. J Cell Biol 159:613–624

Rasola A, Bernardi P (2011) Mitochondrial permeability transition in Ca(2+)-dependent apoptosis and necrosis. Cell Calcium 50:222–233

Rharass T, Lemcke H, Lantow M, Kuznetsov SA, Weiss DG, Panakova D (2014) Ca2+-mediated mitochondrial reactive oxygen species metabolism augments Wnt/beta-catenin pathway activation to facilitate cell differentiation. J Biol Chem 289:27937–27951

Rizzuto R, Pozzan T (2006) Microdomains of intracellular Ca2+: molecular determinants and functional consequences. Physiol Rev 86:369–408

Rizzuto R, Brini M, Murgia M, Pozzan T (1993) Microdomains with high Ca2+ close to IP3-sensitive channels that are sensed by neighboring mitochondria. Science 262:744–747

Rizzuto R, Pinton P, Carrington W, Fay FS, Fogarty KE, Lifshitz LM, Tuft RA, Pozzan T (1998) Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca2+ responses. Science 280:1763–1766

Rizzuto R, De Stefani D, Raffaello A, Mammucari C (2012) Mitochondria as sensors and regulators of calcium signalling. Nat Rev Mol Cell Biol 13:566–578

Rowland AA, Voeltz GK (2012) Endoplasmic reticulum-mitochondria contacts: function of the junction. Nat Rev Mol Cell Biol 13:607–625

Rutter GA, Denton RM (1988) Regulation of NAD+-linked isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase by Ca2+ ions within toluene-permeabilized rat heart mitochondria. Interactions with regulation by adenine nucleotides and NADH/NAD+ ratios. Biochem J 252:181–189

Ryu SY, Beutner G, Kinnally KW, Dirksen RT, Sheu SS (2011) Single channel characterization of the mitochondrial ryanodine receptor in heart mitoplasts. J Biol Chem 286:21324–21329

Salvi M, Toninello A (2004) Effects of polyamines on mitochondrial Ca(2+) transport. Biochim Biophys Acta 1661:113–124

Sancak Y, Markhard AL, Kitami T, Kovacs-Bogdan E, Kamer KJ, Udeshi ND, Carr SA, Chaudhuri D, Clapham DE, Li AA, Calvo SE, Goldberger O, Mootha VK (2013) EMRE is an essential component of the mitochondrial calcium uniporter complex. Science 342:1379–1382

Santo-Domingo J, Vay L, Hernandez-Sanmiguel E, Lobaton CD, Moreno A, Montero M, Alvarez J (2007) The plasma membrane Na+/Ca2+ exchange inhibitor KB-R7943 is also a potent inhibitor of the mitochondrial Ca2+ uniporter. Br J Pharmacol 151:647–654

Sastrasinh M, Weinberg JM, Humes HD (1982) The effect of gentamicin on calcium uptake by renal mitochondria. Life Sci 30:2309–2315

Schellenberg GD, Anderson L, Cragoe EJ Jr, Swanson PD (1985) Inhibition of brain mitochondrial Ca2+ transport by amiloride analogues. Cell Calcium 6:431–447

Schwartz J, Holmuhamedov E, Zhang X, Lovelace GL, Smith CD, Lemasters JJ (2013) Minocycline and doxycycline, but not other tetracycline-derived compounds, protect liver cells from chemical hypoxia and ischemia/reperfusion injury by inhibition of the mitochondrial calcium uniporter. Toxicol Appl Pharmacol 273:172–179

Seidlmayer LK, Kuhn J, Berbner A, Arias-Loza PA, Williams T, Kaspar M, Czolbe M, Kwong JQ, Molkentin JD, Heinze KG, Dedkova EN, Ritter O (2016) Inositol 1,4,5-trisphosphate-mediated sarcoplasmic reticulum-mitochondrial crosstalk influences adenosine triphosphate production via mitochondrial Ca2+ uptake through the mitochondrial ryanodine receptor in cardiac myocytes. Cardiovasc Res 112:491–501

Shanmughapriya S, Rajan S, Hoffman NE, Zhang X, Guo S, Kolesar JE, Hines KJ, Ragheb J, Jog NR, Caricchio R, Baba Y, Zhou Y, Kaufman BA, Cheung JY, Kurosaki T, Gill DL, Madesh M (2015) Ca2+ signals regulate mitochondrial metabolism by stimulating CREB-mediated expression of the mitochondrial Ca2+ uniporter gene MCU. Sci Signal 8:ra23

Sharma VK, Ramesh V, Franzini-Armstrong C, Sheu SS (2000) Transport of Ca2+ from sarcoplasmic reticulum to mitochondria in rat ventricular myocytes. J Bioenerg Biomembr 32:97–104

Sohal RS, Allen RG (1985) Relationship between metabolic rate, free radicals, differentiation and aging: a unified theory. Basic Life Sci 35:75–104

Sparagna GC, Gunter KK, Sheu SS, Gunter TE (1995) Mitochondrial calcium uptake from physiological-type pulses of calcium. A description of the rapid uptake mode. J Biol Chem 270:27510–27515

Szabadkai G, Bianchi K, Varnai P, De Stefani D, Wieckowski MR, Cavagna D, Nagy AI, Balla T, Rizzuto R (2006) Chaperone-mediated coupling of endoplasmic reticulum and mitochondrial Ca2+ channels. J Cell Biol 175:901–911

Szalai G, Csordas G, Hantash BM, Thomas AP, Hajnoczky G (2000) Calcium signal transmission between ryanodine receptors and mitochondria. J Biol Chem 275:15305–15313

Szanda G, Koncz P, Rajki A, Spat A (2008) Participation of p38 mapk and a novel-type protein kinase C in the control of mitochondrial Ca2+ uptake. Cell Calcium 43:250–259

Szanda G, Rajki A, Gallego-Sandin S, Garcia-Sancho J, Spat A (2009) Effect of cytosolic Mg2+ on mitochondrial Ca2+ signaling. Pflugers Arch Eur J Physiol 457:941–954

Tan W, Colombini M (2007) VDAC closure increases calcium ion flux. Biochim Biophys Acta 1768:2510–2515

Tarasov AI, Semplici F, Ravier MA, Bellomo EA, Pullen TJ, Gilon P, Sekler I, Rizzuto R, Rutter GA (2012) The mitochondrial Ca2+ uniporter mcu is essential for glucose-induced atp increases in pancreatic beta-cells. PLoS One 7:e39722

Thayer SA, Miller RJ (1990) Regulation of the intracellular free calcium concentration in single rat dorsal root ganglion neurones in vitro. J Physiol 425:85–115

Tomar D, Dong Z, Shanmughapriya S, Koch DA, Thomas T, Hoffman NE, Timbalia SA, Goldman SJ, Breves SL, Corbally DP, Nemani N, Fairweather JP, Cutri AR, Zhang X, Song J, Jana F, Huang J, Barrero C, Rabinowitz JE, Luongo TS, Schumacher SM, Rockman ME, Dietrich A, Merali S, Caplan J, Stathopulos P, Ahima RS, Cheung JY, Houser SR, Koch WJ, Patel V, Gohil VM, Elrod JW, Rajan S, Madesh M (2016) MCUR1 is a scaffold factor for the MCU complex function and promotes mitochondrial bioenergetics. Cell Rep 15:1673–1685

Tosatto A, Sommaggio R, Kummerow C, Bentham RB, Blacker TS, Berecz T, Duchen MR, Rosato A, Bogeski I, Szabadkai G, Rizzuto R, Mammucari C (2016) The mitochondrial calcium uniporter regulates breast cancer progression via HIF-1alpha. EMBO Mol Med 8:569–585

Tsai MF, Jiang D, Zhao L, Clapham D, Miller C (2014) Functional reconstitution of the mitochondrial Ca2+/H+ antiporter Letm1. J Gen Physiol 143:67–73

Tsai MF, Phillips CB, Ranaghan M, Tsai CW, Wu Y, Willliams C, Miller C (2016) Dual functions of a small regulatory subunit in the mitochondrial calcium uniporter complex. Elife 5

Vais H, Mallilankaraman K, Mak DO, Hoff H, Payne R, Tanis JE, Foskett JK (2016) EMRE is a matrix Ca(2+) sensor that governs gatekeeping of the mitochondrial Ca(2+) uniporter. Cell Rep 14:403–410

Varadi A, Cirulli V, Rutter GA (2004) Mitochondrial localization as a determinant of capacitative Ca2+ entry in hela cells. Cell Calcium 36:499–508

Vasington FD, Murphy JV (1962) Ca ion uptake by rat kidney mitochondria and its dependence on respiration and phosphorylation. J Biol Chem 237:2670–2677

Vecellio Reane D, Vallese F, Checchetto V, Acquasaliente L, Butera G, De Filippis V, Szabo I, Zanotti G, Rizzuto R, Raffaello A (2016) A MICU1 splice variant confers high sensitivity to the mitochondrial Ca2+ uptake machinery of skeletal muscle. Mol Cell 64:760–773

Wang L, Yang X, Li S, Wang Z, Liu Y, Feng J, Zhu Y, Shen Y (2014) Structural and mechanistic insights into MICU1 regulation of mitochondrial calcium uptake. EMBO J 33:594–604

Wu Y, Rasmussen TP, Koval OM, Joiner ML, Hall DD, Chen B, Luczak ED, Wang Q, Rokita AG, Wehrens XH, Song LS, Anderson ME (2015) The mitochondrial uniporter controls fight or flight heart rate increases. Nat Commun 6:6081

Xu S, Chisholm AD (2014) C. elegans epidermal wounding induces a mitochondrial ros burst that promotes wound repair. Dev Cell 31:48–60

Yamamoto T, Yamagoshi R, Harada K, Kawano M, Minami N, Ido Y, Kuwahara K, Fujita A, Ozono M, Watanabe A, Yamada A, Terada H, Shinohara Y (2016) Analysis of the structure and function of emre in a yeast expression system. Biochim Biophys Acta 1857:831–839

Ying WL, Emerson J, Clarke MJ, Sanadi DR (1991) Inhibition of mitochondrial calcium ion transport by an oxo-bridged dinuclear ruthenium ammine complex. Biochemistry 30:4949–4952

Zimanyi I, Pessah IN (1991) Comparison of [3H]ryanodine receptors and Ca++ release from rat cardiac and rabbit skeletal muscle sarcoplasmic reticulum. J Pharmacol Exp Ther 256:938–946

Mitochondrial Fission in Human Diseases

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Contents

Abstract

Mitochondria are an essential component of multicellular life – from primitive organisms, to highly complex entities like mammals. The importance of mitochondria is underlined by their plethora of well-characterized essential functions such as energy production through oxidative phosphorylation (OX-PHOS), calcium and reactive oxygen species (ROS) signaling, and regulation of apoptosis. In addition, novel roles and attributes of mitochondria are coming into focus through the recent years of mitochondrial research. In particular, over the past decade the study of mitochondrial shape and dynamics has achieved special significance, as they are found to impact mitochondrial function. Recent advances indicate that mitochondrial function and dynamics are inter-connected, and maintain the balance between health and disease at a cellular and an organismal level. For example, excessive mitochondrial division (fission) is associated with functional defects, and is implicated in multiple human diseases from neurodegenerative diseases to cancer. In this chapter we examine the recent literature on the mitochondrial dynamics–function

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H. Singh, S.-S. Sheu (eds.), *Pharmacology of Mitochondria*,
Handbook of Experimental Pharmacology 240, DOI 10.1007/164_2016_38

relationship, and explore how it impacts on the development and progression of human diseases. We will also highlight the implications of therapeutic manipulation of mitochondrial dynamics in treating various human pathologies.

Keywords
Cancer • DRP1 • DRP1S616 • Fis1 • Fission • Fusion • Mff • Mfn1 • Mfn2 • MiD49/51 • MiD49/51OPA1 • Mitochondrial dynamics • Neurodegenerative diseases

1 Introduction

Since their first inclusion into cells through endosymbiosis millions of years ago, mitochondria are an essential component of multicellular life – from primitive organisms to highly complex entities like mammals. The importance of mitochondria is underlined by their plethora of well-characterized essential functions such as energy production through oxidative phosphorylation (OX-PHOS), calcium and reactive oxygen species (ROS) signaling, and apoptosis. In addition, novel roles and attributes of mitochondria are coming into focus through the recent years of mitochondrial research. In particular, over the past decade the study of mitochondrial shape and dynamics has achieved special significance, as they are found to impact mitochondrial function. Recent advances indicate that mitochondrial function and dynamics are inter-connected, and maintain the balance between health and disease at a cellular and an organismal level. For example, excessive mitochondrial division (fission) is associated with functional defects, and is implicated in multiple human diseases from neurodegenerative diseases to cancer. The purpose of this chapter is to examine the recent literature on the mitochondrial dynamics–function relationship, and explore how it impacts on the development and progression of human diseases. We will also highlight how therapeutic manipulation of mitochondrial dynamics may be utilized to modulate mitochondrial health with potential in treating various human pathologies.

1.1 Mitochondrial Biology: Functions

Mitochondria are highly versatile organelles with a multitude of vital functions within the cell. Primarily, mitochondria act as the main source of cellular energy production, by hosting the TCA cycle and the OX-PHOS pathway machinery to utilize fuels (e.g., pyruvate and NADH produced via glycolysis, acetyl co-a produced by lipid β-oxidation, and glutamine) to generate energy in the form of ATP (Ward and Thompson 2012b). The OX-PHOS pathway is also known as aerobic respiration as it requires the presence of oxygen. The mitochondrial respiratory function is particularly essential in organs and tissues with high energy demand, such as the heart and tissues of the central nervous system (Ikeda et al. 2014).

Mitochondria also play an important role in the biosynthesis of cellular building blocks such as nucleotides, amino acids, and lipids required for cellular growth and proliferation (Ward and Thompson 2012b). Another important function of mitochondria is calcium buffering and modulating calcium signaling through controlled uptake and release of calcium from cellular stores (Walsh et al. 2009). Ca^{2+} buffering is achieved in part via a close contact between mitochondria and the endoplasmic reticulum (ER) network, creating calcium micro-domains that facilitate Ca^{2+} uptake by mitochondria to maintain cellular calcium homeostasis (de Brito and Scorrano 2008; Walsh et al. 2009). Mitochondrial ATP, NADH, pyruvate, and ROS also modulate Ca^{2+} signaling machinery, while the uptake and release of Ca^{2+} by mitochondria directly influence cytosolic calcium concentrations and Ca^{2+} signaling (Walsh et al. 2009). As the primary site for ROS generation in the cell, mitochondria are also responsible for cellular redox signaling, as well as maintaining oxidative homeostasis through a series of antioxidants (Collins et al. 2012; Hamanaka and Chandel 2010). Signaling pathways that utilize ROS include cellular response to hypoxia, growth factor stimulation for cell proliferation and survival, and generation of inflammatory responses (Hamanaka and Chandel 2010; Schieber and Chandel 2014). Last, but not least, mitochondria play a central role in the apoptotic pathway of cell death. Multiple upstream signaling pathways converge on mitochondria in a complex network of pro- and anti-apoptotic proteins to signal for mitochondrial outer membrane permeabilization (MOMP) and the release of cytochrome c (Chipuk et al. 2010). Mitochondria play a pivotal role in apoptosis, by compartmentalizing the cell death inducing cytochrome c under normal healthy conditions, and orchestrating its release upon stress conditions to undergo apoptosis (Elkholi et al. 2014).

1.2 Mitochondrial Biology: Dynamics

Over the past few decades, an important characteristic feature of mitochondria emerged in the field of mitochondrial research. In place of the original "text book" idea of a static bean-shaped mitochondrion, it is now appreciated that mitochondria exist as a network of highly dynamic organelles and may be present in multiple shapes and sizes within the cell, ranging from small puncta to short and long tubules (Fig. 2). These different shapes are the result of the ability of mitochondria to divide, fuse together, and move through the cell. These processes are collectively termed mitochondrial dynamics, and involve two specific, highly regulated opposing processes known as fission (division) and fusion (Fig. 1). In healthy cells in most tissues, mitochondria continuously undergo these fission and fusion events, changing their shape and size and moving within short distances to do so.

Mitochondrial dynamics are considered important for maintaining healthy functional mitochondria in the cell. Through fusion and fission events, mitochondria are able to mix and exchange their DNA and protein content, or allow for the segregation and elimination of unhealthy components through an autophagic process termed mitophagy. Mitochondrial fission is also required for distribution of

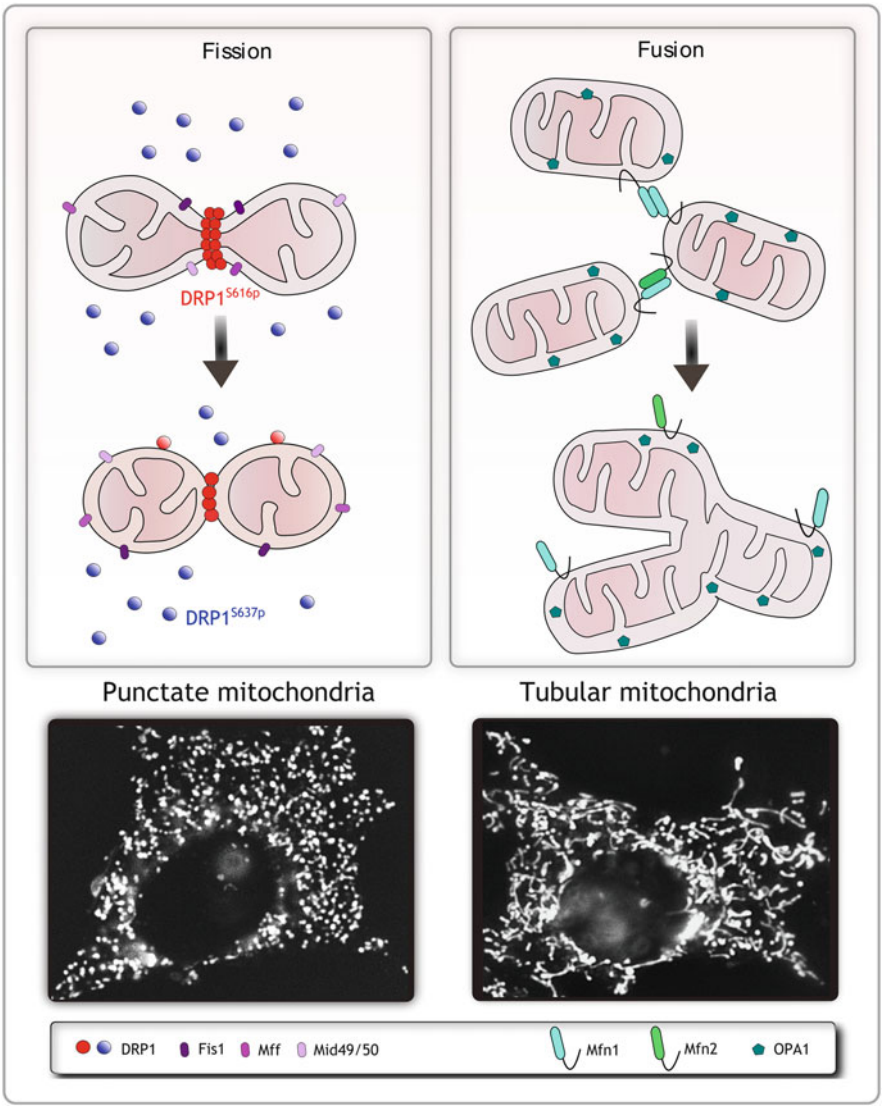

Fig. 1 Dynamic mitochondria. Mitochondrial morphology is maintained through a balance of fission and fusion events and involve the activity of several large GTPases and adapter proteins. Mitochondrial fission is mediated by DRP1 and the OMM anchored adapter proteins Fis1, Mff and MID49/51. Fusion is regulated by the mitofusins Mfn1 and 2 on the outer membrane, and OPA1 in the inner membrane. If increased fission is favored, mitochondria form short punctate structures (fragmented mitochondria). If fusion is favored mitochondria form tubular networks (fused mitochondria)

mitochondria to daughter cells during cell division, as well as distribution of the organelle to distal, energy demanding regions of the cell such as neuronal axons, and lamellipodia during cell motility. More recent developments in mitochondrial research indicate that mitochondrial dynamics are also intricately involved with the functions of mitochondria, and there is emerging evidence that connects mitochondrial dynamics directly to the manifestation of certain pathologies, such as neurodegenerative diseases, cancer, cardiomyopathies, and metabolic disorders. In this section of the chapter we will introduce the major players in mitochondrial dynamics and how cellular signaling controls these processes. We will also discuss how aberrant fission and fusion of mitochondria arise, and how these aberrations result in mitochondrial dysfunction and human diseases.

1.2.1 Mitochondrial Fission: Executors and Regulators

Mitochondrial fission is the process by which a mitochondrion divides into two smaller units. The major executor of fission is the dynamin related protein 1 (DRP1). DRP1 is mainly cytosolic, but translocates to the mitochondrial surface in order to mediate fission of the organelle. DRP1 assembles on the surface of mitochondria, forms helical oligomers, and undergoes self-interaction mediated GTP hydrolysis and subsequent membrane constriction resulting in the scission of both the inner and outer mitochondrial membranes (OMM). In a series of elegant in vitro experiments, both DRP1 and its yeast homolog Dnm1 were shown to self-assemble into helices and tubulate liposomes (Ingerman et al. 2005). These DRP1 helices have a diameter of ~120 nm (Ingerman et al. 2005; Mears et al. 2011), and since the diameter of an average mitochondrion is approximately fivefold to sevenfold larger, it is speculated that already existing membrane constrictions may provide the sites for the self-assembly of DRP1 oligomers. Accordingly, it has been shown that ER–mitochondria contact sites constrict mitochondria to a diameter that is permissive for DRP1 oligomer formation, and therefore may mark future fission sites (Friedman et al. 2011).

As DRP1 is mostly cytosolic, there may be a requirement for a mitochondrial membrane anchored receptor to recruit DRP1 to the mitochondria for fission. Several such receptor proteins have been described, including mitochondrial fission 1 (Fis1), mitochondrial fission factor (Mff), Mid 49/51 (MIEF1/2), and ganglioside-induced differentiation-associated protein 1 (GDAP) (Gandre-Babbe and van der Bliek 2008; Niemann et al. 2005; Palmer et al. 2011; Serasinghe and Yoon 2008; Yoon et al. 2003). Fis1 is the first receptor identified to recruit DRP1, and is thought to interact with DRP1 via its two tetra-tricopeptide repeat (TPR)-like motifs. Fis1 forms oligomers on the OMM which may provide the scaffold for DRP1 recruitment (Serasinghe and Yoon 2008; Yoon et al. 2003). More recently Mff was identified as an adaptor for DRP1 (Gandre-Babbe and van der Bliek 2008). Mff is also an OMM anchored protein which recruits DRP1 via its cytosolic domain. Mid49 and Mid50 were also recently identified as mitochondrial outer membrane anchored adapters of DRP1; however, their exact role in mitochondrial fission remains to be clarified (Liu et al. 2013; Palmer et al. 2011; Zhao et al. 2011).

GDAP1 is an OMM anchored protein that regulates mitochondrial fission in neuronal cells by cooperating with DRP1 and Fis1 (Niemann et al. 2005, 2009). The presence of multiple adaptor proteins for DRP1 suggests that there may be several pathways regulating mitochondrial fission. It also indicates that these proteins may be functionally redundant and have cell type specific or context dependent activity (Loson et al. 2013; Niemann et al. 2005, 2009), further suggesting additional points of control in the fission process.

Transcriptional Regulation of DRP1

Both transcriptional and post-translational modifications of DRP1 regulate mitochondrial fission. Several reports indicate that DRP1 is transcriptionally upregulated in cancer (Choudhary et al. 2011; Serasinghe et al. 2015). While the precise transcriptional regulators of Drp1 are currently not known, several putative transcriptional activators have been reported in the recent literature. For example, the androgen receptor was shown to directly activate Drp1 transcription by binding to the promoter region of the DRP1 gene in prostate cancer, suggesting hormone responsive regulation of Drp1 (Choudhary et al. 2011). Drp1 has also been proposed as a p53 transcriptional target indicating a potential intersection between cellular stress pathways and mitochondrial fission (Li et al. 2010). More recently it was shown that oncogenic RAS-MAPK signaling upregulates Drp1 mRNA levels via ERK, and that blocking RAS-MAPK signaling with targeted inhibitors drastically reduces Drp1 mRNA levels (Serasinghe et al. 2015). It is likely that a transcription factor downstream of ERK is responsible for this upregulation. It is intriguing that certain disease conditions, cancer in particular, are associated with transcriptional upregulation of Drp1, which suggests an important role/function of the protein in disease pathology. Identification of Drp1 specific transcription factors and upstream signaling networks will yield important insights into the function of Drp1 and mitochondrial fission in these diseases.

Post-Translational Regulation of DRP1

Mechanisms of post-translational regulation of DRP1 include phosphorylation, SUMOylation, nitrosylation, and ubiquitinylation. One of the best characterized post-translational modifications of DRP1 leading to the modulation of its function is phosphorylation. Two main phosphorylation sites in DRP1 have been identified, both on serine residues residing on the GTPase effector domain (GED) of DRP1 (Fig. 2). DRP1 serine 616 phosphorylation is an activating event resulting in the OMM localization of DRP1 and subsequent mitochondrial fragmentation. It has been reported that DRP1S616 phosphorylation by calcium/calmodulin-dependent protein kinase-1 (CAMK-1) facilitates DRP1 interaction with Fis1 on the OMM (Han et al. 2008). DRP1S616 is phosphorylated by protein kinase C (PKC) under oxidative stress conditions (Qi et al. 2011), by CDK1/cyclin B during cell cycle progression (Taguchi et al. 2007), and by ERK 1/2 under pathological conditions leading to the activation of fission (Kashatus et al. 2015; Serasinghe et al. 2015; Yu et al. 2011). The specific phosphatases responsible for dephosphorylation on this

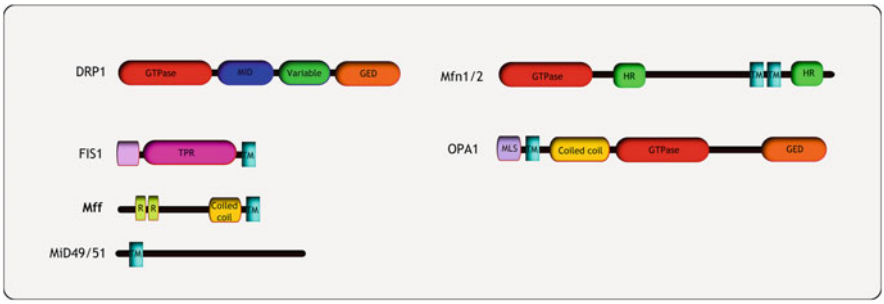

Fig. 2 Regulators of mitochondrial dynamics. Several large GTPases govern mitochondrial dynamics. DRP1 is a cytosolic protein that is recruited to the OMM by membrane anchored adaptor proteins such as Fis1, Mff and Mid49/50. Post translational modifications of DRP1 include phosphorylation, SUMOylation, ubiquitinylation and s-nitrosylation. The outer membrane fusion proteins Mfn1 and Mfn2 are anchored on the OMM via two transmembrane domains. Mfn1 is regulated by phosphorylation as well as ubiquitinylation. The inner membrane fusion GTPase OPA1 is found in the IM space attached to the IMM, and is regulated through proteolytic cleavage to produce fusion competent shorter isoforms. *Abbreviations*: *GED* GTPase effector domain, *HR* heptad repeat, *TPR* tetra-tricopeptide repeat, *R* conserved repeats, *MLS* mitochondrial localization signal, *TM* trans-membrane

site are currently not known. However, it has been reported that dephosphorylation at S616 leads to mitochondrial fusion and protects from autophagy in nutrient starvation conditions (Rambold et al. 2011). Indeed it is interesting to speculate that there may exist a series of thus far unidentified phosphatases having equal significance to kinases, regulating DRP1 with potential implications on mitochondrial shape and function.

While the putative DRP1S616 phosphatases are not known, it has been demonstrated that DRP1 S637 phosphorylation acts as a countermeasure to inactivate DRP1 mediated fission, thereby establishing a balance between active and inactive forms of DRP1. DRP1 S637 when phosphorylated by the protein kinase A (PKA) acts to prevent mitochondrial fission by translocating DRP1 away from mitochondria (Chang and Blackstone 2007). This site is dephosphorylated by calcineurin. On the other hand, DRP1 phosphorylation at the same site by CAMK-1 and rho-associated coiled-coil containing protein kinase 1 (ROCK 1) has been shown to induce mitochondrial fission by activating DRP1 function in cultured hippocampal neurons and induction of fission, leading to diabetic neuropathies, respectively (Han et al. 2008; Wang et al. 2012). These contrasting observations may be reconciled with the argument that the regulatory phosphorylation events of DRP1 may be highly tissue specific (e.g., nervous system), and may depend on the availability and function of DRP1 receptors in these cells (e.g., hippocampal neurons), and dependent on the mitochondrial requirement of the tissue. It also may depend on the cellular context such as specific stress conditions and associated signaling pathways (e.g., calcium signaling). We will discuss these tissue and disease specific regulation of mitochondrial dynamics further in the context of human pathologies in a subsequent section of this chapter. It is important

however to stress that further investigations into these unknowns would enable a better dissection of the function of DRP1 in mitochondrial fission.

A third phosphorylation site with functional consequence has been recently identified at DRP1 serine 693 residue, which resides in the GED (Fig. 2). This site is phosphorylated by glycogen synthase kinase 3 beta (GSK3β), and inhibits DRP1 oligomerization dependent GTP hydrolysis to block mitochondrial fission (Chou et al. 2012). This phosphorylation event, in particular, has been observed in association with apoptosis (Chou et al. 2012).

Another type of post-translational modification that impacts DRP1 function is ubiquitinylation. Membrane associated ring finger 5 (MARCH 5, also known as MITOL) is a mitochondrial outer membrane localized E3 ligase that ubiquitinates DRP1 on the OMM. MARCH 5 mediated ubiquitinylation of DRP1 was originally reported to mark DRP1 for proteolytic degradation leading to reduced fission and elongated mitochondrial networks (Nakamura et al. 2006; Yonashiro et al. 2006). In more recent reports however, MARCH 5 mediated DRP1 ubiquitinylation was shown to increase DRP1 mitochondrial localization and fission (Karbowski et al. 2007; Park et al. 2010) suggesting that this protein modification may be required for mitochondrial fission. These contradictory reports warrant further study to clarify the role of MARCH 5 mediated ubiquitinylation of DRP1. Parkin is another E3 ubiquitin ligase that was shown to ubiquitinylate DRP1 and Fis1 leading to the proteasomal degradation of these proteins (Cui et al. 2010; Wang et al. 2011). These reports indicate that ubiquitinylation by Parkin acts as an inhibitory event for DRP1 mediated mitochondrial fission. The relevance of this DRP1 regulatory pathway in Parkinson's disease will be discussed later in this chapter.

The SUMO-conjugating enzyme Ubc9 and the mitochondrial SUMO E3 ligase MAPL SUMOylate DRP1, resulting in increased DRP1 protein stability on the OMM, leading to increased fission (Braschi et al. 2009; Harder et al. 2004; Prudent et al. 2015; Wasiak et al. 2007) (Fig. 3). Furthermore, it was shown that during apoptosis, the BCL2 associated X (BAX) protein and Bcl2 homologous antagonist killer (BAK) protein oligomerization stabilizes DRP1 on the OMM in a SUMOylation dependent manner, suggesting that this may be a mechanism of apoptotic fragmentation of mitochondria (Wasiak et al. 2007). Conversely, Sentrin/SUMO specific protease 5 (SENP5) was identified as a SUMO-protease that removes the SUMO groups from DRP1 resulting in decreased mitochondrial fragmentation, and knock down of SENP5 was found to fragment mitochondria by stabilizing DRP1 SUMOylation (Zunino et al. 2007). Similar activity was reported recently from SENP3, another member of the SEN protease family, in an in vitro model of ischemia (Guo et al. 2013). Under ischemic conditions, SENP3 is downregulated leading to extended SUMOylation and resulting fragmentation of mitochondria, which is reversed upon re-oxygenation (Guo et al. 2013). However, the depletion of SENP3 in this case, which led to DRP1-sumo stabilization, was shown to suppress cytochrome c release and caspase mediated cell death, thereby acting in a cell protective manner.

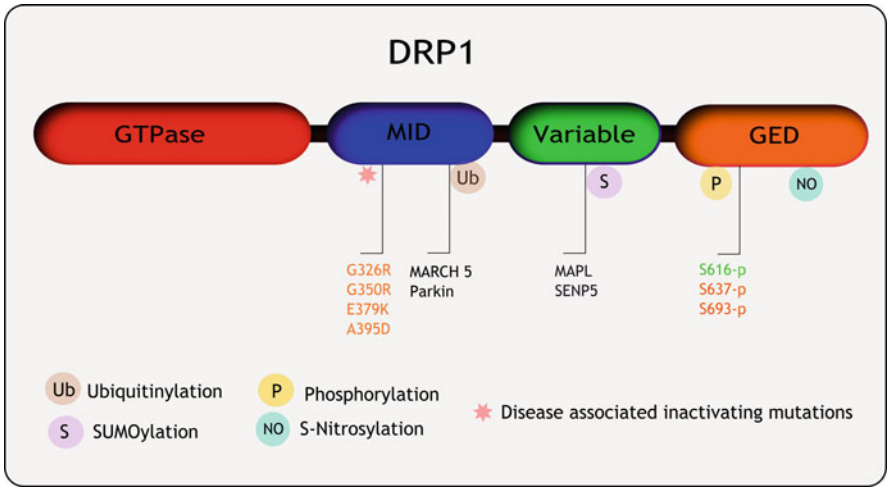

Fig. 3 DRP1 modifications and mutations associated with human diseases. DRP1 activity is regulated by post-translational modifications such as (**a**) Ubiquitinylation: MARCH 5, Parkin (**b**) SUMOylation: MAPL, SENP4 (deubiquitinase) (**c**) Phosphorylation; S616: CAMK-1,CDK1/Cyclin B, ERK; S637: PKA1, CAMK-1, ROCK1, Calcinuerin (phosphatase); S693: GSK3b. De-regulation of these processes affects DRP1 function and manifests in several human pathologies. Additionally, de novo mutations in the DRP1 middle domain which resulted DRP1 loss of function resulting in neuropathological and birth defects are indicated here

It is interesting that the same DRP1 modification can give opposing results under different stimuli, as seen with DRP1 phosphorylation at S637 and ubiquitinylation by MARCH 5 discussed earlier. These differences however could be addressed by taking into consideration the tissue specific context; for example, neuronal cells may have specific requirement for mitochondrial dynamics compared to other tissues, normal vs stress conditions – such as de-SUMOylation under normoxic and ischemic conditions, and involvement of complex signaling networks in regulating multiple fission/fusion factors – e.g., MARCH mediated ubiquitination of DRP1, Fis1 as well as the mitochondrial fusion protein, mitofusin 1 (Mfn1 – to be discussed in the next section). The final phenotypic outcome may be the cumulative effect of several factors, including multiple protein modifications on DRP1 at a given time. It is worth noting that some of these modifications are under normal physiological conditions and others are under disease or stress conditions. This alludes to a highly sophisticated regulatory framework that maintains the fine balance of mitochondrial dynamics in a cell, and can be offset in pathologies. We will discuss this in more detail in Sect. 1.4 of the chapter.

In addition to the modifications occurring in normal cellular conditions, specific disease related post-translational modifications of DRP1 have also been identified. These are S-nitrosylation and O-glucNAcylation, and both were shown to activate DRP1 and increase mitochondrial fragmentation (Bossy et al. 2010; Cho et al. 2009; Gawlowski et al. 2012). Amyloid beta mediated Nitric oxide (NO) production leading to DRP1 S-nitrosylation was shown to fragment mitochondria and

induce neurotoxicity in Alzheimer's disease and Parkinson's disease (Bossy et al. 2010; Cho et al. 2009). O-glucNAcylation was shown to induce mitochondrial fragmentation in rat neonatal cardiomyocytes, with implications in diabetes induced mitochondrial dysfunction and cardiovascular complications (Gawlowski et al. 2012).

1.2.2 Opposing Fission: Mitochondrial Fusion

Unlike fission, mitochondrial fusion has two distinct systems in place for the fusion of inner and outer membranes. Even though these processes are separate, the inner and outer membrane fusion is highly coordinated. OMM fusion is achieved by Mfn1 and 2, which are anchored on the OMM via two transmembrane domains which span the membrane twice (Hoppins et al. 2007) (Fig. 2). Mfn1 and 2 form homo- and heterotypic interactions when two apposing mitochondria come together at a permissive proximity. This initial tethering of mitochondria is followed by the outer membrane fusion through mixing of the lipid bilayers (Koshiba et al. 2004). Mfn1 and 2 perform overlapping functions; however, they are not functionally redundant (Chen and Chan 2004; Chen et al. 2003). Both proteins are generally expressed in cells, and the abundance of each may be cell type dependent.

Regulation of Mitochondrial Fusion Proteins

Both Mfn1 and 2 show regulation at both transcriptional and post-transcriptional levels (Santel et al. 2003). Mfn1 is regulated by peroxisome proliferator-activated receptor gamma co-activator 1α (PGC-1α) during postnatal cardiac growth (Martin et al. 2014). Other reports indicate tissue specific Mfn1 transcriptional repression by the corticosteroid dexamethasone in liver and in hepatoma cells (Hernandez-Alvarez et al. 2013) and by microRNA 140 in cardiomyocytes (Li et al. 2014). Mfn2 transcription is driven by the PGC-1α and PGC-1β, key factors in mitochondrial biogenesis, as well as the transcription factor Sp1 in skeletal and smooth muscle cells (Sorianello et al. 2012). Mfn2 has also shown to have hormone responsive transcriptional regulation as Estrogen-Related Receptor-alpha (ERRα) binds to the human Mfn2 promoter and stimulates transcription (Scarpulla et al. 2012; Soriano et al. 2006). Mfn1 and 2 are also regulated at a post-translational level. One of the best characterized post-translational modifications of these proteins is ubiquitinylation. Mfn1 and 2 are both ubiquitinylated by MARCH-5 and Parkin (Gegg et al. 2010; Park and Cho 2012; Park et al. 2010; Tanaka et al. 2010). Mfn1 is also activated by deacetylation mediated by HDAC6 (Lee et al. 2014). Mfn2, on the other hand, is ubiquitinylated by Parkin and marked for proteasomal degradation (Gegg et al. 2010; Poole et al. 2010; Tanaka et al. 2010; Ziviani and Whitworth 2010). Additional Mfn2 E3 ligases include HUWE1 (regulated by phosphorylation of JNK), and by the ubiquitin ligase Mul1 (Leboucher et al. 2012; Lokireddy et al. 2012) leading to proteasomal degradation. However, MARCH 5 mediated ubiquitinylation of Mfn1 and 2 did not drive proteasomal degradation of these proteins (Sugiura et al. 2013). Mfn2 levels are also regulated by activation of the deubiquitinase USP30 (Yue et al. 2014).

Inner mitochondrial membrane fusion is mediated by OPA1, a GTPase anchored on the inner mitochondrial membrane (Fig. 2). Apart from its function in inner

membrane fusion OPA1 has a distinct function in cristae remodeling and cytochrome c release during apoptosis (Frezza et al. 2006). Eight splice variants of OPA1 have been identified, and categorized as long (L) and short (S) isoforms. Both isoforms localize to the intermembrane space of mitochondria. The L-isoform is membrane anchored and the S-isoform is found peripherally attached to the inner membrane lipids (Olichon et al. 2002; Satoh et al. 2003). The OPA1-L isoform is considered the fusion competent form of OPA1, and further cleavage of the protein at protease sites S1 and S2 generates short forms of the protein that regulate inner membrane fusion (Griparic et al. 2007; Ishihara et al. 2006). Therefore, proteolytic cleavage is the major regulatory mechanism for OPA1 function in membrane fusion (Ishihara et al. 2006).

The intermembrane space rhomboid protease, PARL (presenilin-associated rhomboid-like), cleaves OPA1 at the S1 site to short soluble isoforms, and PRRL knockout resulted in mitochondrial fragmentation and sensitization to apoptosis, suggesting that proteolytic cleavage of OPA1 by PARL is a regulatory mechanism during cell death (Cipolat et al. 2006). However, PARL mediated cleavage of OPA1 does not affect mitochondrial fusion (Cipolat et al. 2006). YME1L another intermembrane space AAA protease was found to cleave OPA1 at the S2 site, resulting in inner membrane fusion. Yme1l is regulated by the OX-PHOS activity of the inner membrane (Anand et al. 2014; Ishihara et al. 2006; Mishra et al. 2014; Song et al. 2007). Under high OX-PHOS conditions, Yme1L cleaves Opa1 with higher efficiency to induce inner mitochondrial membrane fusion, suggesting that mitochondrial inner membrane fusion supports increased OX-PHOS activity, and is required for maintaining this function of mitochondria. Paraplegin is another IMM AAA metalloprotease that participates in OPA1 cleavage to produce a short fusion incompetent form (Duvezin-Caubet et al. 2006; Ishihara et al. 2006). These opposing cleavage events indicate that OPA1 processing is a coordinated event that determines fusion and fission under different physiological conditions.

The requirement of mitochondrial dynamics for cellular function is highlighted by studies from genetic models of fission and fusion including yeast, *Drosophila*, and mouse models. Since the focus of this chapter is on human disease, we will discuss findings from mouse models of genetic knockdown and disease, and correlate these findings with human pathologies presenting aberrant mitochondrial dynamics and resulting functional defects.

1.3 Insights from Genetic Models of Fission and Fusion

1.3.1 DRP1, Mff, Mfn1, and Mfn2 Genetic Knockout Mice

Over the past decade knockout mouse models for the fission protein DRP1 and the fusion proteins Mfn1 and 2 were developed and characterized (Chen et al. 2003; Ishihara et al. 2009; Kageyama et al. 2012; Wakabayashi et al. 2009). The most interesting parallel between all three mouse models is the embryonic lethality that is observed during early mid gestation in the null homozygotes, indicating an essential role of mitochondrial dynamics in embryonic development. In the DRP1 knockout mouse the embryonic lethality was observed at approximately day 11.5, and Mfn1

and Mfn2 knockout mice display a high level of lethality by day 11.5 and 12.5, respectively (Chen et al. 2003; Wakabayashi et al. 2009). Mfn1/2 double knockout mice die sooner, indicating that these two proteins have non-redundant function in the early stages of embryo development (Chen et al. 2003). As expected, all three knockout phenotypes show changes in mitochondrial morphology, dynamics, and function. All three knockouts produced embryos that are smaller than wild type, indicating a requirement of mitochondrial dynamics in cell proliferation, growth, and differentiation (Wakabayashi et al. 2009), while the Mfn1 and 2 knockout embryos showed developmental delays and deformations (Chen et al. 2003). It is noteworthy however that the defects of Mfn1 and 2 knockout phenotypes can be corrected by expressing the dominant negative form of DRP1 (K38A), indicating a possible therapeutic implication for blocking DRP1 function in these conditions where mitochondrial fusion is dysregulated (Chen et al. 2003). More recently an Mff homozygous knockout mouse model was generated and studied (Chen et al. 2015). While the mice were viable at birth, they succumbed prematurely at postnatal 13 weeks to dilated cardiomyopathy leading to heart failure. This phenotype was reversed by deletion of the Mfn1 which restored the fission–fusion balance, demonstrating that re-fusing mitochondria may be a viable therapeutic strategy in diseases with excessive mitochondrial fission (Chen et al. 2015).

1.3.2 Conditional Knockout Mouse Models

DRP1 knockout has the most deleterious effects on the brain, CNS, and heart development. The neonatal brain and heart in particular are organs that express high levels of DRP1, and possibly depend on the protein for their development and function. Accordingly, the most striking phenotypes of DRP1 loss manifest in these organs, suggesting tissue specific requirement of DRP1. In particular, the DRP1 KO embryonic cardiomyocytes show reduced beating rates, and this may be responsible for the death of the embryo (Wakabayashi et al. 2009). To further understand the requirement of DRP1 in the function of these organs in the embryonic and postnatal development, conditional knockout mice have been established (Ishihara et al. 2009; Kageyama et al. 2012; Wakabayashi et al. 2009). The heart-specific knockout of DRP1 in particular showed neonatal lethality as a result of dilated heart, and thin heart walls, displaying clear features of cardiac dysfunction at the time of death at p7–10 after birth (Ishihara et al. 2009) suggesting that Drp1 is essential for neonatal heart development. The brain-specific knockout of DRP1 generated using brain-specific En1-Cre resulted in severe decrease in cerebellum development and neonatal death within 24 h of birth (Wakabayashi et al. 2009). Proliferation of the cerebellar cells, and in particular the number of Purkinje cells, showed dramatic reduction in this conditional knockout model (Wakabayashi et al. 2009). DRP1 deletion in a larger region of the brain using Nes-Cre recombinase resulted in apoptotic cell death in the premature superficial layer neurons and deep cortical layers (Ishihara et al. 2009). These phenotypes are characterized by increased size and reduced number of mitochondria, loss of mitochondria from the synapse, and defective synapse formation in culture (Ishihara et al. 2009). The requirement of DRP1 in synapse formation may be due at least in part to the loss of mitochondrial

fission, but also due to the direct role of DRP1 in the clathrin coated endocytic vesicle formation in response to synaptic stimulation (Li et al. 2013). DRP1 mediated apoptosis in neuronal cells depends on the cell type and physiological context. Cerebellar Purkinje cells and neurons of the forebrain are among specific cell types that are affected severely by the loss of DRP1. In both cases cells undergo initial mitochondrial elongation followed by oxidative damage, resulting in fragmented and swollen mitochondria (Kageyama et al. 2012). In neural tube closure, developmentally regulated apoptosis is decreased by the loss of DRP1 (Wakabayashi et al. 2009), while increased in the neuroepithelium of the brain (Ishihara et al. 2009). Finally, DRP1 was shown to ensure survival of post-mitotic neurons in mice (Kageyama et al. 2012; Zhang et al. 2012). DRP1 null post-mitotic Purkinje cells became elongated and accumulated oxidative damage, became defective in respiration, and gradually degenerated over a period of 6 months. These aberrations manifested in defective motor and coordination behavior in these mice (Kageyama et al. 2012), suggesting that oxidative damage downstream of blocking mitochondrial fission is responsible for the neurodegeneration.

Similarly, Mfn1/2 conditional knockout mice have been developed to study the role of these proteins in the heart and brain function. Cardiomyocyte specific deletion of Mfn2 resulted in enlarged cardiomyocytes, and displayed mild mitochondrial dysfunction and modest cardiac hypertrophy and functional deterioration (Papanicolaou et al. 2011). The absence of Mfn2 prevented ROS mediated mitochondrial permeability transition (MPT), and protected adult cardiomyocytes from a number of cell death inducing stimuli, and better recovery from reperfusion injury. However, an Mfn1/2 double knockout in cardiomyocytes resulted in a much more severe phenotype (Chen et al. 2011; Papanicolaou et al. 2012). The cells displayed abnormally expanded or fragmented mitochondria, cardiomyocyte and respiratory dysfunction and rapidly progressive cardiomyopathies at postnatal day 7. These conditions led to heart failure, resulting in rapid decline in survival and death by 16 days post-birth (Chen et al. 2011; Papanicolaou et al. 2012). These studies established that Mfn1 and 2 are essential for the mitochondrial remodeling and metabolic reprogramming of the heart during neonatal heart development (Papanicolaou et al. 2011, 2012).

Tissue specific knockout mice have also been generated to study the role of Mfn1 and 2 in the nervous system. In particular, Mfn2 knockout and mutant studies were conducted focusing on modeling the Charcot–Marie–Tooth type 2A (CMT2A) disease. Mfn1 and 2 deficiency in the placental giant cell layer is responsible for the embryonic lethality of the Mfn1 and Mfn2 knockout mice (Chen et al. 2003), therefore mice were developed to specifically knockdown these proteins avoiding cells of placental lineage (Chen et al. 2007). These mice were viable for both Mfn1 and 2 knockdown; however, the Mfn2 deficiency showed more dramatic abnormalities. A percentage of Mfn2 knockout pups died post-birth, and the surviving mice showed severe defects in movement and balance, and succumbed at day 17 (Chen et al. 2007). Mfn1 knockout in the same system showed much less severity in phenotype, and the mice survived to adulthood with no obvious defects, indicating lesser significance of this mitofusin in the nervous

system. Cerebellum specific knockout of Mfn2 showed severe defects in postnatal cerebellar growth. With the Mfn2 knockout, the cerebellar Purkinje cells, in particular, showed aberrant mitochondrial distribution, ultrastructure, and ETC activity, and the study showed that Mfn2, but not Mfn1 is required for dendritic outgrowth, spine formation, and cell survival (Chen et al. 2007). Mitochondrial transport also seems to be affected in Mfn2 specific knockout neurons, suggesting a novel function for Mfn2 distinct from its function in OMM fusion (Misko et al. 2010; Pham et al. 2012). A study using the expression of Mfn2 mutant forms in cultured dorsal ganglion neurons to recapitulate the neuronal pathology of the CMT2A disease reported abnormal clustering of small fragmented mitochondria in the cell body as well as axons, along with impaired transport of mitochondria (Baloh et al. 2007). Similar results were observed from a dopaminergic neuron specific Mfn2 knockout mouse model (Pham et al. 2012). Mitochondrial fragmentation in the cell body and neuronal processes as well as markedly reduced mitochondrial mass and transport were observed, and these presumably led to the loss of neurons and movement defects characteristic of the CMT2A disease in these mice (Pham et al. 2012).

Similar to DRP1, Mfn1, and Mfn2 knockout mice, OPA1 knockout mice were also found to be embryonic lethal; death occurring in this case around day 13.5 is indicative of the requirement of this protein in embryonic development. OPA1+/− heterozygotes are viable, and several models were developed to characterize the requirement of OPA1 (Davies et al. 2007; Williams et al. 2010). Many of these studies were developed to model the DOA disease in mice and focus on the effect of OPA1 loss on optic neuronal function. While these studies do not recapitulate effect of the complete loss of OPA1, they represent moderate effects of OPA1 reduction and recapitulate DOA phenotypes. These studies reported that the reduction of OPA1 levels led to Retinal ganglion cell dendritic pruning, increased autophagy, and may contribute to RGC loss and atrophy, a marked reduction in retinal ganglion cell synaptic connectivity (White et al. 2009; Williams et al. 2011, 2012). Study of the heart in this heterozygous mouse model showed alterations in mitochondrial morphology, but not changes to the electron transport chain activity. Delay in Ca^{2+} dependent PTP opening, and associated myocardial hypertrophy induced by pressure overload were also observed (Piquereau et al. 2012). OPA1 loss was examined in the context of embryonic development, and these studies indicate that OPA1 is crucial for the survival of RGCs and essential for early embryonic survival.

1.4 From Mouse Models to Human Diseases

As we discussed in the previous section, genetic disruption of mitochondrial dynamics impacts most severely the tissues and processes that are most energy demanding and dependent on mitochondrial function. For example, the brain and heart are among the organs that are most severely impacted by the loss of mitochondrial dynamics proteins. Of similar importance is embryonic development. It is therefore not surprising that most of the mitochondrial dynamics associated diseases are related to developmental, neurodegenerative, and metabolic disorders, and

cardiomyopathies. These diseases manifest in aberrant mitochondrial function, cell growth, proliferation, and apoptosis. Considering the role of mitochondrial dynamics in cell proliferation in particular, recent advances illuminating these processes in cancer are particularly insightful. It is interesting to note that most of the human diseases are related to increased mitochondrial fragmentation, and in this section we will discuss the role of mitochondrial fission in human diseases.

1.4.1 Cancer: A Novel Role for Mitochondrial Fission

One of the more recent developments on the disease relevance of mitochondrial fission comes from the study of cancer. Increased mitochondrial fission was reported as a pathogenicity factor in several distinct cancer models. It is interesting to note that these reports come from different cancers, originating in distinct organs, and may have entirely different oncogenic signaling circuitry yet result in the upregulation of DRP1 activity leading to mitochondrial fission. Also while some reports indicate a crucial role for DRP1 in cancer initiation and transformation, others show an important role for DRP1 in the later metastatic stages suggesting a role for DRP1 in different stages of the disease progression (Zhao et al. 2013). These observations indicate a fundamental role of DRP1 in cancer.

Mitochondrial fragmentation mediated by the upregulation of DRP1 is implicated in cellular transformation by oncogenic RAS in a mouse embryonic fibroblast (MEF) model (Serasinghe et al. 2015). Excessive mitochondrial fragmentation by oncogenic RAS is crucial for the development of cancer metabolism, and transformation of MEFs. Genetic ablation and pharmacological inhibition of DRP1 was shown to abolish the transformation, suggesting a specific role for this protein in cancer development. The fission reported here is mainly mediated by DRP1S616 phosphorylation by ERK. DRP1S616 is a mitotic phosphorylation site and is responsible for fragmenting mitochondria during mitosis. Extrapolating from this observation, it is possible to envision that RAS mediated phosphorylation of DRP1S616 mimics this mitotic phosphorylation event and may facilitate or drive excessive cell proliferation in these RAS-MAPK driven cancers. Additionally, mitochondrial fission results in cancer metabolism, by preferentially utilizing the glycolytic pathway for energy production and increased biosynthesis of macromolecules. This metabolic switch could also support the development of cancer by supporting growth and proliferation of cancer cells. ERK was found to directly phosphorylate DRP1S616 in BRAFV600E positive melanoma as well as pancreatic cancer (Kashatus et al. 2015; Serasinghe et al. 2015). In melanoma patient samples, DRP1S616 phosphorylation shows a clear correlation with the presence of BRAFV600E, and a strong correlation with increasing DRP1S616 phosphorylation levels with cancer progression (Serasinghe et al. 2015; Wieder et al. 2015).

DRP1 is dysregulated in breast, lung, pancreatic, and thyroid carcinomas (Kashatus et al. 2015; Serasinghe et al. 2015). Recently DRP was found to play a crucial role in the development of glioblastoma (Xie et al. 2015). Brain tumor initiating cells (BTIC) and non-BTIC cells were found to harbor differential post-translational modification patterns of DRP1 enhancing or blocking the development of tumors. BTIC cells had a predominant phosphorylation-activated form of

DRP1S616, while non-BTIC cells showed a predominantly inactive 637 phosphorylation of DRP1. While total DRP1 levels remained unchanged, the presence of DRP1S616 phosphorylation was elevated selectively in a panel of glioblastoma tissue samples compared to non-tumor tissues. The activation of DRP1S616 was found to be regulated by the kinase CDK5. This study was further supported by patient data which indicated an inverse correlation between patient survival and the presence of DRP1S616. This comprehensive study points to the differential regulation of DRP1 post-translational modification as the switch between tumor growth and differentiation of cells, suggesting that DRP1 phosphorylation may be an important point of control in tumorigenesis.

A similar report from the study of lung cancer (Rehman et al. 2012) showed that DRP1 expression is upregulated and present in its activated form (S616p) in cancer cell lines, as well as tissue microarrays. This is associated with a parallel downregulation of the fusion protein Mfn2. Blocking fission using Mdivi-1, a small molecule inhibitor to DRP1, or overexpressing Mfn2 in lung cancer cells blocked cell cycle progression at G2/M stage. This study showed increased expression of DRP1, along with increased serine 616 phosphorylation, and decreased serine 637 phosphorylation in lung cancer cell lines, again drawing attention to the differential regulation of DRP1 post-translational modifications in cancer.

The underlying observation from these cases is that DRP1 expression or activation seems to be the predominant cause for the fragmentation (deferential expression of MFN2 has been noted less frequently) (Ferreira-da-Silva et al. 2015; Rehman et al. 2012), even though the genetic and etiologic factors for these cancers are distinct. Also, the signaling pathways responsible for the activation of DRP1 are distinct between the cancers. Some are directly under the regulation of cell cycle, while others are activated by oncogenic signaling pathways such as the RAS-MAPK pathway. The genotypes of these cancers are not reported except for in a few cases including BRAFV600E in melanoma, so it will be worthwhile to genotype these cancers and cancer cell lines to interrogate whether there exist common components between the signaling circuitry among different cancers. This would enable us to determine if there is indeed a shared component in the upstream DRP1 regulation in cancer or if it is the result of many different factors. In either case it will be interesting to see how this novel, and thus far under explored aspect of mitochondrial dynamics is implicated in cancer.

The fact that different signaling pathways lead to DRP1 activation and mitochondrial fragmentation suggests a fundamental role for mitochondrial fission mediated by DRP1 in cancer. It is possible that excessive mitochondrial fission augments the uncontrolled cell division phenotype seen in cancer. Indeed mitochondrial fission leads to cancer-like metabolism which may be a factor that triggers transformation (Serasinghe et al. 2015), or mitochondrial fragmentation may promote cell cycle progression (Qian et al. 2012; Rehman et al. 2012). Indeed, the end result may be a combination of both these processes which facilitates cells to synthesize the cellular building blocks via a predominant glycolytic metabolic pathway (Ward and Thompson 2012a) and subsequently utilize those components for cellular growth and divide and multiply rapidly as characteristic of cancer.

Another intriguing possibility with mitochondrial dynamics in cancer is the ability of mitochondrial shape to determine the susceptibility to apoptosis. It has been shown that DRP1 depletion sensitizes cancer cells to apoptosis (Inoue-Yamauchi and Oda 2012; Rehman et al. 2012). This suggests that not only does the increased fission lead to enhanced proliferation, but it also enable the cells to evade apoptosis. A key piece of evidence for the ability of mitochondrial shape to determine apoptosis susceptibility come from a recent report, that showed with mechanistic evidence that small mitochondria are more resistant to MOMP compared to intermediate and long mitochondria (Renault et al. 2015). This suggests that the excessive mitochondrial fission may also be a cellular adaptation to avoid apoptosis, and this characteristic maybe selected for in cancer cell survival. This observation, along with the earlier discussed DRP1 involvement in cancer development, reiterates a potential therapeutic intervention point in modulating mitochondrial fission in cancer.

1.4.2 Familial Disorders Arising from Defects in Mitochondrial Dynamics Proteins

It is interesting to note that familial defects of the fusion proteins Mfn1 and OPA1 both lead to disease conditions arising from neuropathies, highlighting the importance of mitochondrial dynamics on the correct functioning of the nervous system. Mutations of OPA1 leading to its loss of function cause Autosomal Dominant Optic Atrophy (ADOA). Mutations in its GTPase domain and C-terminal end result in autosomal dominancy and haplo-insufficiency leading to the loss of function of OPA1 (Olichon et al. 2007). The disease is characterized by progressive loss of vision leading to blindness in the second decade of life (Olichon et al. 2006). Loss of OPA1 and unopposed fission leads to apoptosis of retinal ganglion cells and degradation of the optic nerve. Interestingly rare Mfn2 mutations leading to similar phenotypes and ADOA have been identified, suggesting that aberrant OMM mitochondrial fusion may also contribute to the disease phenotype (Zuchner et al. 2006). On the other hand, autosomal dominant missense mutations in Mfn2 GTPase and coiled-coil domains lead to the development of the CMT2A. This leads to metabolic defects, reduced energy production, and limited mitochondrial transport to synapses, and usually manifests as muscle atrophy of the legs and feet (Klein et al. 2011; Zuchner et al. 2006). While mutations in DRP1 are not affiliated with any familial disease conditions, a DRP1 mutation in the Alanine 395 residue to Aspartic acid was reported to cause death in a new born infant as a result of multiple developmental disorders (Waterham et al. 2007). The infant manifested microcephaly, abnormal brain development, optic atrophy and hypoplasia, persistent lactic acidemia, and a mildly elevated plasma concentration of very-long-chain fatty acids (Waterham et al. 2007). Fibroblasts from this patient showed hyperfused mitochondria due to defective DRP1 function; however, the OX-PHOS and the respiratory complex activity were normal in these cells. This isolated case was due to a random point mutation of the Drp1 gene, but provides insight into the importance of proper functioning of the mitochondrial fission machinery in human health.

1.4.3 Neurodegenerative Disorders

Parkinson's Disease

Parkinson's disease (PD) is characterized by resting tremor, rigidity, and bradykinesia resulting from death of dopaminergic neurons in the *substantia nigra*. While most cases of PD are sporadic, there is a minority of rare heritable juvenile cases resulting from autosomal recessive mutations in PINK1 and Parkin genes (Pickrell and Youle 2015; Valente et al. 2004). Mitochondrial dysfunction is strongly implicated in PD. As we discussed at the beginning of this review PINK1 and Parkin regulate mitochondrial quality control through mitophagy. Knockdown of PINK1 leads to mitochondrial fragmentation and autophagy in neurons through oxidative stress, and PINK1 expression restored tubular mitochondria and suppressed toxin induced autophagy/mitophagy (Dagda et al. 2009). Expression of the dominant negative DRP1 mutant K38A inhibited both fission and mitophagy in PINK1-deficient cells, prevented stress induced mitochondrial depolarization, and apoptosis by reducing oxidative stress and mitochondrial fission (Dagda et al. 2009). Additionally, Pink1 phosphorylates and targets Parkin to depolarize mitochondria and enhances mitophagy (Kim et al. 2008). PINK1/Parkin dysfunction leading to increased DRP1 mediated mitochondrial fission is an important factor contributing to neuronal death in PD (Deng et al. 2008; Wang et al. 2011). For example, loss of PINK1 increases DRP1 levels and results in increased mitochondrial fission, increased oxidative stress, and reduced ATP generation (Dagda et al. 2009; Lutz et al. 2009). These phenotypes were rescued by expression of Mfn1 and 2 and OPA1 and treatment with the DRP1 inhibitor Mdivi-1 (Cui et al. 2010). Mitochondrial fission due to the loss of PINK1 was observed in skin fibroblasts of PD patients (Exner et al. 2007). It has been shown that pesticides such as rotenone and paraquat (inhibitors of mitochondrial respiratory complex I) that promote PD induce mitochondrial fission (Barsoum et al. 2006; Gomez-Lazaro et al. 2008). Reduced complex I activity in the *substantia nigra* of the brain is characteristic of PD, suggesting a mitochondrial fission-function axis on the disease onset and progression of Parkinson's disease (Schapira 2006).

Alzheimer's Disease

Alzheimer's disease is characterized by progressive senile or pre-senile dementia with associated biochemical features such as selective neuronal loss, synaptic alterations leading to loss of connectivity between neurons, neurofibrillary degeneration, and extracellular deposits of αβ plaques (Baloyannis 2006). Alzheimer's disease (AD) displays signs of overall mitochondrial dysfunction such as altered lipid metabolism, calcium homeostasis, decreased energy metabolism, and increased oxidative damage and is considered an early event in disease pathology (Ferreira et al. 2010; Hirai 2000; Supnet and Bezprozvanny 2010). Intriguingly, abnormal mitochondrial shape and dynamics have also been reported from AD studies, and the majority of these studies show a clear association between the form and function of mitochondria in this disease. For example, abnormal small mitochondria with defective cristae structure were observed in an electron microscopy (EM) study of human AD neurons (Baloyannis 2006). Abnormal expression levels

of mitochondrial dynamics proteins, including increased expression of DRP1 and Fis 1, were reported from postmortem brains of Alzheimer patients, along with reduced expression of Mfn1, 2, and OPA1 (Reddy et al. 2012; Wang et al. 2009b). This and other studies in mouse models and APP cell lines indicate that aberrantly fragmented mitochondria in AD are at least in part due to the increased expression of fission factors and decreased expression of fusion proteins, resulting an imbalance in mitochondrial dynamics favoring mitochondrial fission (Barsoum et al. 2006; Reddy et al. 2011; Rui et al. 2006; Wang et al. 2008b). Additionally, many reports implicate post-translational modifications of DRP1 as causative of mitochondrial fragmentation in AD. Accumulation of β amyloid protein leads to the production of nitric oxide (NO), which activates DRP1 function by s-nitrosylating DRP1, resulting in mitochondrial fission, synaptic loss, and neuronal damage (Cho et al. 2009). Mutation of DRP1 to prevent nitrosylation protected neurons from these effects, suggesting a direct role of DRP1 (Cho et al. 2009). This is supported by the observation that exposure to NO rapidly fragments mitochondria in primary cortical neuronal cells, with an associated decline in ATP generation and increase in free radicals (Barsoum et al. 2006). This fission event is thought to precede the neuronal injury and cell death, and is modulated by the regular mitochondrial dynamics machinery (Barsoum et al. 2006). However, in the same system mitochondrial fission was shown to support BAX mediated apoptosis when challenged with nitrosative stress, indicating a direct role of fission in the subsequent neuronal cell death. DRP1 interaction with amyloid precursor protein was reported from AD patient samples and studies done using ABPP (amyloid beta precursor protein) transgenic mice with increased interactions correlating with increased disease pathology (Manczak et al. 2011). Similarly, a study using an AD cybrid cell system showed increased mitochondrial fission mediated by DRP1 and associated mitochondrial dysfunction. ERK signaling facilitated the DRP1 mitochondrial localization and fission, and blocking ERK signaling or DRP1 with Mdivi-1 rescued the morphology and function (Gan et al. 2014). This observation in particular draws a parallel with the ERK-mediated phosphorylation and activation of DRP1 at S616 resulting in increased mitochondrial fission in cancer, described in the previous section, suggesting a similar mode of activation in this system. This also implies an underlying common signaling network governing mitochondrial fission, between these unrelated pathologies. The early onset of mitochondrial dysfunction in AD may be a consequence of altered mitochondrial shape and dynamics, suggesting that interrogation of the mitochondrial dynamics machinery in Alzheimer's disease may be useful in understanding and treating the disease.

Huntington's Disease

Huntington's disease (HD) is an autosomal dominant disease characterized by choreoathetosis, dementia, and premature death. The disease arises due to a mutation in the huntingtin gene (Htt) that results in amplification of a stretch of "CAG" trinucleotide sequences encoding a tract of polyglutamine in the protein (Wang et al. 2009a). This polyglutamine results in mis-folding of the Htt protein and aberrant protein interactions. Mitochondrial dysfunction is one of the prominent

features of the pathophysiology of this neurodegenerative disease (Brouillet et al. 1995; Panov et al. 2002; Schapira and Patel 2014; Tabrizi et al. 1999). Interestingly, increased mitochondrial fission is a characteristic of the disease. Overexpression of mutant Htt was shown to cause mitochondrial fragmentation, and the severity of the fragmentation depended on the length of the polyglutamine repeat (Wang et al. 2008a, 2009a). Upregulation of DRP1 and Fis1 and downregulation of Mfn1, Mfn2, and OPA1 was reported from the frontal cortex of HD patients (Costa et al. 2010; Shirendeb et al. 2011) suggesting that the aberrant phenotype is due to shifting the mitochondrial dynamics to favor fission. In addition, mutant Htt was shown to interact with DRP1, increase its GTPase activity, and promote mitochondrial fission in BAC-HD transgenic mouse neurons (Shirendeb et al. 2012). This resulted in defective anterograde movement of mitochondria on the axons and synaptic defects (Shirendeb et al. 2012). The Htt–DRP1 interaction and mitochondrial fission was associated with increased sensitivity of cells to apoptosis, in both rat neurons and Htt patient derived fibroblasts (Song et al. 2011). Inhibition of DRP1, and increasing Mfn1 and 2 expression restored mitochondrial fusion and prevented cell death. S-Nitrosylation, and activation of DRP1, was reported as a consequence of NO production by the mutant Htt protein in Htt transgenic mice and postmortem brains of HD patients. This draws an interesting parallel with the disease pathology of AD where NO plays an important role in DRP1 activation mediated mitochondrial fission and mitochondrial dysfunction (Cho et al. 2009), suggesting that common disease pathways are shared between these neurodegenerative disorders.

1.4.4 Cardiovascular Diseases

As the major energy demanding organ in the body, the heart relies on mitochondrial function to maintain ATP production to fuel cardiomyocyte metabolism and contractile function. Therefore it is logical to anticipate a role for mitochondrial dynamics in cardiomyocyte function. In support of this notion, increased mitochondrial fission associated with the loss of OPA1 has been reported in failing rat and human hearts (Chen et al. 2009). More recently it was shown that stress induced processing of OPA1 by the protease OMA1 leads to mitochondrial fragmentation, dilated cardiomyopathy, and heart failure in mice (Wai et al. 2015). While defects in mitochondrial fission and fusion factors have not been identified thus far as direct causative factors in cardiac diseases in humans, studies done in mouse models indicate that excessive mitochondrial fission is associated with programmed cardiomyocyte death during heart failure and ischemia–reperfusion injury (Disatnik et al. 2013; Sharp et al. 2014). This provides a therapeutic opportunity for manipulation of mitochondrial dynamics in cardiac diseases to delay or even reverse the cell death occurring during cardiac hypertrophy or heart failure, providing a window of opportunity for the management of the disease. Accordingly, pharmacological inhibition of DRP1 using the small molecule inhibitor Mdivi-1 or siRNA mediated knockdown of DRP1 was shown to rescue mouse hearts from ischemia and reperfusion injury (Ong et al. 2010, 2015; Sharp et al. 2014). DRP1 is activated by dephosphorylation at serine 637 by calcineurin in a cardiac arrest mouse model

(Sharp et al. 2014), and blocking calcineurin function reversed the mitochondrial fission and maintained mitochondrial network connectivity. Similarly, acute inhibition of DRP1–Fis1 interaction by the peptide P110 at the onset of reperfusion blocked mitochondrial fission and resulted in improved bioenergetics leading to long-term benefits postmyocardial infarction (Disatnik et al. 2013). Taken together these observations suggest a therapeutic utility for pharmacologically modulating mitochondrial dynamics in cardiovascular diseases.

1.4.5 Metabolic Disorders and Obesity

Recent evidence suggest that aberrant mitochondria dynamics contribute to metabolic disorders either directly or indirectly. For example, diabetic cardiomyopathy and non-alcoholic liver disease both show gross alterations in mitochondrial morphology with functional consequences such as swollen or misshapen mitochondria in patients (Galloway and Yoon 2013). In DCM patients these defects are associated with reduced Mfn1 levels and correspondingly shorter mitochondria. The mitochondrial network remodeling and associated dysfunction seen in these defects is hyperglycemia driven (Croston et al. 2014).

While a link between obesity and mitochondrial dynamics has not been reported in humans, studies show that this may be the case in mouse models. For example, in the hypothalamic axis of feeding and satiety, neuropeptide Y and agouti-related protein (NPY-AgRP) neurons showed increased mitochondrial fusion via Mfn1 and Mfn2 with feeding and overfeeding, in turn leading to elevated levels of leptin and glucose (Dietrich et al. 2013). Mitochondrial fusion was also shown to regulate neuronal activity via modulation of cellular ATP levels in this mouse model of diet-induced obesity. In proopiomelanocortin (POMC) neurons diet-induced obesity reduced Mfn2 levels impacting on ER–mitochondrial tethering and increased ER stress leading to leptin resistance in these mice (Schneeberger et al. 2013). In mouse and rat models of type I and type II diabetes excessive mitochondrial fragmentation has been reported, with associated detrimental effects such as increased ROS production, reduced mitochondrial function, and increased susceptibility to apoptosis (Makino et al. 2010; Trudeau et al. 2010; Yu et al. 2006). ROS generation in this context was found to be a consequence of mitochondrial fragmentation, providing a direct link to this pathology (Yu et al. 2006).

1.5 Perspectives: Targeting Mitochondrial Fission in Disease Management and Therapy

Considering the role of excessive mitochondrial fission in the development and progression of multiple pathologies as we described in this chapter, it is possible to envision the utility of mitochondrial fission and fusion factors in predicting disease etiology, progression, and maintenance. For example, increased expression of DRP1 is reported in melanoma, lung, breast, and thyroid cancers and linked to the pathology (Ferreira-da-Silva et al. 2015; Serasinghe et al. 2015; Zhao et al. 2013). In addition, DRP1S616 phosphorylation status has been clearly linked to

disease progression in melanoma as well as glioblastoma (Serasinghe et al. 2015; Wieder et al. 2015; Xie et al. 2015). In melanoma, nevus to melanoma progression was correlated with increased DRP1S616 phosphorylation; while in glioblastoma, the differential phosphorylation at S616 vs S637 sites determined the ability of a cell to initiate brain tumors. ERK-mediated DRP1S616 phosphorylation was also reported from human pancreatic cancer (Kashatus et al. 2015). These novel findings foreshadow potential clinical utility of DRP1 as well as DRP1S616/S637 phosphorylation as potential biomarkers for cancer initiation and progression, and may allow novel opportunities for early detection and prevention of the these cancers.

As we discussed throughout this chapter, there is also a large body of evidence to date that show how disease phenotypes resulting from excessive fission can be reversed by blocking fission through pharmacological inhibition of DRP1. Therefore, it is reasonable to predict that future therapies targeting mitochondrial fission may enter into the clinic. Close to a decade ago Mdivi-1, a small molecule inhibitor to Dnm1 – the yeast isoform of DRP1, was identified through a chemical screen (Cassidy-Stone et al. 2008). Mdivi-1 was also found to inhibit mammalian DRP1 function by blocking its GTPase activity and inhibit mitochondrial fission, and has since become a valuable research tool in interrogating mitochondrial dynamics under various contexts. Of note, pharmacological inhibition of DRP1 mediated mitochondrial fission using Mdivi-1 as well as short peptide inhibitors such as p110 has highlighted the potential of blocking mitochondrial fission in multiple disease models, ranging from cancer and neurodegenerative diseases to cardiovascular and metabolic diseases, as discussed earlier in the chapter. While compounds like Mdivi-1 has pioneered the proof-of-principle that DRP1 mediated mitochondrial fission is indeed a viable therapeutic target, it is necessary to develop drugs with higher affinity, specificity, and selectivity in order to achieve clinical benefit. Additionally, depending on the cellular context other fission proteins may serve as viable candidates for blocking mitochondrial fission (Serasinghe et al. 2010). While multiple avenues remain to be explored in terms of effectively blocking excessive mitochondrial fission in the treatment of human diseases, it is evident that mitochondrial fission is a promising target that may reveal exciting clinical outcomes in the near future.

References

Anand R, Wai T, Baker MJ, Kladt N, Schauss AC, Rugarli E, Langer T (2014) The i-AAA protease YME1L and OMA1 cleave OPA1 to balance mitochondrial fusion and fission. J Cell Biol 204: 919–929

Baloh RH, Schmidt RE, Pestronk A, Milbrandt J (2007) Altered axonal mitochondrial transport in the pathogenesis of Charcot-Marie-Tooth disease from mitofusin 2 mutations. J Neurosci 27: 422–430

Baloyannis SJ (2006) Mitochondrial alterations in Alzheimer's disease. J Alzheimers Dis 9: 119–126

Barsoum MJ, Yuan H, Gerencser AA, Liot G, Kushnareva Y, Graber S, Kovacs I, Lee WD, Waggoner J, Cui J, White AD, Bossy B, Martinou JC, Youle RJ, Lipton SA, Ellisman MH, Perkins GA, Bossy-Wetzel E (2006) Nitric oxide-induced mitochondrial fission is regulated by dynamin-related GTPases in neurons. EMBO J 25:3900–3911

Bossy B, Petrilli A, Klinglmayr E, Chen J, Lutz-Meindl U, Knott AB, Masliah E, Schwarzenbacher R, Bossy-Wetzel E (2010) S-Nitrosylation of DRP1 does not affect enzymatic activity and is not specific to Alzheimer's disease. J Alzheimers Dis 20(Suppl 2): S513–S526

Braschi E, Zunino R, McBride HM (2009) MAPL is a new mitochondrial SUMO E3 ligase that regulates mitochondrial fission. EMBO Rep 10:748–754

Brouillet E, Hantraye P, Ferrante RJ, Dolan R, Leroy-Willig A, Kowall NW, Beal MF (1995) Chronic mitochondrial energy impairment produces selective striatal degeneration and abnormal choreiform movements in primates. Proc Natl Acad Sci U S A 92:7105–7109

Cassidy-Stone A, Chipuk JE, Ingerman E, Song C, Yoo C, Kuwana T, Kurth MJ, Shaw JT, Hinshaw JE, Green DR, Nunnari J (2008) Chemical inhibition of the mitochondrial division dynamin reveals its role in Bax/Bak-dependent mitochondrial outer membrane permeabilization. Dev Cell 14:193–204

Chang CR, Blackstone C (2007) Cyclic AMP-dependent protein kinase phosphorylation of Drp1 regulates its GTPase activity and mitochondrial morphology. J Biol Chem 282:21583–21587

Chen H, Chan DC (2004) Mitochondrial dynamics in mammals. Curr Top Dev Biol 59:119–144

Chen H, Detmer SA, Ewald AJ, Griffin EE, Fraser SE, Chan DC (2003) Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. J Cell Biol 160:189–200

Chen H, McCaffery JM, Chan DC (2007) Mitochondrial fusion protects against neurodegeneration in the cerebellum. Cell 130:548–562

Chen L, Gong Q, Stice JP, Knowlton AA (2009) Mitochondrial OPA1, apoptosis, and heart failure. Cardiovasc Res 84:91–99

Chen Y, Liu Y, Dorn GW 2nd (2011) Mitochondrial fusion is essential for organelle function and cardiac homeostasis. Circ Res 109:1327–1331

Chen H, Ren S, Clish C, Jain M, Mootha V, McCaffery JM, Chan DC (2015) Titration of mitochondrial fusion rescues Mff-deficient cardiomyopathy. J Cell Biol 211:795–805

Chipuk JE, Moldoveanu T, Llambi F, Parsons MJ, Green DR (2010) The BCL-2 family reunion. Mol Cell 37:299–310

Cho DH, Nakamura T, Fang J, Cieplak P, Godzik A, Gu Z, Lipton SA (2009) S-nitrosylation of Drp1 mediates beta-amyloid-related mitochondrial fission and neuronal injury. Science 324: 102–105

Chou CH, Lin CC, Yang MC, Wei CC, Liao HD, Lin RC, Tu WY, Kao TC, Hsu CM, Cheng JT, Chou AK, Lee CI, Loh JK, Howng SL, Hong YR (2012) GSK3beta-mediated Drp1 phosphorylation induced elongated mitochondrial morphology against oxidative stress. PLoS One 7, e49112

Choudhary V, Kaddour-Djebbar I, Lakshmikanthan V, Ghazaly T, Thangjam GS, Sreekumar A, Lewis RW, Mills IG, Bollag WB, Kumar MV (2011) Novel role of androgens in mitochondrial fission and apoptosis. Mol Cancer Res 9:1067–1077

Cipolat S, Rudka T, Hartmann D, Costa V, Serneels L, Craessaerts K, Metzger K, Frezza C, Annaert W, D'Adamio L, Derks C, Dejaegere T, Pellegrini L, D'Hooge R, Scorrano L, De Strooper B (2006) Mitochondrial rhomboid PARL regulates cytochrome c release during apoptosis via OPA1-dependent cristae remodeling. Cell 126:163–175

Collins Y, Chouchani ET, James AM, Menger KE, Cocheme HM, Murphy MP (2012) Mitochondrial redox signalling at a glance. J Cell Sci 125:801–806

Costa V, Giacomello M, Hudec R, Lopreiato R, Ermak G, Lim D, Malorni W, Davies KJ, Carafoli E, Scorrano L (2010) Mitochondrial fission and cristae disruption increase the response of cell models of Huntington's disease to apoptotic stimuli. EMBO Mol Med 2: 490–503

Croston TL, Thapa D, Holden AA, Tveter KJ, Lewis SE, Shepherd DL, Nichols CE, Long DM, Olfert IM, Jagannathan R, Hollander JM (2014) Functional deficiencies of subsarcolemmal mitochondria in the type 2 diabetic human heart. Am J Physiol Heart Circ Physiol 307: H54–H65

Cui M, Tang X, Christian WV, Yoon Y, Tieu K (2010) Perturbations in mitochondrial dynamics induced by human mutant PINK1 can be rescued by the mitochondrial division inhibitor mdivi-1. J Biol Chem 285:11740–11752

Dagda RK, Cherra SJ 3rd, Kulich SM, Tandon A, Park D, Chu CT (2009) Loss of PINK1 function promotes mitophagy through effects on oxidative stress and mitochondrial fission. J Biol Chem 284:13843–13855

Davies VJ, Hollins AJ, Piechota MJ, Yip W, Davies JR, White KE, Nicols PP, Boulton ME, Votruba M (2007) Opa1 deficiency in a mouse model of autosomal dominant optic atrophy impairs mitochondrial morphology, optic nerve structure and visual function. Hum Mol Genet 16:1307–1318

de Brito OM, Scorrano L (2008) Mitofusin 2: a mitochondria-shaping protein with signaling roles beyond fusion. Antioxid Redox Signal 10:621–633

Deng H, Dodson MW, Huang H, Guo M (2008) The Parkinson's disease genes pink1 and parkin promote mitochondrial fission and/or inhibit fusion in Drosophila. Proc Natl Acad Sci U S A 105:14503–14508

Dietrich MO, Liu ZW, Horvath TL (2013) Mitochondrial dynamics controlled by mitofusins regulate Agrp neuronal activity and diet-induced obesity. Cell 155:188–199

Disatnik MH, Ferreira JC, Campos JC, Gomes KS, Dourado PM, Qi X, Mochly-Rosen D (2013) Acute inhibition of excessive mitochondrial fission after myocardial infarction prevents long-term cardiac dysfunction. J Am Heart Assoc 2, e000461

Duvezin-Caubet S, Jagasia R, Wagener J, Hofmann S, Trifunovic A, Hansson A, Chomyn A, Bauer MF, Attardi G, Larsson NG, Neupert W, Reichert AS (2006) Proteolytic processing of OPA1 links mitochondrial dysfunction to alterations in mitochondrial morphology. J Biol Chem 281:37972–37979

Ehses S, Raschke I, Mancuso G, Bernacchia A, Geimer S, Tondera D, Martinou JC, Westermann B, Rugarli EI, Langer T (2009) Regulation of OPA1 processing and mitochondrial fusion by m-AAA protease isoenzymes and OMA1. J Cell Biol 187:1023–1036

Elkholi R, Renault TT, Serasinghe MN, Chipuk JE (2014) Putting the pieces together: How is the mitochondrial pathway of apoptosis regulated in cancer and chemotherapy? Cancer Metab 2: 16

Exner N, Treske B, Paquet D, Holmstrom K, Schiesling C, Gispert S, Carballo-Carbajal I, Berg D, Hoepken HH, Gasser T, Kruger R, Winklhofer KF, Vogel F, Reichert AS, Auburger G, Kahle PJ, Schmid B, Haass C (2007) Loss-of-function of human PINK1 results in mitochondrial pathology and can be rescued by parkin. J Neurosci 27:12413–12418

Ferreira IL, Resende R, Ferreiro E, Rego AC, Pereira CF (2010) Multiple defects in energy metabolism in Alzheimer's disease. Curr Drug Targets 11:1193–1206

Ferreira-da-Silva A, Valacca C, Rios E, Populo H, Soares P, Sobrinho-Simoes M, Scorrano L, Maximo V, Campello S (2015) Mitochondrial dynamics protein Drp1 is overexpressed in oncocytic thyroid tumors and regulates cancer cell migration. PLoS One 10, e0122308

Frezza C, Cipolat S, Martins de Brito O, Micaroni M, Beznoussenko GV, Rudka T, Bartoli D, Polishuck RS, Danial NN, De Strooper B, Scorrano L (2006) OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. Cell 126:177–189

Friedman JR, Lackner LL, West M, DiBenedetto JR, Nunnari J, Voeltz GK (2011) ER tubules mark sites of mitochondrial division. Science 334:358–362

Galloway CA, Yoon Y (2013) Mitochondrial morphology in metabolic diseases. Antioxid Redox Signal 19:415–430

Gan X, Huang S, Wu L, Wang Y, Hu G, Li G, Zhang H, Yu H, Swerdlow RH, Chen JX, Yan SS (2014) Inhibition of ERK-DLP1 signaling and mitochondrial division alleviates mitochondrial dysfunction in Alzheimer's disease cybrid cell. Biochim Biophys Acta 1842:220–231

Gandre-Babbe S, van der Bliek AM (2008) The novel tail-anchored membrane protein Mff controls mitochondrial and peroxisomal fission in mammalian cells. Mol Biol Cell 19: 2402–2412

Gawlowski T, Suarez J, Scott B, Torres-Gonzalez M, Wang H, Schwappacher R, Han X, Yates JR 3rd, Hoshijima M, Dillmann W (2012) Modulation of dynamin-related protein 1 (DRP1) function by increased O-linked-beta-N-acetylglucosamine modification (O-GlcNAc) in cardiac myocytes. J Biol Chem 287:30024–30034

Gegg ME, Cooper JM, Chau KY, Rojo M, Schapira AH, Taanman JW (2010) Mitofusin 1 and mitofusin 2 are ubiquitinated in a PINK1/parkin-dependent manner upon induction of mitophagy. Hum Mol Genet 19:4861–4870

Gomez-Lazaro M, Bonekamp NA, Galindo MF, Jordan J, Schrader M (2008) 6-Hydroxydopamine (6-OHDA) induces Drp1-dependent mitochondrial fragmentation in SH-SY5Y cells. Free Radic Biol Med 44:1960–1969

Griparic L, Kanazawa T, van der Bliek AM (2007) Regulation of the mitochondrial dynamin-like protein Opa1 by proteolytic cleavage. J Cell Biol 178:757–764

Guo C, Hildick KL, Luo J, Dearden L, Wilkinson KA, Henley JM (2013) SENP3-mediated deSUMOylation of dynamin-related protein 1 promotes cell death following ischaemia. EMBO J 32:1514–1528

Hamanaka RB, Chandel NS (2010) Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes. Trends Biochem Sci 35:505–513

Han XJ, Lu YF, Li SA, Kaitsuka T, Sato Y, Tomizawa K, Nairn AC, Takei K, Matsui H, Matsushita M (2008) CaM kinase I alpha-induced phosphorylation of Drp1 regulates mitochondrial morphology. J Cell Biol 182:573–585

Harder Z, Zunino R, McBride H (2004) Sumo1 conjugates mitochondrial substrates and participates in mitochondrial fission. Curr Biol 14:340–345

Head B, Griparic L, Amiri M, Gandre-Babbe S, van der Bliek AM (2009) Inducible proteolytic inactivation of OPA1 mediated by the OMA1 protease in mammalian cells. J Cell Biol 187: 959–966

Hernandez-Alvarez MI, Paz JC, Sebastian D, Munoz JP, Liesa M, Segales J, Palacin M, Zorzano A (2013) Glucocorticoid modulation of mitochondrial function in hepatoma cells requires the mitochondrial fission protein Drp1. Antioxid Redox Signal 19:366–378

Hirai S (2000) Alzheimer disease: current therapy and future therapeutic strategies. Alzheimer Dis Assoc Disord 14(Suppl 1):S11–S17

Hoppins S, Lackner L, Nunnari J (2007) The machines that divide and fuse mitochondria. Annu Rev Biochem 76:751–780

Ikeda Y, Sciarretta S, Nagarajan N, Rubattu S, Volpe M, Frati G, Sadoshima J (2014) New insights into the role of mitochondrial dynamics and autophagy during oxidative stress and aging in the heart. Oxid Med Cell Longev 2014:210934

Ingerman E, Perkins EM, Marino M, Mears JA, McCaffery JM, Hinshaw JE, Nunnari J (2005) Dnm1 forms spirals that are structurally tailored to fit mitochondria. J Cell Biol 170:1021–1027

Inoue-Yamauchi A, Oda H (2012) Depletion of mitochondrial fission factor DRP1 causes increased apoptosis in human colon cancer cells. Biochem Biophys Res Commun 421:81–85

Ishihara N, Fujita Y, Oka T, Mihara K (2006) Regulation of mitochondrial morphology through proteolytic cleavage of OPA1. EMBO J 25:2966–2977

Ishihara N, Nomura M, Jofuku A, Kato H, Suzuki SO, Masuda K, Otera H, Nakanishi Y, Nonaka I, Goto Y, Taguchi N, Morinaga H, Maeda M, Takayanagi R, Yokota S, Mihara K (2009) Mitochondrial fission factor Drp1 is essential for embryonic development and synapse formation in mice. Nat Cell Biol 11:958–966

Kageyama Y, Zhang Z, Roda R, Fukaya M, Wakabayashi J, Wakabayashi N, Kensler TW, Reddy PH, Iijima M, Sesaki H (2012) Mitochondrial division ensures the survival of postmitotic neurons by suppressing oxidative damage. J Cell Biol 197:535–551

Karbowski M, Neutzner A, Youle RJ (2007) The mitochondrial E3 ubiquitin ligase MARCH5 is required for Drp1 dependent mitochondrial division. J Cell Biol 178:71–84

Kashatus JA, Nascimento A, Myers LJ, Sher A, Byrne FL, Hoehn KL, Counter CM, Kashatus DF (2015) Erk2 phosphorylation of Drp1 promotes mitochondrial fission and MAPK-driven tumor growth. Mol Cell 57:537–551

Kim Y, Park J, Kim S, Song S, Kwon SK, Lee SH, Kitada T, Kim JM, Chung J (2008) PINK1 controls mitochondrial localization of Parkin through direct phosphorylation. Biochem Biophys Res Commun 377:975–980

Klein CJ, Shi Y, Fecto F, Donaghy M, Nicholson G, McEntagart ME, Crosby AH, Wu Y, Lou H, McEvoy KM, Siddique T, Deng HX, Dyck PJ (2011) TRPV4 mutations and cytotoxic hypercalcemia in axonal Charcot-Marie-Tooth neuropathies. Neurology 76:887–894

Koshiba T, Detmer SA, Kaiser JT, Chen H, McCaffery JM, Chan DC (2004) Structural basis of mitochondrial tethering by mitofusin complexes. Science 305:858–862

Leboucher GP, Tsai YC, Yang M, Shaw KC, Zhou M, Veenstra TD, Glickman MH, Weissman AM (2012) Stress-induced phosphorylation and proteasomal degradation of mitofusin 2 facilitates mitochondrial fragmentation and apoptosis. Mol Cell 47:547–557

Lee JY, Kapur M, Li M, Choi MC, Choi S, Kim HJ, Kim I, Lee E, Taylor JP, Yao TP (2014) MFN1 deacetylation activates adaptive mitochondrial fusion and protects metabolically challenged mitochondria. J Cell Sci 127:4954–4963

Li J, Donath S, Li Y, Qin D, Prabhakar BS, Li P (2010) miR-30 regulates mitochondrial fission through targeting p53 and the dynamin-related protein-1 pathway. PLoS Genet 6:e1000795

Li H, Alavian KN, Lazrove E, Mehta N, Jones A, Zhang P, Licznerski P, Graham M, Uo T, Guo J, Rahner C, Duman RS, Morrison RS, Jonas EA (2013) A Bcl-xL-Drp1 complex regulates synaptic vesicle membrane dynamics during endocytosis. Nat Cell Biol 15:773–785

Li J, Li Y, Jiao J, Wang J, Li Y, Qin D, Li P (2014) Mitofusin 1 is negatively regulated by microRNA 140 in cardiomyocyte apoptosis. Mol Cell Biol 34:1788–1799

Liu T, Yu R, Jin SB, Han L, Lendahl U, Zhao J, Nister M (2013) The mitochondrial elongation factors MIEF1 and MIEF2 exert partially distinct functions in mitochondrial dynamics. Exp Cell Res 319:2893–2904

Lokireddy S, Wijesoma IW, Teng S, Bonala S, Gluckman PD, McFarlane C, Sharma M, Kambadur R (2012) The ubiquitin ligase Mul1 induces mitophagy in skeletal muscle in response to muscle-wasting stimuli. Cell Metab 16:613–624

Loson OC, Song Z, Chen H, Chan DC (2013) Fis1, Mff, MiD49, and MiD51 mediate Drp1 recruitment in mitochondrial fission. Mol Biol Cell 24:659–667

Lutz AK, Exner N, Fett ME, Schlehe JS, Kloos K, Lammermann K, Brunner B, Kurz-Drexler A, Vogel F, Reichert AS, Bouman L, Vogt-Weisenhorn D, Wurst W, Tatzelt J, Haass C, Winklhofer KF (2009) Loss of parkin or PINK1 function increases Drp1-dependent mitochondrial fragmentation. J Biol Chem 284:22938–22951

Makino A, Scott BT, Dillmann WH (2010) Mitochondrial fragmentation and superoxide anion production in coronary endothelial cells from a mouse model of type 1 diabetes. Diabetologia 53:1783–1794

Manczak M, Calkins MJ, Reddy PH (2011) Impaired mitochondrial dynamics and abnormal interaction of amyloid beta with mitochondrial protein Drp1 in neurons from patients with Alzheimer's disease: implications for neuronal damage. Hum Mol Genet 20:2495–2509

Martin OJ, Lai L, Soundarapandian MM, Leone TC, Zorzano A, Keller MP, Attie AD, Muoio DM, Kelly DP (2014) A role for peroxisome proliferator-activated receptor gamma coactivator-1 in the control of mitochondrial dynamics during postnatal cardiac growth. Circ Res 114:626–636

Mears JA, Lackner LL, Fang S, Ingerman E, Nunnari J, Hinshaw JE (2011) Conformational changes in Dnm1 support a contractile mechanism for mitochondrial fission. Nat Struct Mol Biol 18:20–26

Mishra P, Carelli V, Manfredi G, Chan DC (2014) Proteolytic cleavage of Opa1 stimulates mitochondrial inner membrane fusion and couples fusion to oxidative phosphorylation. Cell Metab 19:630–641

Misko A, Jiang S, Wegorzewska I, Milbrandt J, Baloh RH (2010) Mitofusin 2 is necessary for transport of axonal mitochondria and interacts with the Miro/Milton complex. J Neurosci 30:4232–4240

Nakamura N, Kimura Y, Tokuda M, Honda S, Hirose S (2006) MARCH-V is a novel mitofusin 2- and Drp1-binding protein able to change mitochondrial morphology. EMBO Rep 7:1019–1022

Niemann A, Ruegg M, La Padula V, Schenone A, Suter U (2005) Ganglioside-induced differentiation associated protein 1 is a regulator of the mitochondrial network: new implications for Charcot-Marie-Tooth disease. J Cell Biol 170:1067–1078

Niemann A, Wagner KM, Ruegg M, Suter U (2009) GDAP1 mutations differ in their effects on mitochondrial dynamics and apoptosis depending on the mode of inheritance. Neurobiol Dis 36:509–520

Olichon A, Emorine LJ, Descoins E, Pelloquin L, Brichese L, Gas N, Guillou E, Delettre C, Valette A, Hamel CP, Ducommun B, Lenaers G, Belenguer P (2002) The human dynamin-related protein OPA1 is anchored to the mitochondrial inner membrane facing the inter-membrane space. FEBS Lett 523:171–176

Olichon A, Guillou E, Delettre C, Landes T, Arnaune-Pelloquin L, Emorine LJ, Mils V, Daloyau M, Hamel C, Amati-Bonneau P, Bonneau D, Reynier P, Lenaers G, Belenguer P (2006) Mitochondrial dynamics and disease, OPA1. Biochim Biophys Acta 1763:500–509

Olichon A, Elachouri G, Baricault L, Delettre C, Belenguer P, Lenaers G (2007) OPA1 alternate splicing uncouples an evolutionary conserved function in mitochondrial fusion from a vertebrate restricted function in apoptosis. Cell Death Differ 14:682–692

Ong SB, Subrayan S, Lim SY, Yellon DM, Davidson SM, Hausenloy DJ (2010) Inhibiting mitochondrial fission protects the heart against ischemia/reperfusion injury. Circulation 121: 2012–2022

Ong SB, Kalkhoran SB, Cabrera-Fuentes HA, Hausenloy DJ (2015) Mitochondrial fusion and fission proteins as novel therapeutic targets for treating cardiovascular disease. Eur J Pharmacol 763:104–114

Palmer CS, Osellame LD, Laine D, Koutsopoulos OS, Frazier AE, Ryan MT (2011) MiD49 and MiD51, new components of the mitochondrial fission machinery. EMBO Rep 12:565–573

Panov AV, Gutekunst CA, Leavitt BR, Hayden MR, Burke JR, Strittmatter WJ, Greenamyre JT (2002) Early mitochondrial calcium defects in Huntington's disease are a direct effect of polyglutamines. Nat Neurosci 5:731–736

Papanicolaou KN, Khairallah RJ, Ngoh GA, Chikando A, Luptak I, O'Shea KM, Riley DD, Lugus JJ, Colucci WS, Lederer WJ, Stanley WC, Walsh K (2011) Mitofusin-2 maintains mitochondrial structure and contributes to stress-induced permeability transition in cardiac myocytes. Mol Cell Biol 31:1309–1328

Papanicolaou KN, Ngoh GA, Dabkowski ER, O'Connell KA, Ribeiro RF Jr, Stanley WC, Walsh K (2012) Cardiomyocyte deletion of mitofusin-1 leads to mitochondrial fragmentation and improves tolerance to ROS-induced mitochondrial dysfunction and cell death. Am J Physiol Heart Circ Physiol 302:H167–H179

Park YY, Cho H (2012) Mitofusin 1 is degraded at G2/M phase through ubiquitylation by MARCH5. Cell Div 7:25

Park YY, Lee S, Karbowski M, Neutzner A, Youle RJ, Cho H (2010) Loss of MARCH5 mitochondrial E3 ubiquitin ligase induces cellular senescence through dynamin-related protein 1 and mitofusin 1. J Cell Sci 123:619–626

Pham AH, Meng S, Chu QN, Chan DC (2012) Loss of Mfn2 results in progressive, retrograde degeneration of dopaminergic neurons in the nigrostriatal circuit. Hum Mol Genet 21: 4817–4826

Pickrell AM, Youle RJ (2015) The roles of PINK1, parkin, and mitochondrial fidelity in Parkinson's disease. Neuron 85:257–273

Piquereau J, Caffin F, Novotova M, Prola A, Garnier A, Mateo P, Fortin D, le Huynh H, Nicolas V, Alavi MV, Brenner C, Ventura-Clapier R, Veksler V, Joubert F (2012) Down-regulation of OPA1 alters mouse mitochondrial morphology, PTP function, and cardiac adaptation to pressure overload. Cardiovasc Res 94:408–417

Poole AC, Thomas RE, Yu S, Vincow ES, Pallanck L (2010) The mitochondrial fusion-promoting factor mitofusin is a substrate of the PINK1/parkin pathway. PLoS One 5:e10054

Prudent J, Zunino R, Sugiura A, Mattie S, Shore GC, McBride HM (2015) MAPL SUMOylation of Drp1 stabilizes an ER/mitochondrial platform required for cell death. Mol Cell 59:941–955

Qi X, Disatnik MH, Shen N, Sobel RA, Mochly-Rosen D (2011) Aberrant mitochondrial fission in neurons induced by protein kinase C{delta} under oxidative stress conditions in vivo. Mol Biol Cell 22:256–265

Qian W, Choi S, Gibson GA, Watkins SC, Bakkenist CJ, Van Houten B (2012) Mitochondrial hyperfusion induced by loss of the fission protein Drp1 causes ATM-dependent G2/M arrest and aneuploidy through DNA replication stress. J Cell Sci 125:5745–5757

Rambold AS, Kostelecky B, Elia N, Lippincott-Schwartz J (2011) Tubular network formation protects mitochondria from autophagosomal degradation during nutrient starvation. Proc Natl Acad Sci U S A 108:10190–10195

Reddy PH, Reddy TP, Manczak M, Calkins MJ, Shirendeb U, Mao P (2011) Dynamin-related protein 1 and mitochondrial fragmentation in neurodegenerative diseases. Brain Res Rev 67: 103–118

Reddy PH, Tripathi R, Troung Q, Tirumala K, Reddy TP, Anekonda V, Shirendeb UP, Calkins MJ, Reddy AP, Mao P, Manczak M (2012) Abnormal mitochondrial dynamics and synaptic degeneration as early events in Alzheimer's disease: implications to mitochondria-targeted antioxidant therapeutics. Biochim Biophys Acta 1822:639–649

Rehman J, Zhang HJ, Toth PT, Zhang Y, Marsboom G, Hong Z, Salgia R, Husain AN, Wietholt C, Archer SL (2012) Inhibition of mitochondrial fission prevents cell cycle progression in lung cancer. FASEB J 26:2175–2186

Renault TT, Floros KV, Elkholi R, Corrigan KA, Kushnareva Y, Wieder SY, Lindtner C, Serasinghe MN, Asciolla JJ, Buettner C, Newmeyer DD, Chipuk JE (2015) Mitochondrial shape governs BAX-induced membrane permeabilization and apoptosis. Mol Cell 57:69–82

Rui Y, Tiwari P, Xie Z, Zheng JQ (2006) Acute impairment of mitochondrial trafficking by beta-amyloid peptides in hippocampal neurons. J Neurosci 26:10480–10487

Santel A, Frank S, Gaume B, Herrler M, Youle RJ, Fuller MT (2003) Mitofusin-1 protein is a generally expressed mediator of mitochondrial fusion in mammalian cells. J Cell Sci 116: 2763–2774

Satoh M, Hamamoto T, Seo N, Kagawa Y, Endo H (2003) Differential sublocalization of the dynamin-related protein OPA1 isoforms in mitochondria. Biochem Biophys Res Commun 300:482–493

Scarpulla RC, Vega RB, Kelly DP (2012) Transcriptional integration of mitochondrial biogenesis. Trends Endocrinol Metab 23:459–466

Schapira AH (2006) Etiology of Parkinson's disease. Neurology 66:S10–S23

Schapira AH, Patel S (2014) Targeting mitochondria for neuroprotection in Parkinson disease. JAMA Neurol 71:537–538

Schieber M, Chandel NS (2014) ROS function in redox signaling and oxidative stress. Curr Biol 24:R453–R462

Schneeberger M, Dietrich MO, Sebastian D, Imbernon M, Castano C, Garcia A, Esteban Y, Gonzalez-Franquesa A, Rodriguez IC, Bortolozzi A, Garcia-Roves PM, Gomis R, Nogueiras R, Horvath TL, Zorzano A, Claret M (2013) Mitofusin 2 in POMC neurons connects ER stress with leptin resistance and energy imbalance. Cell 155:172–187

Serasinghe MN, Yoon Y (2008) The mitochondrial outer membrane protein hFis1 regulates mitochondrial morphology and fission through self-interaction. Exp Cell Res 314:3494–3507

Serasinghe MN, Seneviratne AM, Smrcka AV, Yoon Y (2010) Identification and characterization of unique proline-rich peptides binding to the mitochondrial fission protein hFis1. J Biol Chem 285:620–630

Serasinghe MN, Wieder SY, Renault TT, Elkholi R, Asciolla JJ, Yao JL, Jabado O, Hoehn K, Kageyama Y, Sesaki H, Chipuk JE (2015) Mitochondrial division is requisite to RAS-induced transformation and targeted by oncogenic MAPK pathway inhibitors. Mol Cell 57:521–536

Sharp WW, Fang YH, Han M, Zhang HJ, Hong Z, Banathy A, Morrow E, Ryan JJ, Archer SL (2014) Dynamin-related protein 1 (Drp1)-mediated diastolic dysfunction in myocardial ischemia-reperfusion injury: therapeutic benefits of Drp1 inhibition to reduce mitochondrial fission. FASEB J 28:316–326

Shirendeb U, Reddy AP, Manczak M, Calkins MJ, Mao P, Tagle DA, Reddy PH (2011) Abnormal mitochondrial dynamics, mitochondrial loss and mutant huntingtin oligomers in Huntington's disease: implications for selective neuronal damage. Hum Mol Genet 20:1438–1455

Shirendeb UP, Calkins MJ, Manczak M, Anekonda V, Dufour B, McBride JL, Mao P, Reddy PH (2012) Mutant huntingtin's interaction with mitochondrial protein Drp1 impairs mitochondrial biogenesis and causes defective axonal transport and synaptic degeneration in Huntington's disease. Hum Mol Genet 21:406–420

Song Z, Chen H, Fiket M, Alexander C, Chan DC (2007) OPA1 processing controls mitochondrial fusion and is regulated by mRNA splicing, membrane potential, and Yme1L. J Cell Biol 178:749–755

Song W, Chen J, Petrilli A, Liot G, Klinglmayr E, Zhou Y, Poquiz P, Tjong J, Pouladi MA, Hayden MR, Masliah E, Ellisman M, Rouiller I, Schwarzenbacher R, Bossy B, Perkins G, Bossy-Wetzel E (2011) Mutant huntingtin binds the mitochondrial fission GTPase dynamin-related protein-1 and increases its enzymatic activity. Nat Med 17:377–382

Sorianello E, Soriano FX, Fernandez-Pascual S, Sancho A, Naon D, Vila-Caballer M, Gonzalez-Navarro H, Portugal J, Andres V, Palacin M, Zorzano A (2012) The promoter activity of human Mfn2 depends on Sp1 in vascular smooth muscle cells. Cardiovasc Res 94:38–47

Soriano FX, Liesa M, Bach D, Chan DC, Palacin M, Zorzano A (2006) Evidence for a mitochondrial regulatory pathway defined by peroxisome proliferator-activated receptor-gamma coactivator-1 alpha, estrogen-related receptor-alpha, and mitofusin 2. Diabetes 55:1783–1791

Sugiura A, Nagashima S, Tokuyama T, Amo T, Matsuki Y, Ishido S, Kudo Y, McBride HM, Fukuda T, Matsushita N, Inatome R, Yanagi S (2013) MITOL regulates endoplasmic reticulum-mitochondria contacts via Mitofusin2. Mol Cell 51:20–34

Supnet C, Bezprozvanny I (2010) Neuronal calcium signaling, mitochondrial dysfunction, and Alzheimer's disease. J Alzheimers Dis 20(Suppl 2):S487–S498

Tabrizi SJ, Cleeter MW, Xuereb J, Taanman JW, Cooper JM, Schapira AH (1999) Biochemical abnormalities and excitotoxicity in Huntington's disease brain. Ann Neurol 45:25–32

Taguchi N, Ishihara N, Jofuku A, Oka T, Mihara K (2007) Mitotic phosphorylation of dynamin-related GTPase Drp1 participates in mitochondrial fission. J Biol Chem 282:11521–11529

Tanaka A, Cleland MM, Xu S, Narendra DP, Suen DF, Karbowski M, Youle RJ (2010) Proteasome and p97 mediate mitophagy and degradation of mitofusins induced by Parkin. J Cell Biol 191:1367–1380

Trudeau K, Molina AJ, Guo W, Roy S (2010) High glucose disrupts mitochondrial morphology in retinal endothelial cells: implications for diabetic retinopathy. Am J Pathol 177:447–455

Valente EM, Abou-Sleiman PM, Caputo V, Muqit MM, Harvey K, Gispert S, Ali Z, Del Turco D, Bentivoglio AR, Healy DG, Albanese A, Nussbaum R, Gonzalez-Maldonado R, Deller T, Salvi S, Cortelli P, Gilks WP, Latchman DS, Harvey RJ, Dallapiccola B, Auburger G, Wood NW (2004) Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science 304:1158–1160

Wai T, Garcia-Prieto J, Baker MJ, Merkwirth C, Benit P, Rustin P, Ruperez FJ, Barbas C, Ibanez B, Langer T (2015) Imbalanced OPA1 processing and mitochondrial fragmentation cause heart failure in mice. Science 350, aad0116

Wakabayashi J, Zhang Z, Wakabayashi N, Tamura Y, Fukaya M, Kensler TW, Iijima M, Sesaki H (2009) The dynamin-related GTPase Drp1 is required for embryonic and brain development in mice. J Cell Biol 186:805–816

Walsh C, Barrow S, Voronina S, Chvanov M, Petersen OH, Tepikin A (2009) Modulation of calcium signalling by mitochondria. Biochim Biophys Acta 1787:1374–1382

Wang CE, Tydlacka S, Orr AL, Yang SH, Graham RK, Hayden MR, Li S, Chan AW, Li XJ (2008a) Accumulation of N-terminal mutant huntingtin in mouse and monkey models implicated as a pathogenic mechanism in Huntington's disease. Hum Mol Genet 17:2738–2751

Wang X, Su B, Siedlak SL, Moreira PI, Fujioka H, Wang Y, Casadesus G, Zhu X (2008b) Amyloid-beta overproduction causes abnormal mitochondrial dynamics via differential modulation of mitochondrial fission/fusion proteins. Proc Natl Acad Sci U S A 105:19318–19323

Wang H, Lim PJ, Karbowski M, Monteiro MJ (2009a) Effects of overexpression of huntingtin proteins on mitochondrial integrity. Hum Mol Genet 18:737–752

Wang X, Su B, Lee HG, Li X, Perry G, Smith MA, Zhu X (2009b) Impaired balance of mitochondrial fission and fusion in Alzheimer's disease. J Neurosci 29:9090–9103

Wang H, Song P, Du L, Tian W, Yue W, Liu M, Li D, Wang B, Zhu Y, Cao C, Zhou J, Chen Q (2011) Parkin ubiquitinates Drp1 for proteasome-dependent degradation: implication of dysregulated mitochondrial dynamics in Parkinson disease. J Biol Chem 286:11649–11658

Wang W, Wang Y, Long J, Wang J, Haudek SB, Overbeek P, Chang BH, Schumacker PT, Danesh FR (2012) Mitochondrial fission triggered by hyperglycemia is mediated by ROCK1 activation in podocytes and endothelial cells. Cell Metab 15:186–200

Ward PS, Thompson CB (2012a) Metabolic reprogramming: a cancer hallmark even warburg did not anticipate. Cancer Cell 21:297–308

Ward PS, Thompson CB (2012b) Signaling in control of cell growth and metabolism. Cold Spring Harb Perspect Biol 4:a006783

Wasiak S, Zunino R, McBride HM (2007) Bax/Bak promote sumoylation of DRP1 and its stable association with mitochondria during apoptotic cell death. J Cell Biol 177:439–450

Waterham HR, Koster J, van Roermund CW, Mooyer PA, Wanders RJ, Leonard JV (2007) A lethal defect of mitochondrial and peroxisomal fission. N Engl J Med 356:1736–1741

White KE, Davies VJ, Hogan VE, Piechota MJ, Nichols PP, Turnbull DM, Votruba M (2009) OPA1 deficiency associated with increased autophagy in retinal ganglion cells in a murine model of dominant optic atrophy. Invest Ophthalmol Vis Sci 50:2567–2571

Wieder SY, Serasinghe MN, Sung JC, Choi DC, Birge MB, Yao JL, Bernstein E, Celebi JT, Chipuk JE (2015) Activation of the mitochondrial fragmentation protein DRP1 correlates with BRAF(V600E) melanoma. J Invest Dermatol 135:2544–2547

Williams PA, Morgan JE, Votruba M (2010) Opa1 deficiency in a mouse model of dominant optic atrophy leads to retinal ganglion cell dendropathy. Brain 133:2942–2951

Williams PA, Morgan JE, Votruba M (2011) Mouse models of dominant optic atrophy: what do they tell us about the pathophysiology of visual loss? Vision Res 51:229–234

Williams PA, Piechota M, von Ruhland C, Taylor E, Morgan JE, Votruba M (2012) Opa1 is essential for retinal ganglion cell synaptic architecture and connectivity. Brain 135:493–505

Xie Q, Wu Q, Horbinski CM, Flavahan WA, Yang K, Zhou W, Dombrowski SM, Huang Z, Fang X, Shi Y, Ferguson AN, Kashatus DF, Bao S, Rich JN (2015) Mitochondrial control by DRP1 in brain tumor initiating cells. Nat Neurosci 18:501–510

Yonashiro R, Ishido S, Kyo S, Fukuda T, Goto E, Matsuki Y, Ohmura-Hoshino M, Sada K, Hotta H, Yamamura H, Inatome R, Yanagi S (2006) A novel mitochondrial ubiquitin ligase plays a critical role in mitochondrial dynamics. EMBO J 25:3618–3626

Yoon Y, Krueger EW, Oswald BJ, McNiven MA (2003) The mitochondrial protein hFis1 regulates mitochondrial fission in mammalian cells through an interaction with the dynamin-like protein DLP1. Mol Cell Biol 23:5409–5420

Yu T, Robotham JL, Yoon Y (2006) Increased production of reactive oxygen species in hyperglycemic conditions requires dynamic change of mitochondrial morphology. Proc Natl Acad Sci U S A 103:2653–2658

Yu T, Jhun BS, Yoon Y (2011) High-glucose stimulation increases reactive oxygen species production through the calcium and mitogen-activated protein kinase-mediated activation of mitochondrial fission. Antioxid Redox Signal 14:425–437

Yue W, Chen Z, Liu H, Yan C, Chen M, Feng D, Yan C, Wu H, Du L, Wang Y, Liu J, Huang X, Xia L, Liu L, Wang X, Jin H, Wang J, Song Z, Hao X, Chen Q (2014) A small natural molecule promotes mitochondrial fusion through inhibition of the deubiquitinase USP30. Cell Res 24: 482–496

Zhang Z, Kageyama Y, Sesaki H (2012) Mitochondrial division prevents neurodegeneration. Autophagy 8:1531–1533

Zhao J, Liu T, Jin S, Wang X, Qu M, Uhlen P, Tomilin N, Shupliakov O, Lendahl U, Nister M (2011) Human MIEF1 recruits Drp1 to mitochondrial outer membranes and promotes mitochondrial fusion rather than fission. EMBO J 30:2762–2778

Zhao J, Zhang J, Yu M, Xie Y, Huang Y, Wolff DW, Abel PW, Tu Y (2013) Mitochondrial dynamics regulates migration and invasion of breast cancer cells. Oncogene 32:4814–4824

Ziviani E, Whitworth AJ (2010) How could Parkin-mediated ubiquitination of mitofusin promote mitophagy? Autophagy 6:660–662

Zuchner S, De Jonghe P, Jordanova A, Claeys KG, Guergueltcheva V, Cherninkova S, Hamilton SR, Van Stavern G, Krajewski KM, Stajich J, Tournev I, Verhoeven K, Langerhorst CT, de Visser M, Baas F, Bird T, Timmerman V, Shy M, Vance JM (2006) Axonal neuropathy with optic atrophy is caused by mutations in mitofusin 2. Ann Neurol 59:276–281

Zunino R, Schauss A, Rippstein P, Andrade-Navarro M, McBride HM (2007) The SUMO protease SENP5 is required to maintain mitochondrial morphology and function. J Cell Sci 120: 1178–1188

Mitochondrial Cholesterol and the Paradox in Cell Death

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H. Singh, S.-S. Sheu (eds.), *Pharmacology of Mitochondria*,
Handbook of Experimental Pharmacology 240, DOI 10.1007/164_2016_110

Abstract

Mitochondria are considered cholesterol-poor organelles, and obtain their cholesterol load by the action of specialized proteins involved in its delivery from extramitochondrial sources and trafficking within mitochondrial membranes. Although mitochondrial cholesterol fulfills vital physiological functions, such as the synthesis of bile acids in the liver or the formation of steroid hormones in specialized tissues, recent evidence indicates that the accumulation of cholesterol in mitochondria may be a key event in prevalent human diseases, in particular in the development of steatohepatitis (SH) and its progression to hepatocellular carcinoma (HCC). Mitochondrial cholesterol accumulation promotes the transition from simple steatosis to SH due to the sensitization to oxidative stress and cell death. However, mitochondrial cholesterol loading in HCC determines apoptosis resistance and insensitivity to chemotherapy. These opposing functions of mitochondrial cholesterol in SH and HCC define its paradoxical role in cell death as a pro- and anti-apoptotic factor. Further understanding of this conundrum may be useful to modulate the progression from SH to HCC by targeting mitochondrial cholesterol trafficking.

Keywords

Cholesterol • Liver cancer • Mitochondria • Steatohepatitis

1 Introduction

Cholesterol is a critical component of biological membranes, which plays a key role in membrane physical properties and in the regulation of signaling pathways. Due to its role in the regulation of membrane fluidity, cholesterol is an important determinant of the relative impermeability of membrane bilayers. Moreover, cholesterol induces membrane packing in specific microdomains of the plasma membrane, providing a platform for a variety of membrane-associated signaling proteins (Maxfield and Tabas 2005). Not all cell bilayers exhibit the same levels of cholesterol in their composition; cholesterol is particularly abundant in plasma membrane. Due to these essential functions of cholesterol, cells satisfy their need for cholesterol from different sources and mechanisms, including its uptake from cholesterol-rich low-density lipoproteins (LDL) or its De novo synthesis from acetyl-CoA in the endoplasmic reticulum (ER) (Ikonen 2008). Once synthesized in the ER cholesterol is distributed to other membranes, including mitochondria (Garcia-Ruiz et al. 2009). Mitochondria are not only the powerhouses of cells providing the energy required for multiple functions but, in addition, play a central role in various forms of cell death, such as apoptosis (caspase-dependent and -independent), necrosis, and autophagy which are characterized by differential biochemical features (Ribas et al. 2014). A key event in this role is the breakage of mitochondrial membranes, particularly the outer mitochondrial membrane, which allows the release of proapoptotic factors that engage the apoptosome leading to apoptotic cell death. Although the overaccumulation of cholesterol in mitochondria decreases mitochondrial membranes' fluidity and impairs their permeabilization, this

effect can be counterbalanced by the oxidation of cardiolipin species, which are in turn regulated by the availability of mitochondrial GSH (mGSH) (Marí et al. 2008). Thus, mitochondrial cholesterol plays a paradoxical role in cell death, which depends on the regulation of mitochondrial GSH (mGSH) transport, mGSH homeostasis, and subsequent protection of cardiolipin from peroxidation. Thus, emerging evidence points to the small pool of mitochondrial cholesterol as a key factor in several human-prevalent diseases, including the transition from steatosis to steatohepatitis (SH) and its progression to hepatocellular carcinoma (HCC), as well as in neurodegeneration. In this chapter we briefly summarize the regulation of cholesterol, particularly focusing on the trafficking of cholesterol to mitochondria and describe the basis for paradoxical role of this event in cell death and implications in disease.

2 Cholesterol Synthesis

Given the role of cholesterol in the regulation of membrane dynamics, physical properties, and signal transduction the synthesis and supply of membrane cholesterol are tightly regulated processes. There are two general mechanisms whereby cells meet their need for cholesterol, namely its De novo synthesis from acetyl-CoA and its supply from the diet (Grundy 1983). Moreover, factors that regulate the endogenous synthetic rate, absorption, or dietary cholesterol load can modulate the relative balance between both pathways in the supply of cholesterol.

2.1 De Novo Cholesterol Synthesis

It has been estimated that about half of the cholesterol in the body arises by its de novo synthesis. Biosynthesis in the liver accounts for approximately 10% of the amount produced each day and occurs mainly in the ER. Synthesis of cholesterol begins from the two-carbon acetate group of acetyl-CoA. Cells synthesize cholesterol De novo from acetyl-CoA in the mevalonate pathway (Fig. 1). The enzyme 3-hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase (HMG-CoAR) catalyzes the reduction of HMG-CoA to mevalonate, the rate-limiting step in the synthesis of cholesterol and non-sterol isoprenoids that are indispensable for cell function (Goldstein and Brown 1990). Mevalonate is then converted to isopentenyl pyrophosphate (IPP), which can be reversibly transformed to dimethylallyl pyrophosphate (DMAPP). IPP and DMAPP combine to form the isoprenoids, geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP), which regulate proteins posttranslationally (Edwards and Ericsson 1999). The synthesis of squalene, from FPP, represents the first cholesterol-specific step in the cholesterol synthesis pathway. The synthesis of squalene is catalyzed by the farnesyl-diphosphate farnesyltransferase 1, also known as squalene synthase (SS), and requires NADPH. Squalene is sequentially converted to lanosterol before being transformed into cholesterol. Inhibition of HMG-CoA reductase by statins prevents the formation of both mevalonate and its downstream product, IPP, and this inhibition can be reversed completely by mevalonate. As one can deduct from this pathway, the therapeutic effects of statins can be cholesterol independent, which may

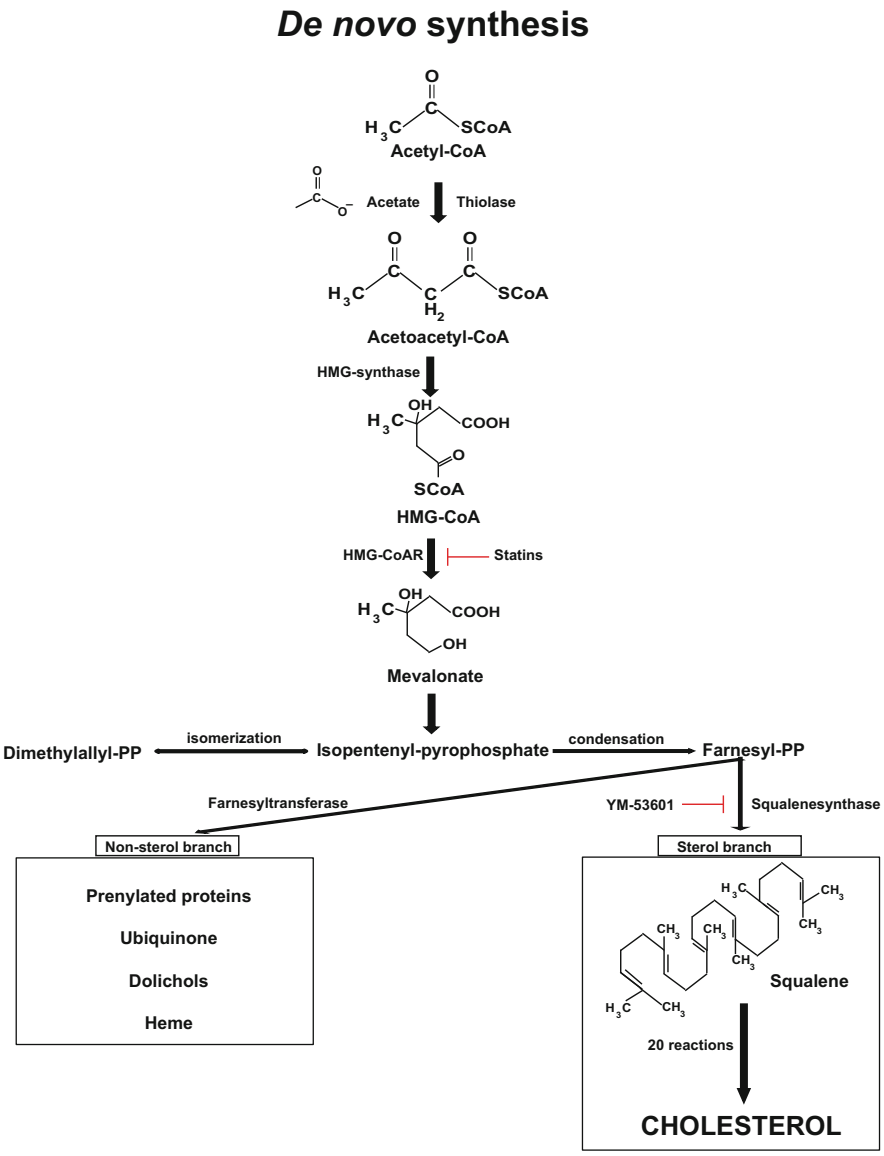

Fig. 1 De novo cholesterol synthesis in the mevalonate pathway. The de novo synthesis of cholesterol proceeds via mevalonate formation from acetyl-CoA. The generation of farnesyl pyrophosphate branches out into the non-sterol arm comprising isoprenoids, which can modify proteins posttranslationally, and the sterol arm in which the formation of farnesyl pyrophosphate into squalene is committed to cholesterol synthesis. Statins block very upstream in the pathway, preventing mevalonate formation from HMG-COA, while the inhibition of squalene synthase may be more specific to prevent cholesterol synthesis without perturbing isoprenoid synthesis

reflect the modification of protein isoprenylation, essential for their final function. Recent studies have shown a paradoxical increase in hepatic cholesterol synthesis rates and the dissociation between plasma cholesterol precursor levels and cholesterol synthesis during statin treatment in mice (Schonewille et al. 2016). In contrast to this caveat with statins, the inhibition of SS can result in selective cholesterol downregulation without exerting major effects on the isoprenylation of proteins (Brusselmans et al. 2007; Joo et al. 2007, 2015; Sun et al. 2007). Normal healthy adults synthesize cholesterol at a rate of approximately 1 g/day and consume approximately 0.3 g/day. A relatively constant level of cholesterol in the blood (150–200 mg/dL) is maintained primarily by controlling the level of De novo synthesis through the regulation of HMG-CoAR. HMG-CoAR is controlled by several mechanisms, including feedback inhibition, control of gene expression, rate of enzyme degradation, and phosphorylation-dephosphorylation. A key mechanism for feedback control of HMG-CoA reductase involves the rapid degradation of HMG-CoAR, which is mediated by ER-residing proteins, Insig-1 and Insig-2 (Sever et al. 2003; Song et al. 2005a, b). Recent studies have designated sterol-accelerated degradation of HMG-CoAR as a potential therapeutic target for prevention of atherosclerosis and associated cardiovascular disease (Hwang et al. 2016).

The rate of synthesis of HMG-CoAR mRNA is controlled by the sterol regulatory element-binding proteins (SREBPs), a family of ER membrane-bound transcription factors, which play an essential role in lipogenesis, and that constitute another mechanism for feedback regulation of HMG-CoAR (Goldstein and Brown 1990). SREBP-2 exhibits preference at controlling the expression of genes involved in cholesterol homeostasis, including all of the genes encoding the sterol biosynthetic enzymes. In addition, SREBP-2 controls expression of the LDL receptor (LDLR) gene. Regulated expression of the SREBPs is complex in that the effects of sterols are different on the SREBP-1 gene versus the SREBP-2 gene. High sterols activate expression of the SREBP-1 gene but do not exert this effect on the SREBP-2 gene.

Translocation of SREBP-2 from the ER to Golgi requires the sterol-responsive escort protein Scap (Rawson et al. 1999). Like HMG-CoAR, Scap contains a hydrophobic N-terminal domain that spans the ER membranes and a C-terminal domain located in the cytosol, which mediates association with SREBPs (Nohturfft et al. 1998; Yang et al. 2002; Yabe et al. 2002; Ikonen 2008). Cholesterol directly binds to the membrane domain of Scap, inducing a conformational change that triggers Insig binding, thus blocking the exit of Scap-SREBP from the ER (Brown et al. 2002; Adams et al. 2004). Unlike cholesterol, lanosterol, the first sterol intermediate in cholesterol synthesis, neither binds Scap nor blocks the proteolytic activation of SREBPs, but induces Insig-dependent ubiquitination and degradation of HMG-CoAR (Song et al. 2005a). In addition, although farnesoid X receptor is known to regulate multiple metabolic processes (Wang et al. 2008), it also controls cholesterol synthesis via induction of hepatic Insig-2 (Hubbert et al. 2007). Finally, another level of regulation of cholesterol synthesis is exerted by the availability of oxygen, as cholesterol synthesis requires oxygen, which is needed in the biotransformation of squalene to cholesterol. Furthermore, hypoxia has been reported to inhibit cholesterol synthesis in rabbit skin fibroblasts, although the efflux of cholesterol was also

suppressed (Mukodani et al. 1990). More recent findings have reported that hypoxia stimulates degradation of HMG-CoAR through both accumulation of lanosterol and HIF-1α-mediated Insig induction (Nguyen et al. 2007).

2.2 *Dietary* Cholesterol

The predominant dietary sterols include cholesterol and β-sitosterol, the major plant sterol. Although β-sitosterol accounts for up to 25% of dietary sterols, this plant sterol is not readily absorbed under physiological conditions. Efficient absorption ensures that dietary fat is available to be used as a source of energy to support various cellular functions or be stored as a reservoir for lipoprotein trafficking, bile acid synthesis, steroidogenesis, membrane formation and maintenance, and epidermal integrity in various mammalian cells. Excess fat is stored in cytosolic lipid droplets until it is needed to support intracellular processes (Ikonen 2008). In addition to the finely tuned pathway of cholesterol synthesis, cells acquire cholesterol from the diet, with the liver playing a key role (Getz and Reardon 2006). The daily intake of cholesterol in humans on a typical Western diet is ~300–500 mg, and the daily secretion of cholesterol into bile is estimated to be ~800–1,200 mg. To attenuate the burden of energy-consuming De novo cholesterol biosynthesis, a physiological process (intestinal cholesterol absorption) has evolved to take up readily available cholesterol from the gut lumen. Cholesterol from the diet is first transported from the gut to the liver, from where it is delivered to other tissues. Enterocytes and hepatocytes package cholesterol and cholesteryl esters into lipoproteins, which are further modified in the circulation. Dietary cholesterol is absorbed by enterocytes, which package it with triglycerides to form chylomicrons. Limited triglyceride hydrolysis occurs in the circulation and new apoproteins (e.g., ApoE) are added to generate chylomicron remnants that are taken up by hepatocytes. Hepatocytes, in turn, secrete lipids in very-low-density lipoprotein (VLDL) particles that are processed in the circulation into LDL, which functions as the main vehicle to deliver cholesterol to peripheral tissues (Ikonen 2008). When cholesterol is in excess in extrahepatic tissues it can be packed into HDL and returned to the liver in a process called reverse cholesterol transport. Liver secretes both unesterified cholesterol and cholesterol-derived bile acids into bile and thus is important for cholesterol disposal. All these pathways in the liver are protein-mediated processes that are metabolically integrated on several levels in response to fluctuations in dietary, systemic, and local cholesterol pools. Cholesterol and bile salts from the intestine are either reabsorbed (enterohepatic cycle) or excreted into feces. Several mechanisms exist for the uptake of cholesterol or its shuttling between organelles, which contribute to the regulation of the levels of membrane cholesterol to avoid its accumulation, as it may have adverse effects in membrane biophysics and function (Trapani et al. 2012). After cholesterol is synthesized in the ER it will be delivered to other organelles by a combination of vesicular and non-vesicular transport processes (Ikonen 2008; Maxfield and Tabas 2005). Of relevance for this chapter, we focus on the trafficking of cholesterol to mitochondria.

3 Cholesterol Trafficking

3.1 Intracellular Distribution

To ensure adequate levels within cell bilayers cholesterol is transferred between different membrane compartments by either vesicular or non-vesicular mechanisms (Fig. 2). Cholesterol membrane trafficking occurs through vesicular and tubular intermediates that transport membrane components between subcellular organelles

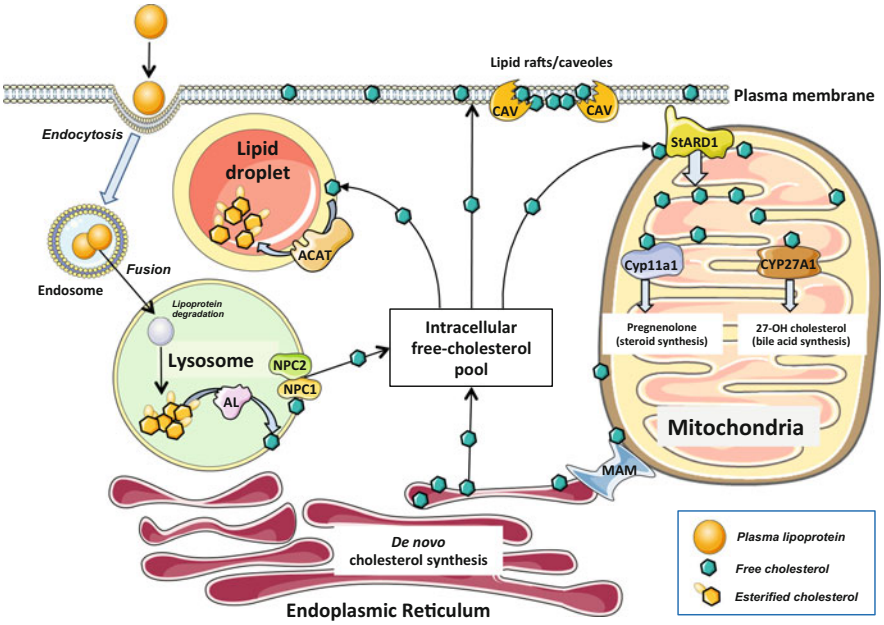

Fig. 2 Intracellular cholesterol distribution and mitochondrial trafficking. De novo cholesterol biosynthesis occurs in the ER from its precursor acetyl-CoA cholesterol and leaves the ER by non-vesicular transport without crossing the Golgi membrane transport. The newly synthesized cholesterol equilibrates with the preexisting cholesterol pool in different cellular membrane compartments. To reduce unesterified sterol levels within the cell, acyl-CoA cholesterol acyl-transferase (ACAT) esterifies cholesterol with fatty acids given the more hydrophobic sterol esters which can be stored in lipid droplets. Unesterified cholesterol is mainly located in the plasma membrane where specific domains (lipid rafts, caveolae) form within this membrane. Caveolin has been shown to be a crucial component of caveolae and play a role in cholesterol trafficking to plasma membrane. Mitochondrial cholesterol transport is preferentially performed by StARD1, which mediates the initial transport of cholesterol from the outer to the inner mitochondrial membrane. In steroidogenic tissues, mitochondrial cholesterol is metabolized by CYP11A1 yielding pregnenolone which is a central precursor of steroids. In other tissues with Cyp27a1 expression, this enzyme converts the mitochondrial cholesterol in 27-hydroxy-cholesterol which is a precursor of the acidic bile acid synthesis pathway. It has been proposed that some START family proteins and the MAM domains may be involved in transferring free cholesterol to the mitochondrial outer membrane

along the cytoskeleton by specific carriers, some of which are cholesterol enriched (Maxfield and Tabas 2005; Ikonen 2008; Soccio and Breslow 2003). Non-vesicular cholesterol transfer occurs by the action of cytosolic lipid transfer proteins.

Once synthesized in the ER from acetyl-CoA cholesterol leaves the ER, mostly by non-vesicular mechanisms that bypass ER-Golgi membrane transport, which accounts for the low ER sterol content (Baumann et al. 2005). The biosynthetic sterols, including cholesterol and a considerable fraction of its precursors are rapidly targeted to the plasma membrane (Lange et al. 1991; Ikonen 2006, 2008). Alternatively, the newly synthesized cholesterol equilibrates with the preexisting cholesterol pool in different sites, including endosomal compartments (Cruz and Chang 2000; Maxfield and Tabas 2005). An important mechanism to reduce unesterified sterol levels in the ER is esterification, which is catalyzed by acyl-CoA cholesterol acyltransferase (ACAT). Fatty acid sterol esters are stored in lipid droplets, which have been considered as passive fat deposits, although current evidence points to these bodies as dynamic regulated organelles (Martin and Parton 2006). At least in adipocytes, considerable amounts of unesterified cholesterol are stored in lipid droplets (Prattes et al. 2000). Cholesterol feeding to cells has been shown to induce the trafficking of plasma membrane caveolin to lipid droplets, where it plays a key role in regulating unesterified cholesterol levels (Le Lay et al. 2006).

As an alternative mechanism to De novo synthesis, cells acquire cholesterol from extracellular lipoproteins, which are taken up by receptor-mediated endocytosis. LDL receptors are present in the plasma membrane of most cells and exhibit affinity for particles that contain ApoB or ApoE proteins, such as chylomicron remnants, VLDL and LDL. These particles are endocytosed by clathrin-coated vesicles and transported to acidic endocytic compartments, where cholesteryl esters are hydrolyzed by acid lipase within lysosomes to provide unesterified cholesterol. The membranes of multivesicular late endosomes are enriched in the phospholipid lysobisphosphatidic acid/bismonoacylglycerophosphate (LBPA/BMP) and function in the regulation of cholesterol transport by controlling the fusion of internal vesicles with late endosomes (Kobayashi et al. 1999). The multivesicular late endosomes harbor two proteins, NPC1 and NPC2, which play a crucial role in cholesterol trafficking out of the endosomal system. Indeed, deficiency of either of these proteins leads to the accumulation of LDL-derived unesterified cholesterol in late endocytic organelles, which is characteristic of the lysosomal disorder Niemann–Pick type C disease (Sturley et al. 2004). Another site in the endocytic pathway for active sterol exchange is the recycling compartment. Recycling endosomes are enriched in sterol- and sphingolipid and function as acceptors for non-vesicular sterol flux through the cytosol (Gagescu et al. 2000). Several Rab proteins, including Rab7, Rab9, and Rab11, exert a key role in the regulation of vesicular dependent intracellular lipid trafficking. Once released from the endolysosomal system, cholesterol is delivered to other membranes, such as the plasma membrane, ER, recycling endosomes, and mitochondria.

3.2 Mitochondrial Cholesterol Trafficking

Mitochondria are cholesterol-poor organelles, with estimates ranging from 0.5 to 3% of the content found in other cellular compartments (van Meer et al. 2008; Ikonen 2008). The regulated transport of cholesterol in mitochondria plays physiological role in the synthesis of bile acids in liver and steroid hormones in hepatic and steroidogenic tissues, respectively (Garcia-Ruiz et al. 2009; Montero et al. 2008). This process involves the initial transport of cholesterol to the inner mitochondrial membrane (IMM) for its metabolism through P450scc (CYP11A1), the rate-limiting step in steroidogenesis. Mitochondrial sterol 27-hydroxylase (CYP27A1) is widely distributed in numerous tissues and plays a critical role in the regulation of cholesterol homeostasis. In non-hepatic (peripheral) tissues, the 27-hydroxylation of cholesterol by CYP27A1 has been proposed to participate in the reverse cholesterol transport to the liver. Within the liver, CYP27A1 initiates an alternative pathway of bile acid biosynthesis, the so-called acidic pathway (Stravitz et al. 1996; Hall et al. 2005). Both in vivo and in vitro studies have shown that this acidic pathway may be responsible for close to 50% of bile acid synthesis.

Given its lipophilic properties and water insolubility, non-vesicular transport by specific carriers stands as the major mechanism of cholesterol transport between organelles. In particular, mitochondrial cholesterol transport is primarily regulated by the steroidogenic acute regulatory domain StARD1, the founding member of a family of lipid-transporting proteins that contain StAR-related lipid transfer (START) domains (Fig. 2) (Miller 2007). StARD1 is an outer mitochondrial membrane (OMM) protein, which was first described and best characterized in steroidogenic cells where it plays an essential role in cholesterol transfer to the IMM for metabolism by cholesterol side-chain cleavage enzyme (CYP11A1) as described above. Pregnenolone synthesis in mitochondria is limited by the availability of cholesterol in the IMM (Clark 2012). Other members of the START family have been shown to have sterol transport activity. MLN64/StARD3 is widely expressed and its transmembrane domain localizes to late endosomes with the StART domain facing the cytosol. While full-length MLN64 is relatively inactive in steroidogenic assays, its proteolysis could release the StART domain, allowing delivery of cholesterol to mitochondria. However, targeted mutation of the MLN64 StART domain has been shown to exert minor alterations in cellular sterol metabolism (Kishida et al. 2004). StARD4 and 5 are structurally related to StARD1, although they lack signal sequences to target them to specific subcellular organelles. Mouse StARD4 but not D5 is regulated by SREBPs, and both exert low levels of StAR-like activity in COS-1 cells co-transfected with the cholesterol side-chain cleavage enzyme system (Miller 2007). Thus, in view of the current evidence it appears that specific members of the StARD family, e.g., StARD3, are primarily responsible for delivering cholesterol to OMM from elsewhere in the cell (endoplasmic reticulum, lipid droplets). Despite similar properties with StARD1, other StART members cannot replace StARD1, as germline StARD1 deficiency is lethal due to adrenocortical lipoid hyperplasia (Caron et al. 1997).

StARD1 activation and regulation are complex and poorly understood. Its activation is regulated at the transcriptional and posttranslational levels. StARD1 phosphorylation

at serine 194 has been shown to enhance the trafficking of cholesterol to IMM in murine steroidogenic cells, resulting in increased steroidogenesis (Arakane et al. 1997; Kil et al. 2012). Moreover, the role of ER stress in the regulation of StART family members has been limited to StARD5 with conflicting results reported for StARD4. However, recent data have provided evidence that ER stress induces the transcriptional upregulation of StARD1 independently of SREBP regulation (Fernandez et al. 2013). High cholesterol feeding caused the repression of SREBP-2-regulated genes, HMG-CoA reductase, but not that of StARD1.

TSPO, a protein particularly abundant in steroidogenic tissues and primarily localized in the OMM, has been suggested to play an important role in steroidogenesis via the transport of cholesterol to the IMM (Papadopoulos and Miller 2012; Miller 2007). However, quite interestingly, recent studies using tissue-specific genetic deletion of TSPO demonstrated that TSPO is dispensable for steroidogenesis in Leydig cells (Morohaku et al. 2014), questioning the relevance of previous findings on TSPO in mitochondrial cholesterol homeostasis using pharmacological ligands and inhibitors. These data suggest that TSPO does not appear to play a significant role in the trafficking of cholesterol to IMM, and highlight the relevance of StARD1 in this process. Intriguingly, the generation of conditional steroidogenic cell-targeted deletion of TSPO indicates a crucial role of this protein in viability and hormone-dependent steroid formation (Fan et al. 2015), thus leaving the possibility that TSPO may contribute to hormone-stimulated trafficking of cholesterol to mitochondria.

Besides StART family members, a role for caveolin-1 (CAV1) in mitochondrial cholesterol has been recently reported. CAV1 is a key component of caveolae, specialized membrane domains particularly enriched in cholesterol and sphingolipids, and CAV is known to bind cholesterol with high affinity (Murata et al. 1995; Pol et al. 2005). Using immunoelectron microscopy analyses, CAV1 has been localized in mitochondria from a variety of epithelial cells, including hepatocytes (Li et al. 2001). In addition, current evidence has shown that CAV resides in the MAM comportment (Sala-Vila et al. 2016). CAV's ability to move between cell compartments, mitochondria-ER and plasma membrane, might contribute to regulation of cholesterol fluxes and distributions within cells (Pol et al. 2001, 2005; Parton and Simons 2007; Bosch et al. 2011). In line with these features, CAV1 deficiency has been shown to increase mitochondrial cholesterol in hepatocytes causing perturbations in mitochondrial membrane dynamics and function and mitochondria GSH depletion (Bosch et al. 2011), which is a direct consequence of cholesterol loading (see below). The mitochondrial dysfunction sensitizes CAV1 null mice to steatohepatitis and neurodegeneration. Whether the trafficking of mitochondrial cholesterol in the absence of caveolin-1 occurs via MAM or StARD1 remains to be further investigated.

4 Mitochondrial Cholesterol in Steatohepatitis: Evidence for a Proapoptotic Role

4.1 Cholesterol Regulates Mitochondrial GSH Transport

As mentioned above, cholesterol is known to regulate membrane organization. In particular cholesterol modulates the coexistence within membranes of lipid-disordered and lipid-ordered phases, which regulates membrane permeability and function of resident proteins. In this regard, it has been shown that cholesterol loading in mitochondria results in increased membrane order parameter, which impacts negatively specific membrane carriers, such as the GSH transport system without effect in others, including the S-adenosyl-L-methionine transport system or the adenine nucleotide translocator (Fernández et al. 2009; Colell et al. 2003). Since GSH is synthesized De novo exclusively in the cytosol but not in mitochondria, the mitochondrial source of GSH depends on its transport from cytosol by the 2-oxoglutarate and dicarboxylate carriers, which have been previously characterized (Garcia-Ruiz et al. 2009). Functional expression studies in *Xenopus laevis* oocytes provided clear evidence that the 2-oxoglutarate carrier is highly sensitive to mitochondrial membrane fluidity loss induced by cholesterol loading (Coll et al. 2003). Coupled with previous findings showing low- and high-affinity components for the mitochondrial transport of GSH into rat liver mitochondria, the 2-oxoglutarate carrier could account for the low-affinity transport site with the dicarboxylate playing a minor role. Thus, one of the functional consequences of mitochondrial cholesterol enrichment is the impairment of mGSH transport, which results in its depletion in the mitochondrial matrix, promoting the stimulation of mitochondrial ROS induced by different stimuli, including death ligands (e.g., TNF), hypoxia, or ischemia/reperfusion. Replenishment of mGSH may thus stand as a promising approach for the treatment of liver diseases. However, the use of GSH precursors such as N-acetylcysteine (NAC) may be limited, given that the cytosolic GSH generated from NAC would not be effectively transported into the mitochondrial matrix via the 2-oxoglutarate carrier as its activity is dependent on mitochondrial membrane properties. To overcome this limitation, the use of GSH precursor that freely diffuses through membranes would be more effective, such as GSH ethyl ester (GSH-EE). The protective role of GSH-EE against oxidative stress has been recently illustrated in steatohepatitis (von Montfort et al. 2012).

4.2 Mitochondrial Cholesterol-Mediated mGSH Depletion in Steatohepatitis

The role of cholesterol in steatohepatitis has attracted increasing attention in recent years and it is currently considered a key player in both alcoholic (ASH) and non-alcoholic steatohepatitis (NASH) (Garcia-Ruiz et al. 2009; Marí et al. 2010). While the pathogenesis of these diseases is different they exhibit indistinguishable histological features and common underlying mechanisms that begin with hepatic steatosis. Although fat infiltration in these diseases is heterogeneous consisting

primarily of triglyceride, free fatty acids, and cholesterol, recent studies have pointed to an emerging role for cholesterol, particularly in mitochondria, in the progression from steatosis to steatohepatitis as it sensitizes hepatocytes to inflammatory cytokines (e.g., TNF/Fas ligand) and stimulates JNK1 recruitment to mitochondria (Gan et al. 2014).

The trafficking of cholesterol to mitochondrial membranes sensitizes hepatocytes to TNF-mediated steatohepatitis and cell death by promoting mGSH depletion (Marí et al. 2006). Cholesterol accumulation in the ER or the plasma membrane did not cause ER stress or alter TNF signaling. Rather, we observed that the trafficking of cholesterol to mitochondria accounted for the hepatocellular susceptibility to TNF due to mGSH depletion (Fig. 3). In addition, hepatocytes from NPC1 knockout mice exhibit accumulation of cholesterol into mitochondria resulting in mGSH depletion and susceptibility to TNF (Marí et al. 2006). Boosting the pool of mGSH or preventing its depletion by blocking cholesterol synthesis with atorvastatin, the susceptibility of obese ob/ob mice to LPS-mediated liver injury was blunted, highlighting the relevance of mitochondrial mediated oxidative stress in the susceptibility to TNF-induced liver injury. Importantly, it has been reported that alcohol consumption depletes mGSH selectively (Colell et al. 2001; Fernandez-Checa and Kaplowitz 2005; García-Ruiz et al. 1994, 1995; Hirano et al. 1992), and this effect is rapidly reversed upon alcohol withdrawal (Zhao and Slattery 2002; Zhao et al. 2002). Furthermore, recent findings in rats fed with an ethanol-polyunsaturated fatty acid diet confirmed the depletion of mGSH due to mitochondrial cholesterol loading, effects that were prevented by betaine treatment (Varatharajalu et al. 2014).

Alcohol feeding caused mitochondrial cholesterol accumulation by stimulating the expression of the mitochondrial cholesterol carrier StARD1 (Fernandez et al. 2013). Emerging evidence has demonstrated that StARD1 is a previously unrecognized target of the UPR and ER stress signaling, as indicated above. Indeed, tunicamycin, an ER stress trigger, induces the expression of StARD1 in isolated hepatocytes and this effect is prevented by TUDCA treatment (Fernandez et al. 2013). Moreover, mice fed a high-cholesterol diet (HC) exhibited increased expression of StARD1. However, HC feeding downregulates the expression of SREBP-2-regulated target genes, including hydroxymethylglutaryl Co-A reductase, demonstrating that StARD1 is an ER stress but not SREBP-2-regulated gene. As the UPR comprises three transducers, namely inositol requiring (IRE) 1α, PKR-like ER kinase (PERK), and activating transcription factor (ATF) 6α, which are controlled by the master regulator glucose-regulated protein 78 (GRP78 also known as BiP), further work is needed to examine the relative contribution of IRE-1α, PERK, and ATF6α arms in the regulation of StARD1. Besides ER stress, StARD1 activation is regulated at the transcriptional and posttranslational levels. In murine steroidogenic cells StARD1 activity and subsequent steroidogenesis increase upon StARD1 phosphorylation at serine residues (Arakane et al. 1997; Manna et al. 2011). Whether or not StARD1 phosphorylation by alcohol regulates mitochondrial cholesterol homeostasis remains to be explored. If so, then the identification of putative kinases that phosphorylate and activate StARD1 may be of potential relevance in ASH.

Despite its location in endosomes, MLN64 regulates the trafficking of cholesterol to mitochondria (Alpy et al. 2001). For instance, adenovirus-mediated overexpression of

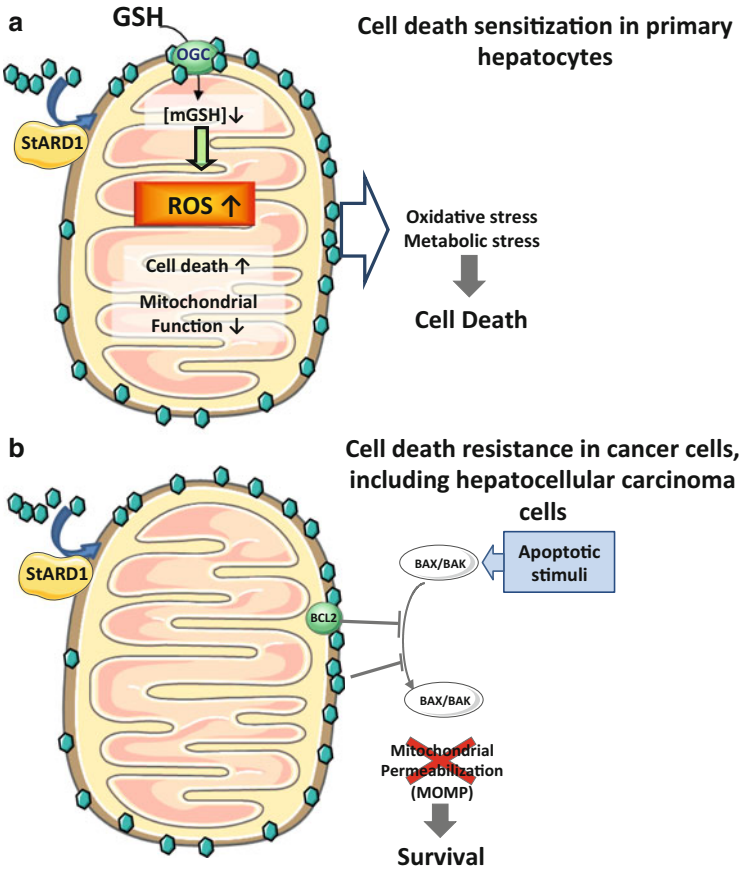

Fig. 3 Dual role of mitochondrial cholesterol in cell death. (**a**) In normal cells mGSH levels can cope with the ROS generated in physiological conditions; however, in cells with cholesterol-enriched mitochondria, mGSH import through the OGC is impaired resulting in mGSH depletion which compromises ROS detoxification, leading to oxidative stress and resulting ultimately in higher susceptibility to cell death, as has been shown in several pathologies such as steatohepatitis. (**b**) Paradoxically, cancer cells, through the overexpression of anti-apoptotic proteins or inactivation of proapoptotic proteins, counteract the action of BAX/BAK and evade MOMP formation. Besides this effect, mitochondrial cholesterol loading shields mitochondrial membrane impairing BAK/BAX oligomerization in MOM and subsequent MOMP and therefore it represents an additional mechanism of cell death resistance in tumor cells

MLN64 induces an increase in hepatic free cholesterol that is associated with apoptosis and liver damage (Tichauer et al. 2007). Additionally, MLN64 overexpression causes an increase in mitochondrial cholesterol content in the liver and correlates with decreased ATPase activity and decreased maximal respiration rates (Arguello et al. 2015). Knockdown of MLN64 by siRNA reduced cholesterol transport of the mitochondrial inner membrane in both wild-type and NPC1-deficient cells, demonstrating a significant role for MLN64 in the mitochondrial delivery of cholesterol (Charman et al.

2010). These data suggest that MLN64 plays a role in the response of hepatic cells to different insults. By contrast, recent studies in rats have shown that loss of hepatic expression of MLN64 induced by genetic obesity may contribute to the pathogenesis of dyslipidemia and steatosis (Soffientini et al. 2014). MLN64 overexpression increased lipidation of exogenous apoA-I and facilitated the De novo biosynthetic pathways for neutral lipids in hepatic cell lines. These effects potentiated TG accumulation but possibly offered protection against lipotoxicity. Thus, it is conceivable that MLN64 may increase circulating levels of HDL and protect the liver against lipotoxicity. Furthermore, JNK1 activation has been found as a relevant consequence of mitochondrial FC deposition in experimental models. Indeed, it has been demonstrated that JNK1 drives mitochondrial cell death pathways leading to release of high-mobility-group box 1 (HMGB1) protein, which in turn activates TLR4 in neighboring hepatocytes, thus promoting hepatocellular injury (Gan et al. 2014). This work is in agreement with recent findings examining the role of HMGB1 and TLR4 in the pathogenesis of NASH (Li et al. 2011). Thus, JNK1 and TLR4 seem to be relevant pathways in free cholesterol lipotoxicity that may be eventually amenable of modulation with specific inhibitors (Gan et al. 2014). In conclusion, the increase in mitochondrial cholesterol content contributes to mitochondrial dysfunction, sensitizes hepatocytes to cytokine signaling, and leads to mGSH depletion contributing to hepatocyte cell death by apoptosis and necrosis (Gan et al. 2014; Hirsova and Gores 2015). Altered expression of mitochondrial cholesterol transporters, which may contribute to the increase in mitochondrial cholesterol levels and JNK1 activation, could be potential therapeutic targets in NAFLD.

5 Mitochondrial Cholesterol in Cancer: Evidence for an Anti-Apoptotic Role

5.1 Mitochondrial Cholesterol Accumulation in Cancer Cells

Cancer cells exhibit critical metabolic transformations induced by mutations leading to gain of function of oncogenes and loss of function of tumor-suppressor genes that result in altered cell metabolism, deregulated growth, resistance to cell death, invasion, and metastasis (Hanahan and Weinberg 2011). Some of these features are governed by changes in mitochondrial function and may be modified by cholesterol metabolism and mitochondrial trafficking.

Experimental evidence indicates that high cell proliferation (Bensinger et al. 2008; Lo Sasso et al. 2010) and tumor growth (Clendening et al. 2010; Dang 2012) are closely associated with enhanced cholesterol requirement. Cholesterol synthesis and regulation are altered at several levels in cancer cells to meet the unrestricted growth needs (Nguyen et al. 2007; Garcia-Ruiz et al. 2009; Guo et al. 2011; Casey et al. 2015). Indeed, tumor cells exhibit increased cholesterol levels compared to surrounding cells; moreover, cancer tissues display increased upregulation of HMGCoAR, loss of feedback inhibition, decreased expression of cholesterol exporter ATP-binding cassette transporter A1 (ABCA1), and increased extracellular cholesterol uptake via LDL

receptor (Casey et al. 2015). Moreover, analyses of the Cancer Genome Atlas (TCGA) database indicated the correlation between increased activity of the cholesterol synthesis pathway and decreased survival in patients with sarcoma, acute myeloid leukemia, and melanoma (Cancer Genome Atlas Research Network et al. 2013; Kuzu et al. 2016), indicating that increased cellular cholesterol promotes carcinogenesis.

Besides these findings of cholesterol deregulation in cancer, there is evidence of increased mitochondrial cholesterol trafficking and levels in solid tumors. For instance, mitochondrial cholesterol levels of tumors from Buffalo rats bearing transplanted Morris hepatomas are two- to fivefold higher than the content found in mitochondria prepared from host liver, and correlated with the degree of tumor growth and malignancy (Crain et al. 1983; Feo et al. 1975). Furthermore, analyses of the TCGA database further support a role for StARD1 and MLN64 and subsequent mitochondrial cholesterol enrichment in cancer development (Cancer Genome Atlas Research Network et al. 2013). Linked to decreased release of cellular cholesterol, low ABCA1 activity has been reported in colorectal cancer cells either through loss of function or gene downregulation and ABCA1 downregulation promoted cancer cell survival by increased mitochondrial cholesterol accumulation (Smith and Land 2012). Thus, these findings indicate that the trafficking and accumulation of cholesterol in mitochondria is a characteristic feature of many types of cancer. The stimulation of mitochondrial cholesterol trafficking may represent a key event in carcinogenesis underlying some of typical biological effects of solid tumors, such as the aerobic glycolysis and resistance to cell death and chemotherapy.

5.2 Mitochondrial Cholesterol and Cell Death Resistance in Cancer Cells

Given the key role of mitochondria in many vital cell functions, bioenergetics, and cell death regulation, the fact that cancer cells accumulate mitochondrial cholesterol may have a significant impact on cancer cell biology and cell death resistance (Montero et al. 2008; Lucken-Ardjomande et al. 2008). In particular, mitochondrial cholesterol loading in cancer cells may account for the resistance to BAX-mediated cell death induced by chemotherapeutic agents that target mitochondria to elicit mitochondrial outer membrane permeabilization (MOMP) (Fig. 3). In line with this, treatments that resulted in mitochondrial cholesterol loading in tumor cells impaired stress-induced apoptosis, while StARD1 knockdown or treatments that resulted in downregulation of cholesterol loading sensitized HCC cells to chemotherapy (Montero et al. 2008). Isolated mitochondria from HCC with increased cholesterol levels have been reported to be resistant to MOMP and release of cytochrome c or smac/DIABLO in response to various stimuli, such as MPT triggers and active BAX. In agreement with these findings, HeLa cells treated with the amphiphilic amine U18666, which perturbs intracellular cholesterol trafficking and stimulates mitochondrial cholesterol accumulation, impair MOMP and the release of cytochrome c in response to BAX (Lucken-Ardjomande et al. 2008). Furthermore, ABCA1 downregulation determines resistance to chemotherapy through increased mitochondrial cholesterol accumulation (Smith and Land 2012). Similar

behavior was observed in cholesterol-enriched mitochondria or liposomes and reversed by restoring mitochondrial membrane order or cholesterol extraction. Cholesterol inhibited the membrane-permeabilizing activity of tBID/BAX or BAX pre-oligomerized with octylglucoside in a dose-dependent manner. Similar to the effect found on BAX, cholesterol also decreased the permeabilizing activity of melittin, a widely studied antimicrobial peptide, which induces membrane permeabilization by forming lipid-containing toroidal pores rather than through the formation of protein channels (Montero et al. 2008). These findings indicate that cholesterol-mediated decrease in membrane fluidity of the bilayer directly modulates BAX proapoptotic activity by reducing the capacity of BAX to insert into the lipid matrix of the membrane, underlying the anti-apoptotic role of mitochondrial cholesterol accumulation in cancer cells. Thus, mitochondrial cholesterol contributes to chemotherapy resistance in HCC by increasing membrane order and resistance of OMM to its permeabilization. As StARD1 regulates mitochondrial cholesterol trafficking, it is conceivable that this member of the StART family stands as a novel target to regulate cancer cell death and chemotherapy response. As cholesterol regulates mGSH transport, it is likely that the accumulation of cholesterol in mitochondria from cancer cells may negatively impact the mGSH levels, as described in steatohepatitis. This is a critical question that remains to be established as mGSH depletion has been shown to sensitize cancer cell to hypoxia (Lluis et al. 2007), implying that mGSH regulation may be an important strategic target for cancer cell biology. As peroxidized cardiolipin regulates MOMP through the restructuring of the lipid bilayer (Marí et al. 2008), it would be interesting to examine the cardiolipin species in HCC and if these species correlate with mGSH levels. In addition to GSH, cancer cells rely on the control of the redox environment to sustain anabolic processes for synthesis of lipids, proteins, and DNA to maintain uncompromised cell growth (Castaldo et al. 2016).

6 Concluding Remarks

Although mitochondria exhibit a limited amount of cholesterol in their membrane bilayers, particularly in the inner membrane, the trafficking of cholesterol to this bilayer fulfills an important physiological role following its metabolism by cytochrome c P540 isoenzymes located in the mitochondrial matrix. The accumulation of cholesterol in mitochondria perturbs critical mitochondrial functions due to changes in mitochondrial membrane dynamics. In particular, this event results in the perturbations in specific carrier proteins located in the mitochondrial inner membrane, such as the mitochondrial GSH carrier, resulting in mGSH depletion. While this process has been shown to contribute to the progression and transition from steatosis to SH, HCC cells also exhibit a significant load of mitochondrial cholesterol, which in this case contributes to cell death resistance and chemotherapy refractoriness due to the protection of MOM against permeabilization by Bax and oxidative stress elicited by hypoxia. This scenario defines a paradoxical and antagonistic role of mitochondrial cholesterol in SH and HCC, which is of advantage to ensure disease progression. The underlying mechanism whereby mitochondrial cholesterol accumulation in HCC protects against cell death, as opposed to what

has been described in SH, remains to be fully understood. Whether this event is related to the differential regulation of mitochondrial GSH transport and status of cardiolipin peroxidation is currently under investigation. Despite this limitation, the current evidence suggests that targeting the trafficking of cholesterol to mitochondria may be useful not only in attenuating SH progression but also in sensitizing HCC to chemotherapy and hypoxia. Although the availability of pharmacological inhibitors of StARD1 could be useful in SH and HCC, unfortunately there has not been much progress in the identification of specific StARD1 inhibitors. In this regard, recent findings modeling the START domain of StARD1 have allowed the identification of small-molecule inhibitors of steroid formation based on cAMP-induced, but not 22R-hydroxycholesterol-supported, inhibition of steroid synthesis by 50% (Akula et al. 2012), which may serve as the foundation to perform screening assays to identify specific inhibitors of StARD1 in the near future.

Acknowledgments The work was supported by CIBEREHD, Fundacio la Marato TV3, SGR2014-785, and Grants SAF2014-57674-R (MINECO/FEDER) and SAF2015-69944-R (MINECO/FEDER) from the Ministerio de Economía y Competitividad and Fondo Europeo de Desarrollo Regional (FEDER), and the center grant P50-AA-11999 (Research Center for Liver and Pancreatic Diseases, NIAAA/NIH).

References

Adams CM, Reitz J, De Brabander JK, Feramisco JD, Li L, Brown MS, Goldstein JL (2004) Cholesterol and 25- hydroxycholesterol inhibit activation of SREBPs by different mechanisms, both involving SCAP and Insigs. J Biol Chem 279:52772–52780

Akula N, Midzak A, Lecanu L, Papadopoulos V (2012) Identification of small-molecule inhibitors of the steroidogenic acute regulatory protein (STARD1) by structure-based design. Bioorg Med Chem Lett 22:4139–4143

Alpy F, Stoeckel ME, Dierich A, Escola JM, Wendling C, Chenard MP, Vanier MT, Gruenberg J, Tomasetto C, Rio MC (2001) The steroidogenic acute regulatory protein homolog MLN64, a late endosomal cholesterol-binding protein. J Biol Chem 276:4261–4269

Arakane F, King SR, Du Y, Kallen CB, Walsh LP, Watari H, Stocco DM, Strauss JF (1997) Phosphorylation of steroidogenic acute regulatory protein (StAR) modulates its steroidogenic activity. J Biol Chem 272:32656–32662

Arguello G, Balboa E, Arrese M, Zanlungo S (2015) Recent insights on the role of cholesterol in non-alcoholic fatty liver disease. Biochim Biophys Acta 1852:1765–1778

Baumann NA, Sullivan DP, Ohvo-Rekila H, Simonot C, Pottekat A, Klaassen Z, Beh CT, Menon AK (2005) Transport of newly synthesized sterol to the sterol-enriched plasma membrane occurs via nonvesicular equilibration. Biochemistry 44(15):5816–5826

Bensinger SJ, Bradley MN, Joseph SB, Zelcer N, Janssen EM, Hausner MA, Shih R, Parks JS, Edwards PA, Jamieson BD, Tontonoz P (2008) LXR signaling couples sterol metabolism to proliferation in the acquired immune response. Cell 134(1):97–111

Bosch M, Mari M, Herms A, Fernandez A, Fajardo A, Kassan A, Giralt A, Colell A, Balgoma D, Barbero E, Gonzalez-Moreno E, Matias N, Tebar F, Balsinde J, Camps M, Enrich C, Gross SP, Garcia-Ruiz C, Perez-Navarro E, Fernandez-Checa JC, Pol A (2011) Caveolin-1 deficiency causes cholesterol-dependent mitochondrial dysfunction and apoptotic susceptibility. Curr Biol 21(8):681–686

Brown AJ, Sun L, Feramisco JD, Brown MS, Goldstein JL (2002) Cholesterol addition to ER membranes alters conformation of SCAP, the SREBP escort protein that regulates cholesterol metabolism. Mol Cell 10:237–245

Brusselmans K, Timmermans L, Sande TV, Veldhove PP, Guan G, Shechter I, Claessens F, Verhoven G, Swinnen JV (2007) Squalene synthase, a determinant of raft-associated cholesterol and modulator of cancer cell proliferation. J Biol Chem 282:18777–18785

Cancer Genome Atlas Research Network, Weinstein JN, Collisson EA, Mills GB, Shaw KR, Ozenberger BA, Ellrott K, Shmulevich I, Sander C, Stuart JM (2013) The Cancer Genome Atlas Pan-Cancer analysis project. Nat Genet 45(10):1113–1120

Caron KM, Soo SC, Wetsel WC, Stocco DM, Clark BJ, Parker KL (1997) Targeted disruption of the mouse gene encoding steroidogenic acute regulatory protein provides insights into congenital lipoid adrenal hyperplasia. Proc Natl Acad Sci U S A 94(21):11540–11545

Casey SC, Amedei A, Aquilano K, Azmi AS, Benencia F, Bhakta D, Bilsland AE, Boosani CS, Chen S, Ciriolo MR, Crawford S, Fujii H, Georgakilas AG, Guha G, Halicka D, Helferich WG, Heneberg P, Honoki K, Keith WN, Kerkar SP, Mohammed SI, Niccolai E, Nowsheen S, Vasantha Rupasinghe HP, Samadi A, Singh N, Talib WH, Venkateswaran V, Whelan RL, Yang X, Felsher DW (2015) Cancer prevention and therapy through the modulation of the tumor microenvironment. Semin Cancer Biol 35(Suppl):S199–S223

Castaldo SA, Freitas JR, Conchinha NV, Madureira PA (2016) The tumorigenic roles of the cellular REDOX regulatory systems. Oxid Med Cell Longev 2016:8413032

Charman M, Kennedy BE, Osborne N, Karten B (2010) MLN64 mediates egress of cholesterol from endosomes to mitochondria in the absence of functional Niemann-Pick Type C1 protein. J Lipid Res 51:1023–1034

Clark BJ (2012) The mammalian START domain protein family in lipid transport in health and disease. J Endocrinol 212(3):257–275

Clendening JW, Pandyra A, Boutros PC, El Ghamrasni S, Khosravi F, Trentin GA, Martirosyan A, Hakem A, Hakem R, Jurisica I, Penn LZ (2010) Dysregulation of the mevalonate pathway promotes transformation. Proc Natl Acad Sci U S A 107(34):15051–15056

Colell A, Coll O, García-Ruiz C, París R, Tiribelli C, Kaplowitz N, Fernández-Checa JC (2001) Tauroursodeoxycholic acid protects hepatocytes from ethanol-fed rats against tumor necrosis factor-induced cell death by replenishing mitochondrial glutathione. Hepatology 34:964–971

Colell A, García-Ruiz C, Lluis JM, Coll O, Mari M, Fernández-Checa JC (2003) Cholesterol impairs the adenine nucleotide translocator-mediated mitochondrial permeability transition through altered membrane fluidity. J Biol Chem 278:33928–33935

Coll O, Colell A, García-Ruiz C, Kaplowitz N, Fernández-Checa JC (2003) Sensitivity of the 2-oxoglutarate carrier to alcohol intake contributes to mitochondrial glutathione depletion. Hepatology 38(3):692–702

Crain RC, Clark RW, Harvey BE (1983) Role of lipid transfer proteins in the abnormal lipid content of Morris hepatoma mitochondria and microsomes. Cancer Res 43(7):3197–3202

Cruz JC, Chang TY (2000) Fate of endogenously synthesized cholesterol in Niemann-Pick type C1 cells. J Biol Chem 275(52):41309–41316

Dang CV (2012) Links between metabolism and cancer. Genes Dev 26(9):877–890

Edwards PA, Ericsson J (1999) Sterols and isoprenoids: signaling molecules derived from the cholesterol biosynthetic pathway. Annu Rev Biochem 68:157–185

Fan J, Campioli E, Midzak A, Culty M, Papadopoulos V (2015) Conditional steroidogenic cell-targeted deletion of TSPO unveils a crucial role in viability and hormone-dependent steroid formation. Proc Natl Acad Sci U S A 112(23):7261–7266

Feo F, Canuto RA, Garcea R, Gabriel L (1975) Effect of cholesterol content on some physical and functional properties of mitochondria isolated from adult rat liver, fetal liver, cholesterol-enriched liver and hepatomas AH-130, 3924A and 5123. Biochim Biophys Acta 413(1):116–134

Fernández A, Colell A, Caballero F, Matías N, García-Ruiz C, Fernández-Checa JC (2009) Mitochondrial S-adenosyl-L-methionine transport is insensitive to alcohol-mediated changes in membrane dynamics. Alcohol Clin Exp Res 33:1169–1180

Fernandez A, Matias N, Fucho R, Ribas V, Von Montfort C, Nuño N, Baulies A, Martinez L, Tarrats N, Mari M et al (2013) ASMase is required for chronic alcohol induced hepatic endoplasmic reticulum stress and mitochondrial cholesterol loading. J Hepatol 59:805–813

Fernandez-Checa JC, Kaplowitz N (2005) Hepatic mitochondrial glutathione: transport and role in disease and toxicity. Toxicol Appl Pharmacol 204:263–273

Gagescu R, Demaurex N, Parton RG, Hunziker W, Huber LA, Gruenberg J (2000) The recycling endosome of Madin-Darby canine kidney cells is a mildly acidic compartment rich in raft components. Mol Biol Cell 11(8):2775–2791

Gan LT, Van Rooyen DM, Koina ME, McCuskey RS, Teoh NC, Farrell GC (2014) Hepatocyte free cholesterol lipotoxicity results from JNK1-mediated mitochondrial injury and is HMGB1 and TLR4-dependent. J Hepatol 61:1376–1384

García-Ruiz C, Morales A, Ballesta A, Rodés J, Kaplowitz N, Fernández-Checa JC (1994) Effect of chronic ethanol feeding on glutathione and functional integrity of mitochondria in periportal and perivenous rat hepatocytes. J Clin Invest 94:193–201

García-Ruiz C, Morales A, Colell A, Ballesta A, Rodés J, Kaplowitz N, Fernández-Checa JC (1995) Feeding S-adenosyl-L-methionine attenuates both ethanol-induced depletion of mitochondrial glutathione and mitochondrial dysfunction in periportal and perivenous rat hepatocytes. Hepatology 21:207–214

Garcia-Ruiz C, Mari M, Colell A, Morales A, Caballero F, Montero J, Terrones O, Basañez G, Fernández-Checa JC (2009) Mitochondrial cholesterol in health and disease. Histol Histopathol 24:117–132

Getz GS, Reardon CA (2006) Diet and murine atherosclerosis. Arterioscler Thromb Vasc Biol 26:242–249

Goldstein JL, Brown MS (1990) Regulation of the mevalonate pathway. Nature 343:425–430

Grundy SM (1983) Absorption and metabolism of dietary cholesterol. Annu Rev Nutr 3:71–96

Guo D, Reinitz F, Youssef M, Hong C, Nathanson D, Akhavan D, Kuga D, Amzajerdi AN, Soto H, Zhu S, Babic I, Tanaka K, Dang J, Iwanami A, Gini B, Dejesus J, Lisiero DD, Huang TT, Prins RM, Wen PY, Robins HI, Prados MD, Deangelis LM, Mellinghoff IK, Mehta MP, James CD, Chakravarti A, Cloughesy TF, Tontonoz P, Mischel PS (2011) An LXR agonist promotes glioblastoma cell death through inhibition of an EGFR/AKT/SREBP-1/LDLR-dependent pathway. Cancer Discov 1(5):442–456

Hall EA, Ren S, Hylemon PB, Redford K, del Castillo A, Gil G, Pandak WM (2005) Mitochondrial cholesterol transport: a possible target in the management of hyperlipidemia. Lipids 40(12):1237–1244

Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144(5):646–674

Hirano T, Kaplowitz N, Tsukamoto H, Kamimura S, Fernandez-Checa JC (1992) Hepatic mitochondrial glutathione depletion and progression of experimental alcoholic liver disease in rats. Hepatology 16:1423–1427

Hirsova P, Gores GJ (2015) Death receptor-mediated cell death and proinflammatory signaling in nonalcoholic steatohepatitis. Cell Mol Gastroenterol Hepatol 1:17–27

Hubbert ML, Zhang Y, Lee FY, Edwards PA (2007) Regulation of hepatic Insig-2 by the farnesoid X-receptor. Mol Endocrinol 21:1359–1369

Hwang S, Hartman IZ, Calhoun LN, Garland K, Young GA, Mitsche MA, McDonald J, Xu F, Engelking L, DeBose-Boyd RA (2016) Contribution of accelerated degradation to feedback regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase and cholesterol metabolism in the liver. J Biol Chem 291:13479–13494

Ikonen E (2006) Mechanisms for cellular cholesterol transport: defects and human disease. Physiol Rev 86(4):1237–1261

Ikonen E (2008) Cellular cholesterol trafficking and compartmentalization. Nat Rev Mol Cell Biol 9:125–138

Joo J, Liao G, Collins JB, Grisson SF, Jetten AM (2007) Farnesol-induced apoptosis in human lung carcinoma cells is coupled to the endoplasmic reticulum stress response. Cancer Res 67:7929–7936

Joo J, Ueda E, Bortner C, Yang X, Liao G, Jetten AM (2015) Farnesol activates the intrinsic pathway of apoptosis and the ATF4-ATF3-CHOP cascade of ER stress in human T lymphoblastic leukemia Molt4 cells. Biochem Pharmacol 97:256–268

Kil IS, Lee SK, Ryu KW, Woo HA, Hu MC, Bae SH, Rhee SG (2012) Feedback control of adrenal steroidogenesis via H2O2-dependent, reversible inactivation of peroxiredoxin III in mitochondria. Mol Cell 46(5):584–594

Kishida T, Kostetskii I, Zhang Z, Martinez F, Liu P, Walkley SU, Dwyer NK, Blanchette-Mackie EJ, Radice GL, Strauss JF 3rd (2004) Targeted mutation of the MLN64 START domain causes only modest alterations in cellular sterol metabolism. J Biol Chem 279(18):19276–19285

Kobayashi T, Beuchat MH, Lindsay M, Frias S, Palmiter RD, Sakuraba H, Parton RG, Gruenberg J (1999) Late endosomal membranes rich in lysobisphosphatidic acid regulate cholesterol transport. Nat Cell Biol 1(2):113–118

Kuzu OF, Noory MA, Robertson GP (2016) The role of cholesterol in cancer. Cancer Res 76 (8):2063–2070

Lange Y, Echevarria F, Steck TL (1991) Movement of zymosterol, a precursor of cholesterol, among three membranes in human fibroblasts. J Biol Chem 266(32):21439–21443

Le Lay S, Hajduch E, Lindsay MR, Le Liepvre X, Thiele C, Ferre P, Parton RG, Kurzchalia T, Simons K, Dugail I (2006) Cholesterol-induced caveolin targeting to lipid droplets in adipocytes: a role for caveolar endocytosis. Traffic 7(5):549–561

Li WP, Liu P, Pilcher BK, Anderson RG (2001) Cell-specific targeting of caveolin-1 to caveolae, secretory vesicles, cytoplasm or mitochondria. J Cell Sci 114(Pt 7):1397–1408

Li L, Chen L, Hu L, Liu Y, Sun H-Y, Tang J, Hou Y-J, Chang Y-X, Tu Q-Q, Feng G-S et al (2011) Nuclear factor high-mobility group box1 mediating the activation of Toll-like receptor 4 signaling in hepatocytes in the early stage of nonalcoholic fatty liver disease in mice. Hepatology 54:1620–1630

Lluis JM, Buricchi F, Chiarugi P, Morales A, Fernandez-Checa JC (2007) Dual role of mitochondrial reactive oxygen species in hypoxia signaling: activation of nuclear factor-{kappa}B via c-SRC and oxidant-dependent cell death. Cancer Res 67(15):7368–7377

Lo Sasso G, Celli N, Caboni M, Murzilli S, Salvatore L, Morgano A, Vacca M, Pagliani T, Parini P, Moschetta A (2010) Down-regulation of the LXR transcriptome provides the requisite cholesterol levels to proliferating hepatocytes. Hepatology 51(4):1334–1344

Lucken-Ardjomande S, Montessuit S, Martinou JC (2008) Bax activation and stress-induced apoptosis delayed by the accumulation of cholesterol in mitochondrial membranes. Cell Death Differ 15(3):484–493

Manna PR, Soh J-W, Stocco DM (2011) The involvement of specific PKC isoenzymes in phorbol ester-mediated regulation of steroidogenic acute regulatory protein expression and steroid synthesis in mouse Leydig cells. Endocrinology 152:313–325

Marí M, Caballero F, Colell A, Morales A, Caballeria J, Fernandez A, Enrich C, Fernandez-Checa JC, García-Ruiz C (2006) Mitochondrial free cholesterol loading sensitizes to TNF- and Fas-mediated steatohepatitis. Cell Metab 4:185–198

Marí M, Colell A, Morales A, Caballero F, Moles A, Fernández A, Terrones O, Basañez G, Antonsson B, García-Ruiz C, Fernández-Checa JC (2008) Mechanism of mitochondrial glutathione-dependent hepatocellular susceptibility to TNF despite NF-kappaB activation. Gastroenterology 134(5):1507–1520

Marí M, Colell A, Morales A, von Montfort C, Garcia-Ruiz C, Fernández-Checa JC (2010) Redox control of liver function in health and disease. Antioxid Redox Signal 12(11):1295–1331

Martin S, Parton RG (2006) Lipid droplets: a unified view of a dynamic organelle. Nat Rev Mol Cell Biol 7(5):373–378

Maxfield FR, Tabas I (2005) Role of cholesterol and lipid organization in disease. Nature 438:612–621

Miller WL (2007) Steroidogenic acute regulatory protein (StAR), a novel mitochondrial cholesterol transporter. Biochim Biophys Acta 1771:663–676

Montero J, Morales A, Llacuna L, Lluis JM, Terrones O, Basañez G, Antonsson B, Prieto J, García-Ruiz C, Colell A, Fernández-Checa JC (2008) Mitochondrial cholesterol contributes to chemotherapy resistance in hepatocellular carcinoma. Cancer Res 68:5246–5256

Morohaku K, Pelton SH, Daugherty DJ, Butler WR, Deng W, Selvaraj V (2014) Translocator protein/peripheral benzodiazepine receptor is not required for steroid hormone biosynthesis. Endocrinology 155(1):89–97

Mukodani J, Ishikawa Y, Fukuzaki H (1990) Effects of hypoxia on sterol synthesis, acyl-CoA: cholesterol acyltransferase activity, and efflux of cholesterol in cultured rabbit skin fibroblasts. Arteriosclerosis 10:106–110

Murata M, Peranen J, Schreiner R, Wieland F, Kurzchalia TV, Simons K (1995) VIP21/caveolin is a cholesterol-binding protein. Proc Natl Acad Sci U S A 92(22):10339–10343

Nguyen AD, McDonald JG, Bruick RK, DeBose-Boyd RA (2007) Hypoxia stimulates degradation of 3-hydroxy-3-methylglutaryl coenzyme A reductase through accumulation of lanosterol and hypoxia-inducible factor (HIF)-mediated induction of Insigs. J Biol Chem 282:27436–27446

Nohturfft A, Brown MS, Goldstein JL (1998) Topology of SREBP cleavage-activating protein, a polytopic membrane protein with a sterol-sensing domain. J Biol Chem 273:17243–17250

Papadopoulos V, Miller WL (2012) Role of mitochondria in steroidogenesis. Best Pract Res Clin Endocrinol Metab 26(6):771–790

Parton RG, Simons K (2007) The multiple faces of caveolae. Nat Rev Mol Cell Biol 8(3):185–194

Pol A, Luetterforst R, Lindsay M, Heino S, Ikonen E, Parton RG (2001) A caveolin dominant negative mutant associates with lipid bodies and induces intracellular cholesterol imbalance. J Cell Biol 152(5):1057–1070

Pol A, Martin S, Fernandez MA, Ingelmo-Torres M, Ferguson C, Enrich C, Parton RG (2005) Cholesterol and fatty acids regulate dynamic caveolin trafficking through the Golgi complex and between the cell surface and lipid bodies. Mol Biol Cell 16(4):2091–2105

Prattes S, Horl G, Hammer A, Blaschitz A, Graier WF, Sattler W, Zechner R, Steyrer E (2000) Intracellular distribution and mobilization of unesterified cholesterol in adipocytes: triglyceride droplets are surrounded by cholesterol-rich ER-like surface layer structures. J Cell Sci 113(Pt 17):2977–2989

Rawson RB, DeBose-Boyd R, Goldstein JL, Brown MS (1999) Failure to cleave sterol regulatory element-binding proteins (SREBPs) causes cholesterol auxotrophy in Chinese hamster ovary cells with genetic absence of SREBP cleavage-activating protein. J Biol Chem 274:28549–28556

Ribas V, García-Ruiz C, Fernandez-Checa JC (2014) Glutathione and mitochondria. Front Pharmacol 5:151

Sala-Vila A, Navarro-Lérida I, Sánchez-Alvarez M, Bosch M, Calvo C, López JA, Calvo E, Ferguson C, Giacomello M, Serafini A, Scorrano L, Enriquez JA, Balsinde J, Parton RG, Vázquez J, Pol A, Del Pozo MA (2016) Interplay between hepatic mitochondria-associated membranes, lipid metabolism and caveolin-1 in mice. Sci Rep 6:27351

Schonewille M, de Boer JF, Mele L, Wolters H, Bloks VW, Wolters JC, Kuivenhoven JA, Tietge UJ, Brufau G, Groen AK (2016) Statins increase hepatic cholesterol synthesis and stimulate fecal cholesterol elimination in mice. J Lipid Res 57:1455–1464

Sever N, Yang T, Brown MS, Goldstein JL, DeBose-Boyd RA (2003) Accelerated degradation of HMG-CoA reductase mediated by binding of insig-1 to its sterol-sensing domain. Mol Cell 11:25–33

Smith B, Land H (2012) Anticancer activity of the cholesterol exporter ABCA1 gene. Cell Rep 2(3):580–590

Soccio RE, Breslow JL (2003) StAR-related lipid transfer (START) proteins: mediators of intracellular lipid metabolism. J Biol Chem 278:22183–22186

Soffientini U, Caridis A-M, Dolan S, Graham A (2014) Intracellular cholesterol transporters and modulation of hepatic lipid metabolism: implications for diabetic dyslipidaemia and steatosis. Biochim Biophys Acta 1842:1372–1382

Song BL, Javitt NB, DeBoyse-Boyd RA (2005a) Insig-mediated degradation of HMG-CoA reductase stimulated by lanosterol, an intermediate in the synthesis of cholesterol. Cell Metab 1:179–189

Song BL, Sever N, DeBose-Boyd RA (2005b) Gp78, a membrane-anchored ubiquitin ligase, associates with Insig-1 and couples sterol-regulated ubiquitination to degradation of HMG-CoA reductase. Mol Cell 19:829–840

Stravitz RT, Vlahcevic ZR, Russell TL, Heizer ML, Avadhani NG, Hylemon PB (1996) Regulation of sterol 27-hydroxylase and an alternative pathway of bile acid biosynthesis in primary cultures of rat hepatocytes. J Steroid Biochem Mol Biol 57(5–6):337–347

Sturley SL, Patterson MC, Balch W, Liscum L (2004) The pathophysiology and mechanisms of NP-C disease. Biochim Biophys Acta 1685(1–3):83–87

Sun SY, Liu X, Zou W, Yue P, Marcus AI, Khuri FR (2007) The farnesyltransferase inhibitor lonafarnib induces CCAAT/enhancer-binding protein homologous protein-dependent expression of death receptor 5, leading to induction of apoptosis in human cancer cells. J Biol Chem 282:18800–18809

Tichauer J-E, Morales M-G, Amigo L, Galdames L, Klein A, Quinones V, Ferrada C, Alvarez A-R, Rio M-C, Miquel J-F et al (2007) Overexpression of the cholesterol-binding protein MLN64 induces liver damage in the mouse. World J Gastroenterol 13:3071–3079

Trapani L, Segatto M, Pallottini V (2012) Regulation and deregulation of cholesterol homeostasis: the liver as a metabolic power station. World J Hepatol 4:184–190

van Meer G, Voelker DR, Feigenson GW (2008) Membrane lipids: where they are and how they behave. Nat Rev Mol Cell Biol 9(2):112–124

Varatharajalu R, Garige M, Leckey LC, Arellanes-Robledo J, Reyes-Gordillo K, Shah R, Lakshman MR (2014) Adverse signaling of scavenger receptor class B1 and PGC1s in alcoholic hepatosteatosis and steatohepatitis and protection by betaine in rat. Am J Pathol 184:2035–2044

von Montfort C, Matias N, Fernandez A, Fucho R, Conde de la Rosa L, Martinez-Chantar ML, Mato JM, Machida K, Tsukamoto H, Murphy MP, Mansouri A, Kaplowitz N, Garcia-Ruiz C, Fernandez-Checa JC (2012) Mitochondrial GSH determines the toxic or therapeutic potential of superoxide scavenging in steatohepatitis. J Hepatol 57(4):852–859

Wang YD, Chen WD, Huang W (2008) FXR, a target for different diseases. Histol Histopathol 23:621–627

Yabe D, Brown MS, Goldstein JL (2002) Insig-2, a second endoplasmic reticulum protein that binds SCAP and blocks export of sterol regulatory element-binding proteins. Proc Natl Acad Sci U S A 99:12753–12758

Yang T, Espenshade PJ, Wright ME, Yabe D, Gong Y, Aebersold R, Goldstein JL, Brown MS (2002) Crucial step in cholesterol homeostasis: sterols promote binding of SCAP to INSIG-1, a membrane protein that facilitates retention of SREBPs in ER. Cell 110:489–500

Zhao P, Slattery JT (2002) Effects of ethanol dose and ethanol withdrawal on rat liver mitochondrial glutathione: implication of potentiated acetaminophen toxicity in alcoholics. Drug Metab Dispos 30:1413–1417

Zhao P, Kalhorn TF, Slattery JT (2002) Selective mitochondrial glutathione depletion by ethanol enhances acetaminophen toxicity in rat liver. Hepatology 36(2):326–335

Mitochondrial Changes in Cancer

Shubha Gururaja Rao

Contents

Abstract

Mitochondrial structural and functional integrity defines the health of a cell by regulating cellular metabolism. Thus, mitochondria play an important role in both cell proliferation and cell death. Cancer cells are metabolically altered compared to normal cells for their ability to survive better and proliferate faster. Resistance to apoptosis is an important characteristic of cancer cells and given the contribution of mitochondria to apoptosis, it is imperative that mitochondria could behave differently in a tumor situation. The other feature associated with cancer cells is the Warburg effect, which engages a shift in metabolism. Although the Warburg effect often occurs in conjunction with dysfunctional mitochondria, the relationship between mitochondria, the Warburg effect, and cancer cell metabolism is not clearly decoded. Other than these changes, several mitochondrial gene mutations occur in cancer cells, mitochondrial biogenesis is affected and mitochondria see structural and functional variations. In cancer

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H. Singh, S.-S. Sheu (eds.), *Pharmacology of Mitochondria*,
Handbook of Experimental Pharmacology 240, DOI 10.1007/164_2016_40

pharmacology, targeting mitochondria and mitochondria associated signaling pathways to reduce tumor proliferation is a growing field of interest. This chapter summarizes various changes in mitochondria in relevance to cancer, behavior of mitochondria during tumorigenesis, and the progress on using mitochondria as a therapeutic target for cancer.

Keywords
Apoptosis • Cancer • Mitochondria • Mitochondrial fission • Mitochondrial fusion • Reactive oxygen species

1 Introduction

In routine clinical diagnosis of cancer, a glucose analogue (2-18 fluoro-2-deoxy-Glucose) is used to trace tumor tissue that would uptake more glucose than normal tissues due to its increased necessity for sugars. This technique is based on a hypothesis by Otto Warburg in the 1930s that cancer cells choose a different metabolic route than normal cells (Koppenol et al. 2011; Warburg 1956). This school of thought led to major studies on aerobic glycolysis in tumor cells where cells adapt to a glycolytic pathway to make adenosine triphosphate (ATP) instead of using the regular mitochondrial electron transport chain (ETC). Thus, cancer cells involve increased breakdown of glucose generating raw materials for the synthesis for other macromolecules, helping their rapid growth. Although tumor hypoxia is hypothesized to be a trigger (Gatenby and Gillies 2004), there are evidences where there is a metabolic shift to aerobic glycolysis in free availability of oxygen (Christofk et al. 2008). It is speculated that in cancer, there is a reprogramming of the cells driven by oncogenes into a proliferative metabolism, resembling an embryonic program (Vander Heiden et al. 2009) with upregulation of glycolytic enzymes (Christofk et al. 2008). Glycolysis under aerobic conditions makes cells acidic due to increased production of lactate as a result of glycolytic cycles, but this excess lactate is postulated to be a fuel for mitochondrial oxidative phosphorylation (Sonveaux et al. 2008). This phenomenon is proposed to be used by certain cancer cells in a reverse Warburg effect, where cancer cells induce Warburg effect in neighboring stromal cells and in turn receive lactate and pyruvate for oxidative phosphorylation (Pavlides et al. 2009). All these metabolic altercations point to an altered mitochondrial function in tumor cells that has led to years of research in this field with respect to ATP production and beyond (Boland et al. 2013). This chapter summarizes the important changes in mitochondria associated with onset and progression of cancer such as mitochondrial DNA (mtDNA) mutations, mitochondrial reactive oxygen species (ROS), mitochondrial mass regulation, and mitochondrial dynamics. The chapter also discusses and summarizes major cancer drug classes that target mitochondria (Table 1).

Table 1 List of drugs targeting mitochondria in cancer

Drug classes	Targeted mitochondrial component/process
1-methyl-4-phenylpyridinium, Vitamin K	mtDNA/mtDNA replication-copy number inhibition (Sasaki et al. 2008; Umeda et al. 2000; Neuzil et al. 2007)
ABT-263, Gossypol, antimycin A, alpha-tocopheryl	Bcl-2 family/BH3 domain mimetics (Kang et al. 2010; Neuzil et al. 2007)
Metformin (biguanide)	Mitochondrial complex I/inhibition of ATP production (El-Mir et al. 2000; Owen et al. 2000)
Phenformin (biguanide)	Mitochondrial complex I/inhibition of ATP production (Birsoy et al. 2014)
VLX600	ETC inhibitor/inhibition of ATP production (Zhang et al. 2014)
Tigecycline	ETC inhibitor/inhibition of ATP production (Skrtic et al. 2011)
bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide)	Glutaminase inhibitor/inhibition of biosynthetic pathways (Le et al. 2012)
Compound 968	Glutaminase inhibitor/inhibition of biosynthetic pathways (Le et al. 2012)
Chloroquine	Autophagy inhibitor/inhibition of mitophagy (Balic et al. 2014)
Antioxidants	ROS inhibition/mitochondrial ROS scavenging (Bjelakovic and Gluud 2007)
Inhibitors of antioxidants	Antioxidant inhibition/selective tumor cell death (Raj et al. 2011; Glasauer et al. 2014)

2 Mitochondrial Mutations in Cancer Cells

Each cell contains numerous mitochondria, and every mitochondrion has its own DNA (mtDNA) in multiple copies. Mammalian cells contain about 1000–10,000 copies of mtDNA which can replicate independent of cellular division (Lightowlers et al. 1997). Mitochondria can accumulate somatic mutations and lead to a heteroplasmic state where mitochondria with dissimilar DNA content co-exist. Mutations in mitochondrial DNA contribute to mitochondrial function, especially ROS production, and hence it is important to consider mitochondrial DNA as an important factor in tumorigenesis.

The mitochondrial genome has been sequenced and characterized (Blanchard and Schmidt 1996; Grivell 1983), and several mutations in mtDNA have been associated with cancers in human tissues (Chatterjee et al. 2006). A list of major cancers and associated mtDNA mutations is provided in Table 2 (Abu-Amero et al. 2005; Fliss et al. 2000; Habano et al. 1999; Jeronimo et al. 2001; Jones et al. 2001; Maximo et al. 2002; Polyak et al. 1998; Sanchez-Cespedes et al. 2001; Canter et al. 2005; Parrella et al. 2001; Petros et al. 2005; Wong et al. 2003). Mitochondrial mutations (both homoplasmic and heteroplasmic) have been

Table 2 List of mitochondrial gene mutations and relevant cancers

Major cancer types	Mitochondrial gene affected
Bladder	Cyt b, ND3 (Fliss et al. 2000)
Colon	ND1, ND5, COX I, COX II, COX III, Cyt b (Polyak et al. 1998; Jones et al. 2001; Habano et al. 1999)
Pancreas	ND1, ND2, ND3, ND4, ND6, COX I, COX II, COX III, ATPase, Cyt b (Jones et al. 2001; Jeronimo et al. 2001)
Ovary	Cyt b (Liu et al. 2013)
Thyroid	Cyt b, ND1, ND2, ND3, ND4, ND5, ND6, COX II, COX III (Abu-Amero et al. 2005)
Breast	ND4, ND5 (Canter et al. 2005; Parrella et al. 2001)
Head and neck	ND4 (Fliss et al. 2000)
Medulloblastoma	ND4L (Wong et al. 2003)
Prostrate	ND1, ND5 COX I (Jeronimo et al. 2001; Petros et al. 2005)

detected in body fluids of cancer patients (Chinnery et al. 2002; Fliss et al. 2000). Although normal subjects are reported to display age-associated accumulation of mitochondrial mutations (Cormio et al. 2005), mtDNA mutations are highly prevalent in cancer tissues. mtDNA coded enzymes contributing to mitochondrial oxidative phosphorylation (Luciakova and Kuzela 1992) are reported defective in tumor situations, which could possibly lead to deregulation of ROS production from the mitochondria. Thus, mtDNA mutations could contribute to solid tumors by favoring the Warburg phenomenon and by playing a role in apoptosis. These mutations can be traced along with tumors and thus could be used as markers for identifying types of tumors. However, it is not clear if mtDNA mutations themselves can drive tumor growth or merely provide an advantage to cancer cells. This is one key area awaiting extensive research in order to understand the mtDNA mutations and their relation to tumorigenesis.

3 Mitochondrial Reactive Oxygen Species

ROS are a by-product of the electron transport chain at the level of complex I and complex II/III where electrons escape (leak) the canonical pathway of electron transport, and are established to play a role in cellular signaling (Brand 2010; Chen et al. 2003). Reduced levels of antioxidants can also contribute to increased ROS levels in the cells (Hamanaka and Chandel 2010; Schumacker 2006). ROS can have both deleterious and favorable effects on cancer cells depending on the amount as well as rate of generation.

ROS can activate several signaling pathways promoting tumorigenesis and thus play a factor favoring the cancer cells. ROS stabilize hypoxia inducible factor (HIF)-α, an important protein for the survival of tumor cells in extremely hypoxic tumor environment (Jung et al. 2008). Intriguingly, antioxidant treatment of cancer cells can cause suppression of HIF1-α, again indicating the importance of ROS

regulation in tumor microenvironment (Gao et al. 2009). Activation of the hypoxia pathway in cancer cells is also related to metabolic changes. HIF1-α induces the expression of glycolytic enzymes driving the cancer cells to adapt to an alternative ATP generation mechanism (Kim et al. 2006). The other way by which ROS regulate metabolism in cancer cells is by driving the activation of NRF2, a nuclear factor involved in increased production of anabolic enzymes (Mitsuishi et al. 2012). ROS also oxidize pyruvate kinase M2, which in turn drives a pentose pathway flux and increases glutathione levels promoting tumorigenesis (Anastasiou et al. 2011; Israelsen et al. 2013).

ROS regulate signaling pathways in cancer; the most studied one amongst them is the PI3Kinase pathway—a major growth-promoting signal in normal as well as cancer cells (Cantley 2002). The target of ROS within the context of cancer is the phosphatase PTEN, a negative regulator of the PI3Kinase pathway. ROS oxidize an active site cysteine on PTEN leading to the hyper-activation of the pathway (Lee et al. 2002; Leslie et al. 2003). ROS, on the other hand, can also inhibit the phosphatases of this pathway, namely PP2A and PTP1B, that negatively regulate Akt (Ostman et al. 2011), fostering the pathway's activity and thus promoting cell survival and proliferation.

Another role of ROS in cancer cells is induction of oxidative DNA damage leading to the development and progression of tumor in several examples. Patients with increased oxidative damage are more likely to develop tumors (Hagen et al. 1994; Shimoda et al. 1994). The origin of ROS in tumor cells could often be oncogenes themselves (Irani et al. 1997) or increased activity of oxidases or peroxisome activity (Liou and Storz 2010). Mutations in mitochondrial DNA or mitochondrial dysfunction also cause increased levels of ROS. Heteroplasmic mitochondrial DNA mutations in ND (NADH Dehydrogenase) genes are shown to increase ROS production from the mitochondria (Larman et al. 2012). Tumor cells also have an elevated antioxidant response to balance the increased ROS, avoiding apoptosis (Liou and Storz 2010).

As mentioned above, although ROS is a tumor-promoting signal, the levels of ROS clearly define if it is playing a deleterious effect for the cancer cells or an advantage. Given cancer cells carry high levels of ROS, they can be targeted to death by further elevating the levels of ROS using chemicals that produce ROS. Small molecules and alkaloids have been used to target cancer cells (Raj et al. 2011; Shaw et al. 2011; Trachootham et al. 2009). However, a major disadvantage of using ROS as a target for cancer cells stems from the fact that normal cells are also affected by increased ROS, especially in cells that utilize ROS as a physiological molecule (Sena and Chandel 2012; Nagaraj et al. 2012; Owusu-Ansah and Banerjee 2009). Moreover, not all cancer cells elevate ROS levels (Nagaraj et al. 2012; Shaw et al. 2011). Hence, ROS offers a tumor type specific therapeutic scope in cancer biology (discussed at the end of this chapter).

4 Mitochondrial Dynamics in Cancer

Mitochondria are dynamic organelles undergoing fusion and fission events constantly. The outer membrane consists of mitofusins, Mfn1 and Mfn2 and the inner membrane consists of Opa1, which facilitates fusion and fission in their respective membranes (Karbowski and Youle 2003). Drp1, a dynamin related GTPase is another protein required for mitochondrial fission, which forms rings where mitochondria pinch off from each other (Detmer and Chan 2007).

The shape of mitochondria changes throughout the cell cycle and apoptosis, which relates to their role in cancer (Van den Bogert et al. 1988). During G1-S phase there is an increased oxidative phosphorylation and the function of mitochondria to facilitate cell division. However during S-M phase, given the need of the cell to divide, the mitochondria become fragmented as they are distributed between the daughter cells (Margineantu et al. 2002). It is believed that the G1-S phase networking of mitochondria can regulate the cyclin E levels and thus is essential for cell cycle progression. This also involves membrane polarization and hyperfusion of mitochondria (Mitra et al. 2009). Although cyclin E expression is important for cell cycle progression, how this is driven by mitochondrial hyperfusion is not yet proved. However, ATP and ROS have been ruled out to be playing a major role (Qian et al. 2012). Glycolysis and glutaminolysis are noted to be increased around G1/S phase and which could be linked to the change in mitochondrial dynamics (Qian et al. 2012; Dang 2010). Thus, mitochondrial structural status can contribute to cell cycle progression and a deregulation would affect cell division.

Other than cell cycle, stress signals are documented to alter mitochondrial dynamics. Signaling pathways interact with mitochondrial dynamics and regulate the structure and function of the mitochondria in order to sustain stress. Drugs, UV, production of increased amounts of ATP, and higher rates of oxidative phosphorylation are all known to cause stress-induced hyperfusion to prevent apoptosis and mitophagy (Mitra et al. 2009; Rambold et al. 2011; Tondera et al. 2009). During glucose deprivation, cells switch to oxidative phosphorylation with increased mitochondrial fusion and cristae density as an adaptation for cell survival (Rossignol et al. 2004) in normal as well as tumor producing cells. Hence, such structural changes are relevant to cell survival and division. This was shown in an *in vivo* model Drosophila (Nagaraj et al. 2012), where the oncogene Yorkie (Yki) transcriptionally regulated mitochondrial proteins Opa1 and mitofusin causing increased mitochondrial fusion. This fusion was essential throughout proliferation mediated by Yorkie. This study was extended into human cells where Yap2 expression, a homologue of Yorkie showed hyperfusion. Mitochondrial fusion is also indirectly correlated with decreased levels of ROS (Nagaraj et al. 2012), a contradiction from the general notion that ROS are elevated in tumor cells. Given ROS can induce apoptosis, it can be postulated that mitochondrial fusion is pro-survival and thus helps cancer cell endurance.

Conversely, inhibiting mitochondrial fusion impedes cell growth and proliferation as well as oxidative metabolism (Bach et al. 2003). Hence, it is imperative that

fusion promotes mitochondrial function by increasing the efficiency of oxidative phosphorylation as well as ATP production, and reducing ROS generation. Thus, fusion could also promote mitochondria related metabolic pathways such as the Kreb's cycle, fatty acid oxidation, etc.

Mitochondrial fission, the opposite phenomenon to fusion, has opposite effects on metabolism. It is acknowledged to stunt growth and increase ROS (Nagaraj et al. 2012; Yarosh et al. 2008), which could be the result of compromised respiratory activity. Loss of Drp1, a protein required for fission is also shown to cause deregulation of cyclin E, leading to aberrant proliferation (Parker et al. 2015). Dysregulated Drp1 expression is linked to tumor cell fission (Rehman et al. 2012). Given fission increases ROS production, and the ability of ROS to regulate hypoxia and lactate production, it is an interesting problem to answer how fission contributes to the Warburg effect.

Mitochondrial fission also promotes membrane depolarization, cytochrome c release, and apoptosis. Drp1 promotes Bax oligomerization, possibly allowing fragmented mitochondria to form Bax/Bak openings (Brooks et al. 2007; Montessuit et al. 2010). Other classes of apoptotic proteins, such as Bcl2 family members (Bcl-X_L), promote mitochondrial fission (Li H 2008) and are overexpressed in tumors (Kelly and Strasser 2011). These are predicted to promote a Warburg shift by reducing oxidative phosphorylation. However, their mechanistic connection to mitochondrial activity and metabolic changes is to be explored.

Mitochondrial dynamics (both fusion and fission) contribute to tumor cell proliferation by different mechanisms. However, if these changes can act as cause for tumor progression or if they are a consequence of tumor growth is yet to be understood.

5 Mitochondrial Content in Cancer

Mitochondrial content is decided by two factors in cancer: mitochondrial biogenesis and mitophagy. Signaling pathways, especially the oncogenes, control mitochondrial biogenesis, as established in cell culture systems. However, there is no clear evidence of altered mitochondrial biogenesis in tumors. Yet, given the established roles of oncogenes such as Myc in mitochondrial biogenesis (Li et al. 2005; Morrish and Hockenbery 2014), it is vital to understand the importance of mitochondrial amounts in tumor cells.

Mitochondrial biogenesis involves nuclear factors as well as mitochondrial genes; most of the mitochondrial proteins are synthesized by the nuclear factor and imported to mitochondria, while some enzymes in the ETC are encoded by the mitochondrial genes themselves (Chacinska et al. 2009). It is an essential process for the cells, and the mitochondrial content of the cells depends on the nutrient availability, dividing status and physiological (and/or pathophysiological) state of the cells (Wenz 2013). Mitochondrial biogenesis is controlled by peroxisome-proliferator activator receptor-alpha and gamma (PPARα, PPARγ), nuclear respiratory factor 1 (NRF1), nuclear respiratory factor 2 (NRF2), and also estrogen

related receptors (ERR) α, β, γ (Scarpulla et al. 2012). PPAR-γ co-activator 1-alpha (PGC-1α), with its partners (PGC-1β, and PRC-PGC related co-activator) termed as the master regulator of mitochondrial biogenesis. It forms a protein complex, controlling and maintaining the expression of mitochondrial biogenesis factors, antioxidants, and several metabolic genes (Dominy et al. 2010).

The role of mitochondrial biogenesis in cancer comes to picture with the discoveries of oncogenes regulating mitochondrial biogenesis through the PGC family. The most well-known oncogene that triggers mitochondrial biogenesis is c-Myc, which control biogenesis through PGC-1β (Zhang et al. 2007). However, tumor related genes such as HIF-1α negatively regulate mitochondrial biogenesis by inhibiting c-Myc (Zhang et al. 2007). PGC-1α activity is upregulated in a subset of melanoma where oxidative phosphorylation dependency is seen (Vazquez et al. 2013). PGC-1β and its targets are upregulated in ALT (Alternative Lengthening of Telomeres) positive tumors (Hu et al. 2012). p53, the tumor suppressor protein negatively regulates mitochondrial biogenesis, again indicating a relation between cancer and mitochondrial function (Sahin et al. 2011).

Taken together, it is not clear whether mitochondrial biogenesis is beneficial for tumor cells or is inhibiting rapid proliferation. Not all tumor cells carry the same metabolic profiles. Thus, tumor mitochondrial biogenesis needs to be studied in specification to the oncogenes involved and the nature of the tumors.

The other process that regulated mitochondrial mass in cancer cells is mitochondrial autophagy. Autophagy is activated in a situation of nutrient deprivation to the cells, and mitophagy is a specific process where mitochondria are targeted for degradation to provide nutrient for cell survival (Rabinowitz and White 2010). Mitochondrial fusion and fission events control mitophagy, where fission facilitates mitophagy by marking them for degradation and fusion keeps the mitochondria healthy, protecting them from mitophagy (Twig et al. 2008). Mitophagy is regulated by two pathways postulated to be tumor suppressors: the Parkin pathway and the BNIP3/NIX pathway (Youle and Narendra 2011; Zhang and Ney 2009). Parkin promotes mitochondrial turnover via fission and assists in mitochondrial transport via microtubules (Narendra et al. 2010; Narendra et al. 2008). Parkin, although is a gene associated with Parkinson's syndrome (Youle and Narendra 2011), is also identified as a tumor suppressor gene in several cancers, and Parkin mutant mice are susceptible to tumors (Cesari et al. 2003; Fujiwara et al. 2008). Parkin promotes oxidative metabolism as a p53 target while inhibiting the development of Warburg effect (Zhang et al. 2011). Although direct evidences of Parkin involved in a tumor scenario via mitophagy are unclear, it can be postulated that Parkin maintains healthy mitochondria balancing metabolism in cells and thus can cause deregulation of metabolism in cancer cells upon its mutation.

BNIP3 and NIX are redox-sensing hypoxia inducible genes promoting mitophagy (Zhang and Ney 2009). These proteins directly interact with the autophagy protein LC3 as adaptors targeting mitochondria for degradation (Hanna et al. 2012). They reduce mitochondrial mass in hypoxic condition by inducing mitophagy helping the cells regulate excessive ROS production in a state of hypoxia (Tracy et al. 2007). BNIP3 and NIX are characterized as

dysregulated in several tumors. In certain malignant tumors, the expression of these genes decreases, whereas pre-malignant stages see an increase (Okami et al. 2004; Tan et al. 2007; Sowter et al. 2003). Loss of BNIP3 promotes tumors in mouse models, and hence it is considered to be a tumor suppressor (Chourasia and Macleod 2015). Similar to Parkin, BNIP3 and NIX proteins are considered to control mitochondrial turnover. Although it is established that Parkin and BNIP3/NIX pathways control mitophagy in tumor scenarios, a definitive mechanism for the effect of mitophagy in cancer is yet to emerge. Mitophagy is also directly related to aging as cancer mutations accumulate with age and the development of tumor progresses. Hence mitophagy and aging work *in tandem* in cancer situations. However, targeting mitophagy offers an advantage in tumor therapy over apoptosis and general autophagy due to specificity.

Along with the above discussed major mitochondrial changes, retrograde signals from mitochondria to the nucleus (Wallace 2012), and oncogenic control of mitochondria by oncogenes, such as Myc and KRas, and tumors suppressors, such as p53 and RB (Sherr and McCormick 2002; Vousden and Prives 2009; Dang 2010), also bring about mitochondrial changes in tumor situations. Although it is now established that mitochondrial changes occur in cancer cells, how these changes affect tumor growth and how they can be manipulated to achieve cancer therapy still require specific research.

6 Targeting Mitochondria for Cancer Therapy

Given the multilevel involvement of mitochondria in cancer, researchers have used several ways to target mitochondria for cancer therapy.

Mitochondrial DNA is targeted by drugs that reduce the copy number of mitochondrial DNA or by inhibiting replication. Vitamin K3 (menadione) (Sasaki et al. 2008) inhibits DNA polymerase gamma, which is important for replication of mitochondrial DNA. Parkinsonian toxin 1-methyl-4-phenylpyridinium reduces the copy number of mtDNA by destabilizing the structure (Umeda et al. 2000; Neuzil et al. 2007).

Dysregulated ROS production from mitochondria in cancer is another target for therapy. However, the human trials have not had great success (Bjelakovic and Gluud 2007) as they fail to inhibit the mitochondrial generated ROS. Another problem with the antioxidant drugs is that normal cells such as immune cells also produce ROS for physiological functions. Nevertheless, another approach in ROS based therapeutic experiments is using the ability of cancer cells to produce more antioxidants and ROS. Hence, inhibition of antioxidants in cancer cells could lead to excessive ROS in cancer cells and cause selective killing of cancer cells (Raj et al. 2011; Glasauer et al. 2014).

The Bcl-2 family of proteins consists of pro and anti-survival factors (Youle and Strasser 2008). Cell death is favored in the absence of pro-survival factors. Mimetic drugs that target BH3 domains of these proteins, the domain that interacts with Bax/Bak proteins (Youle and Strasser 2008), are used to target tumors. Drugs such

as ABT-263, Gossypol, antimycin A, alpha-tocopheryl succinates are some examples of Bcl-2 family targets that induce mitochondria mediated cell death, partly due to their interaction with the BH3 domain (Kang et al. 2010; Neuzil et al. 2007).

Given ATP generation is essential for normal cells as well as tumor cells (Zu and Guppy 2004), mitochondrial bioenergetic targeting drugs need to be specifically targeted to tumor cells. Poorly perfused tumors which make ATP (Rumsey et al. 1990) are an apt target for drugs that inhibit ATP production given it would cause cell death in such poorly perfused tumors only. The antidiabetic drug, metformin, a biguanide, is thought to be such a candidate to target ATP production without affecting the normal tissue. Metformin decreases hepatic gluconeogenesis and brings down insulin levels (Bailey and Turner 1996). Metformin has many effects on cancer cells. It decreases blood glucose and insulin levels, and inhibits the growth of tumors that are insulin dependent (Pollak 2014). It acts on the mitochondrial complex I (El-Mir et al. 2000; Owen et al. 2000). It also impairs glycolysis by decreasing the activity of enzyme hexokinase 2, an important enzyme to carry out glycolysis (Salani et al. 2013). Thus, metformin is believed to inhibit tumor growth by lowering glucose supply, acting on complex I and thereby reducing ATP production. The dosages and complete mechanism of action for this drug are still under investigation in clinical trials. Another drug similar to metformin is phenformin, a biguanide that also inhibits mitochondrial complex I (Birsoy et al. 2014). It has higher affinity to mitochondria and recently shown to work better than metformin in breast tumors (Appleyard et al. 2012). Although lactose acidosis is a drawback of phenformin, there are evidences that the drug can be used in combination with BRAF inhibitors to control melanomas effectively (Yuan et al. 2013). Hence, phenformin also makes an attractive candidate for clinical trials.

One of the classes of drugs also tried on inhibiting ATP production is VLX600, an ETC inhibitor. This is shown to reduce colon cancer tumor growth (Zhang et al. 2014) at experimental levels. Mitochondrial protein translation is targeted by certain drugs such as tigecycline that reduces the expression of 13 subunits of ETC. This drug has been efficient on leukemic cells that survive primarily on mitochondrial ATP production (Skrtic et al. 2011). Mitochondrial chaperones, such as heat shock proteins, are targeted by drugs such as Gamitrinib, which are modified to accumulate in mitochondria and reduce the activity of HSP90 and ATPase-1, thus reducing energy production of mitochondria.

Another target in mitochondrial cancer drugs is biosynthetic pathways. The glutamine addicted tumors are formed mostly due to Myc and Kras (Gaglio et al. 2011). Such tumors can be targeted by using inhibitors of glumatinases that use glutamine in their reaction to continue the tricarboxylic acid cycle. Glutaminase inhibitors such as bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide) or compound 968 (Le et al. 2012) already attenuate tumor growth. Mitophagy also produces raw materials for the mitochondrial TCA cycle as discussed in the review before. Several autophagy inhibitors are on trials and chloroquine is one such drug

(Balic et al. 2014). Targeting mitophagy specifically would be a safer option for such treatments given the toxicity associated with autophagy drugs.

Several mitochondria related protein specific inhibitors, such as hexokinase inhibitors (Ben Sahra et al. 2010; Chen et al. 2009; Mathupala et al. 2006), VDAC, and ANT inhibitors, are also reportedly used as mitochondrial targets in cancers (Belzacq et al. 2001; Don et al. 2003).

In conclusion, although there are several attempts to target mitochondria in cancer, the efficiency has been low due to the lack of complete understanding of the role of mitochondria in cancer. Mitochondria are complex organelles and they undergo drastic structural and functional changes during tumor development. As every cancer is defined by its own oncogenic signals, and every signal will have a different effect on mitochondria, there is a tremendous difference in how mitochondria are affected in each scenario. Like the saying—*one glove does not fit all*, there is a need to understand specific changes in mitochondria in specific cancers and target processes with one or a combination of drugs effectively.

Acknowledgments I thank Drs. Harpreet Singh and James Barrett (Drexel University College of Medicine) for helpful discussions, and Dr. Frances Munoz and Kajol Shah for proof reading of the book chapter. The work was supported by a CURE grant.

References

Abu-Amero KK, Alzahrani AS, Zou M, Shi Y (2005) High frequency of somatic mitochondrial DNA mutations in human thyroid carcinomas and complex I respiratory defect in thyroid cancer cell lines. Oncogene 24:1455–1460

Anastasiou D, Poulogiannis G, Asara JM, Boxer MB, Jiang JK, Shen M, Bellinger G, Sasaki AT, Locasale JW, Auld DS et al (2011) Inhibition of pyruvate kinase M2 by reactive oxygen species contributes to cellular antioxidant responses. Science 334:1278–1283

Appleyard MV, Murray KE, Coates PJ, Wullschleger S, Bray SE, Kernohan NM, Fleming S, Alessi DR, Thompson AM (2012) Phenformin as prophylaxis and therapy in breast cancer xenografts. Br J Cancer 106:1117–1122

Bach D, Pich S, Soriano FX, Vega N, Baumgartner B, Oriola J, Daugaard JR, Lloberas J, Camps M, Zierath JR et al (2003) Mitofusin-2 determines mitochondrial network architecture and mitochondrial metabolism. A novel regulatory mechanism altered in obesity. J Biol Chem 278:17190–17197

Bailey CJ, Turner RC (1996) Metformin. N Engl J Med 334:574–579

Balic A, Sorensen MD, Trabulo SM, Sainz B Jr, Cioffi M, Vieira CR, Miranda-Lorenzo I, Hidalgo M, Kleeff J, Erkan M et al (2014) Chloroquine targets pancreatic cancer stem cells via inhibition of CXCR4 and hedgehog signaling. Mol Cancer Ther 13:1758–1771

Belzacq AS, El Hamel C, Vieira HL, Cohen I, Haouzi D, Metivier D, Marchetti P, Brenner C, Kroemer G (2001) Adenine nucleotide translocator mediates the mitochondrial membrane permeabilization induced by lonidamine, arsenite and CD437. Oncogene 20:7579–7587

Ben Sahra I, Laurent K, Giuliano S, Larbret F, Ponzio G, Gounon P, Le Marchand-Brustel Y, Giorgetti-Peraldi S, Cormont M, Bertolotto C et al (2010) Targeting cancer cell metabolism: the combination of metformin and 2-deoxyglucose induces p53-dependent apoptosis in prostate cancer cells. Cancer Res 70:2465–2475

Birsoy K, Possemato R, Lorbeer FK, Bayraktar EC, Thiru P, Yucel B, Wang T, Chen WW, Clish CB, Sabatini DM (2014) Metabolic determinants of cancer cell sensitivity to glucose limitation and biguanides. Nature 508:108–112

Bjelakovic G, Gluud C (2007) Surviving antioxidant supplements. J Natl Cancer Inst 99:742–743

Blanchard JL, Schmidt GW (1996) Mitochondrial DNA migration events in yeast and humans: integration by a common end-joining mechanism and alternative perspectives on nucleotide substitution patterns. Mol Biol Evol 13:537–548

Boland ML, Chourasia AH, Macleod KF (2013) Mitochondrial dysfunction in cancer. Front Oncol 3:292

Brand MD (2010) The sites and topology of mitochondrial superoxide production. Exp Gerontol 45:466–472

Brooks C, Wei Q, Feng L, Dong G, Tao Y, Mei L, Xie ZJ, Dong Z (2007) Bak regulates mitochondrial morphology and pathology during apoptosis by interacting with mitofusins. Proc Natl Acad Sci U S A 104:11649–11654

Canter JA, Kallianpur AR, Parl FF, Millikan RC (2005) Mitochondrial DNA G10398A polymorphism and invasive breast cancer in African-American women. Cancer Res 65:8028–8033

Cantley LC (2002) The phosphoinositide 3-kinase pathway. Science 296:1655–1657

Cesari R, Martin ES, Calin GA, Pentimalli F, Bichi R, McAdams H, Trapasso F, Drusco A, Shimizu M, Masciullo V et al (2003) Parkin, a gene implicated in autosomal recessive juvenile parkinsonism, is a candidate tumor suppressor gene on chromosome 6q25-q27. Proc Natl Acad Sci U S A 100:5956–5961

Chacinska A, Koehler CM, Milenkovic D, Lithgow T, Pfanner N (2009) Importing mitochondrial proteins: machineries and mechanisms. Cell 138:628–644

Chatterjee A, Mambo E, Sidransky D (2006) Mitochondrial DNA mutations in human cancer. Oncogene 25:4663–4674

Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ (2003) Production of reactive oxygen species by mitochondria: central role of complex III. J Biol Chem 278:36027–36031

Chen Z, Zhang H, Lu W, Huang P (2009) Role of mitochondria-associated hexokinase II in cancer cell death induced by 3-bromopyruvate. Biochim Biophys Acta 1787:553–560

Chinnery PF, Samuels DC, Elson J, Turnbull DM (2002) Accumulation of mitochondrial DNA mutations in ageing, cancer, and mitochondrial disease: is there a common mechanism? Lancet 360:1323–1325

Chourasia AH, Macleod KF (2015) Tumor suppressor functions of BNIP3 and mitophagy. Autophagy 11:1937–1938

Christofk HR, Vander Heiden MG, Wu N, Asara JM, Cantley LC (2008) Pyruvate kinase M2 is a phosphotyrosine-binding protein. Nature 452:181–186

Cormio A, Milella F, Vecchiet J, Felzani G, Gadaleta MN, Cantatore P (2005) Mitochondrial DNA mutations in RRF of healthy subjects of different age. Neurobiol Aging 26:655–664

Dang CV (2010) Rethinking the Warburg effect with Myc micromanaging glutamine metabolism. Cancer Res 70:859–862

Detmer SA, Chan DC (2007) Functions and dysfunctions of mitochondrial dynamics. Nat Rev Mol Cell Biol 8:870–879

Dominy JE Jr, Lee Y, Gerhart-Hines Z, Puigserver P (2010) Nutrient-dependent regulation of PGC-1alpha's acetylation state and metabolic function through the enzymatic activities of Sirt1/GCN5. Biochim Biophys Acta 1804:1676–1683

Don AS, Kisker O, Dilda P, Donoghue N, Zhao X, Decollogne S, Creighton B, Flynn E, Folkman J, Hogg PJ (2003) A peptide trivalent arsenical inhibits tumor angiogenesis by perturbing mitochondrial function in angiogenic endothelial cells. Cancer Cell 3:497–509

El-Mir MY, Nogueira V, Fontaine E, Averet N, Rigoulet M, Leverve X (2000) Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. J Biol Chem 275:223–228

Fliss MS, Usadel H, Caballero OL, Wu L, Buta MR, Eleff SM, Jen J, Sidransky D (2000) Facile detection of mitochondrial DNA mutations in tumors and bodily fluids. Science 287:2017–2019

Fujiwara M, Marusawa H, Wang HQ, Iwai A, Ikeuchi K, Imai Y, Kataoka A, Nukina N, Takahashi R, Chiba T (2008) Parkin as a tumor suppressor gene for hepatocellular carcinoma. Oncogene 27:6002–6011

Gaglio D, Metallo CM, Gameiro PA, Hiller K, Danna LS, Balestrieri C, Alberghina L, Stephanopoulos G, Chiaradonna F (2011) Oncogenic K-Ras decouples glucose and glutamine metabolism to support cancer cell growth. Mol Syst Biol 7:523

Gao P, Tchernyshyov I, Chang TC, Lee YS, Kita K, Ochi T, Zeller KI, De Marzo AM, Van Eyk JE, Mendell JT et al (2009) c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. Nature 458:762–765

Gatenby RA, Gillies RJ (2004) Why do cancers have high aerobic glycolysis? Nat Rev Cancer 4:891–899

Glasauer A, Sena LA, Diebold LP, Mazar AP, Chandel NS (2014) Targeting SOD1 reduces experimental non-small-cell lung cancer. J Clin Invest 124:117–128

Grivell LA (1983) Mitochondrial DNA. Sci Am 248:78–89

Habano W, Sugai T, Yoshida T, Nakamura S (1999) Mitochondrial gene mutation, but not large-scale deletion, is a feature of colorectal carcinomas with mitochondrial microsatellite instability. Int J Cancer 83:625–629

Hagen TM, Huang S, Curnutte J, Fowler P, Martinez V, Wehr CM, Ames BN, Chisari FV (1994) Extensive oxidative DNA damage in hepatocytes of transgenic mice with chronic active hepatitis destined to develop hepatocellular carcinoma. Proc Natl Acad Sci U S A 91:12808–12812

Hamanaka RB, Chandel NS (2010) Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes. Trends Biochem Sci 35:505–513

Hanna RA, Quinsay MN, Orogo AM, Giang K, Rikka S, Gustafsson AB (2012) Microtubule-associated protein 1 light chain 3 (LC3) interacts with Bnip3 protein to selectively remove endoplasmic reticulum and mitochondria via autophagy. J Biol Chem 287:19094–19104

Hu J, Hwang SS, Liesa M, Gan B, Sahin E, Jaskelioff M, Ding Z, Ying H, Boutin AT, Zhang H et al (2012) Antitelomerase therapy provokes ALT and mitochondrial adaptive mechanisms in cancer. Cell 148:651–663

Irani K, Xia Y, Zweier JL, Sollott SJ, Der CJ, Fearon ER, Sundaresan M, Finkel T, Goldschmidt-Clermont PJ (1997) Mitogenic signaling mediated by oxidants in Ras-transformed fibroblasts. Science 275:1649–1652

Israelsen WJ, Dayton TL, Davidson SM, Fiske BP, Hosios AM, Bellinger G, Li J, Yu Y, Sasaki M, Horner JW et al (2013) PKM2 isoform-specific deletion reveals a differential requirement for pyruvate kinase in tumor cells. Cell 155:397–409

Jeronimo C, Nomoto S, Caballero OL, Usadel H, Henrique R, Varzim G, Oliveira J, Lopes C, Fliss MS, Sidransky D (2001) Mitochondrial mutations in early stage prostate cancer and bodily fluids. Oncogene 20:5195–5198

Jones JB, Song JJ, Hempen PM, Parmigiani G, Hruban RH, Kern SE (2001) Detection of mitochondrial DNA mutations in pancreatic cancer offers a "mass"-ive advantage over detection of nuclear DNA mutations. Cancer Res 61:1299–1304

Jung SN, Yang WK, Kim J, Kim HS, Kim EJ, Yun H, Park H, Kim SS, Choe W, Kang I et al (2008) Reactive oxygen species stabilize hypoxia-inducible factor-1 alpha protein and stimulate transcriptional activity via AMP-activated protein kinase in DU145 human prostate cancer cells. Carcinogenesis 29:713–721

Kang N, Zhang JH, Qiu F, Tashiro S, Onodera S, Ikejima T (2010) Inhibition of EGFR signaling augments oridonin-induced apoptosis in human laryngeal cancer cells via enhancing oxidative stress coincident with activation of both the intrinsic and extrinsic apoptotic pathways. Cancer Lett 294:147–158

Karbowski M, Youle RJ (2003) Dynamics of mitochondrial morphology in healthy cells and during apoptosis. Cell Death Differ 10:870–880

Kelly PN, Strasser A (2011) The role of Bcl-2 and its pro-survival relatives in tumourigenesis and cancer therapy. Cell Death Differ 18:1414–1424

Kim JW, Tchernyshyov I, Semenza GL, Dang CV (2006) HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. Cell Metab 3:177–185

Koppenol WH, Bounds PL, Dang CV (2011) Otto Warburg's contributions to current concepts of cancer metabolism. Nat Rev Cancer 11:325–337

Larman TC, DePalma SR, Hadjipanayis AG, Cancer Genome Atlas Research Network, Protopopov A, Zhang J, Gabriel SB, Chin L, Seidman CE, Kucherlapati R et al (2012) Spectrum of somatic mitochondrial mutations in five cancers. Proc Natl Acad Sci U S A 109:14087–14091

Le A, Lane AN, Hamaker M, Bose S, Gouw A, Barbi J, Tsukamoto T, Rojas CJ, Slusher BS, Zhang H et al (2012) Glucose-independent glutamine metabolism via TCA cycling for proliferation and survival in B cells. Cell Metab 15:110–121

Lee SR, Yang KS, Kwon J, Lee C, Jeong W, Rhee SG (2002) Reversible inactivation of the tumor suppressor PTEN by H2O2. J Biol Chem 277:20336–20342

Leslie NR, Bennett D, Lindsay YE, Stewart H, Gray A, Downes CP (2003) Redox regulation of PI 3-kinase signalling via inactivation of PTEN. EMBO J 22:5501–5510

Li F, Wang Y, Zeller KI, Potter JJ, Wonsey DR, O'Donnell KA, Kim JW, Yustein JT, Lee LA, Dang CV (2005) Myc stimulates nuclearly encoded mitochondrial genes and mitochondrial biogenesis. Mol Cell Biol 25:6225–6234

Li H, Chen Y, Jones AF, Sanger RH, Collis LP, Flannery R, McNay EC, Yu T, Schwarzenbacher R, Bossy B et al (2008) Bcl-xL induces Drp1-dependent synapse formation in cultured hippocampal neurons. Proc Natl Acad Sci U S A 105:2169–2174

Lightowlers RN, Chinnery PF, Turnbull DM, Howell N (1997) Mammalian mitochondrial genetics: heredity, heteroplasmy and disease. Trends Genet 13:450–455

Liou GY, Storz P (2010) Reactive oxygen species in cancer. Free Radic Res 44:479–496

Liu Z, Li D, Zheng X, Wang E, Wang J (2013) Selective induction of apoptosis: promising therapy in pancreatic cancer. Curr Pharm Des 19:2259–2268

Luciakova K, Kuzela S (1992) Increased steady-state levels of several mitochondrial and nuclear gene transcripts in rat hepatoma with a low content of mitochondria. Eur J Biochem 205:1187–1193

Margineantu DH, Gregory Cox W, Sundell L, Sherwood SW, Beechem JM, Capaldi RA (2002) Cell cycle dependent morphology changes and associated mitochondrial DNA redistribution in mitochondria of human cell lines. Mitochondrion 1:425–435

Mathupala SP, Ko YH, Pedersen PL (2006) Hexokinase II: cancer's double-edged sword acting as both facilitator and gatekeeper of malignancy when bound to mitochondria. Oncogene 25:4777–4786

Maximo V, Soares P, Lima J, Cameselle-Teijeiro J, Sobrinho-Simoes M (2002) Mitochondrial DNA somatic mutations (point mutations and large deletions) and mitochondrial DNA variants in human thyroid pathology: a study with emphasis on Hurthle cell tumors. Am J Pathol 160:1857–1865

Mitra K, Wunder C, Roysam B, Lin G, Lippincott-Schwartz J (2009) A hyperfused mitochondrial state achieved at G1-S regulates cyclin E buildup and entry into S phase. Proc Natl Acad Sci U S A 106:11960–11965

Mitsuishi Y, Motohashi H, Yamamoto M (2012) The Keap1-Nrf2 system in cancers: stress response and anabolic metabolism. Front Oncol 2:200

Montessuit S, Somasekharan SP, Terrones O, Lucken-Ardjomande S, Herzig S, Schwarzenbacher R, Manstein DJ, Bossy-Wetzel E, Basanez G, Meda P et al (2010) Membrane remodeling induced by the dynamin-related protein Drp1 stimulates Bax oligomerization. Cell 142:889–901

Morrish F, Hockenbery D (2014) MYC and mitochondrial biogenesis. Cold Spring Harb Perspect Med 4

Nagaraj R, Gururaja-Rao S, Jones KT, Slattery M, Negre N, Braas D, Christofk H, White KP, Mann R, Banerjee U (2012) Control of mitochondrial structure and function by the Yorkie/YAP oncogenic pathway. Genes Dev 26:2027–2037

Narendra D, Tanaka A, Suen DF, Youle RJ (2008) Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. J Cell Biol 183:795–803

Narendra D, Kane LA, Hauser DN, Fearnley IM, Youle RJ (2010) p62/SQSTM1 is required for Parkin-induced mitochondrial clustering but not mitophagy; VDAC1 is dispensable for both. Autophagy 6:1090–1106

Neuzil J, Dyason JC, Freeman R, Dong LF, Prochazka L, Wang XF, Scheffler I, Ralph SJ (2007) Mitocans as anti-cancer agents targeting mitochondria: lessons from studies with vitamin E analogues, inhibitors of complex II. J Bioenerg Biomembr 39:65–72

Okami J, Simeone DM, Logsdon CD (2004) Silencing of the hypoxia-inducible cell death protein BNIP3 in pancreatic cancer. Cancer Res 64:5338–5346

Ostman A, Frijhoff J, Sandin A, Bohmer FD (2011) Regulation of protein tyrosine phosphatases by reversible oxidation. J Biochem 150:345–356

Owen MR, Doran E, Halestrap AP (2000) Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. Biochem J 348 (Pt 3):607–614

Owusu-Ansah E, Banerjee U (2009) Reactive oxygen species prime Drosophila haematopoietic progenitors for differentiation. Nature 461:537–541

Parker DJ, Iyer A, Shah S, Moran A, Hjelmeland AB, Basu MK, Liu R, Mitra K (2015) A new mitochondrial pool of cyclin E, regulated by Drp1, is linked to cell-density-dependent cell proliferation. J Cell Sci 128:4171–4182

Parrella P, Xiao Y, Fliss M, Sanchez-Cespedes M, Mazzarelli P, Rinaldi M, Nicol T, Gabrielson E, Cuomo C, Cohen D et al (2001) Detection of mitochondrial DNA mutations in primary breast cancer and fine-needle aspirates. Cancer Res 61:7623–7626

Pavlides S, Whitaker-Menezes D, Castello-Cros R, Flomenberg N, Witkiewicz AK, Frank PG, Casimiro MC, Wang C, Fortina P, Addya S et al (2009) The reverse Warburg effect: aerobic glycolysis in cancer associated fibroblasts and the tumor stroma. Cell Cycle 8:3984–4001

Petros JA, Baumann AK, Ruiz-Pesini E, Amin MB, Sun CQ, Hall J, Lim S, Issa MM, Flanders WD, Hosseini SH et al (2005) mtDNA mutations increase tumorigenicity in prostate cancer. Proc Natl Acad Sci U S A 102:719–724

Pollak M (2014) Overcoming drug development bottlenecks with repurposing: repurposing biguanides to target energy metabolism for cancer treatment. Nat Med 20:591–593

Polyak K, Li Y, Zhu H, Lengauer C, Willson JK, Markowitz SD, Trush MA, Kinzler KW, Vogelstein B (1998) Somatic mutations of the mitochondrial genome in human colorectal tumours. Nat Genet 20:291–293

Qian W, Choi S, Gibson GA, Watkins SC, Bakkenist CJ, Van Houten B (2012) Mitochondrial hyperfusion induced by loss of the fission protein Drp1 causes ATM-dependent G2/M arrest and aneuploidy through DNA replication stress. J Cell Sci 125:5745–5757

Rabinowitz JD, White E (2010) Autophagy and metabolism. Science 330:1344–1348

Raj L, Ide T, Gurkar AU, Foley M, Schenone M, Li X, Tolliday NJ, Golub TR, Carr SA, Shamji AF et al (2011) Selective killing of cancer cells by a small molecule targeting the stress response to ROS. Nature 475:231–234

Rambold AS, Kostelecky B, Elia N, Lippincott-Schwartz J (2011) Tubular network formation protects mitochondria from autophagosomal degradation during nutrient starvation. Proc Natl Acad Sci U S A 108:10190–10195

Rehman J, Zhang HJ, Toth PT, Zhang Y, Marsboom G, Hong Z, Salgia R, Husain AN, Wietholt C, Archer SL (2012) Inhibition of mitochondrial fission prevents cell cycle progression in lung cancer. FASEB J 26:2175–2186

Rossignol R, Gilkerson R, Aggeler R, Yamagata K, Remington SJ, Capaldi RA (2004) Energy substrate modulates mitochondrial structure and oxidative capacity in cancer cells. Cancer Res 64:985–993

Rumsey WL, Schlosser C, Nuutinen EM, Robiolio M, Wilson DF (1990) Cellular energetics and the oxygen dependence of respiration in cardiac myocytes isolated from adult rat. J Biol Chem 265:15392–15402

Sahin E, Colla S, Liesa M, Moslehi J, Muller FL, Guo M, Cooper M, Kotton D, Fabian AJ, Walkey C et al (2011) Telomere dysfunction induces metabolic and mitochondrial compromise. Nature 470:359–365

Salani B, Marini C, Rio AD, Ravera S, Massollo M, Orengo AM, Amaro A, Passalacqua M, Maffioli S, Pfeffer U et al (2013) Metformin impairs glucose consumption and survival in Calu-1 cells by direct inhibition of hexokinase-II. Sci Rep 3:2070

Sanchez-Cespedes M, Parrella P, Nomoto S, Cohen D, Xiao Y, Esteller M, Jeronimo C, Jordan RC, Nicol T, Koch WM et al (2001) Identification of a mononucleotide repeat as a major target for mitochondrial DNA alterations in human tumors. Cancer Res 61:7015–7019

Sasaki R, Suzuki Y, Yonezawa Y, Ota Y, Okamoto Y, Demizu Y, Huang P, Yoshida H, Sugimura K, Mizushina Y (2008) DNA polymerase gamma inhibition by vitamin K3 induces mitochondria-mediated cytotoxicity in human cancer cells. Cancer Sci 99:1040–1048

Scarpulla RC, Vega RB, Kelly DP (2012) Transcriptional integration of mitochondrial biogenesis. Trends Endocrinol Metab 23:459–466

Schumacker PT (2006) Reactive oxygen species in cancer cells: live by the sword, die by the sword. Cancer Cell 10:175–176

Sena LA, Chandel NS (2012) Physiological roles of mitochondrial reactive oxygen species. Mol Cell 48:158–167

Shaw AT, Winslow MM, Magendantz M, Ouyang C, Dowdle J, Subramanian A, Lewis TA, Maglathin RL, Tolliday N, Jacks T (2011) Selective killing of K-ras mutant cancer cells by small molecule inducers of oxidative stress. Proc Natl Acad Sci U S A 108:8773–8778

Sherr CJ, McCormick F (2002) The RB and p53 pathways in cancer. Cancer Cell 2:103–112

Shimoda R, Nagashima M, Sakamoto M, Yamaguchi N, Hirohashi S, Yokota J, Kasai H (1994) Increased formation of oxidative DNA damage, 8-hydroxydeoxyguanosine, in human livers with chronic hepatitis. Cancer Res 54:3171–3172

Skrtic M, Sriskanthadevan S, Jhas B, Gebbia M, Wang X, Wang Z, Hurren R, Jitkova Y, Gronda M, Maclean N et al (2011) Inhibition of mitochondrial translation as a therapeutic strategy for human acute myeloid leukemia. Cancer Cell 20:674–688

Sonveaux P, Vegran F, Schroeder T, Wergin MC, Verrax J, Rabbani ZN, De Saedeleer CJ, Kennedy KM, Diepart C, Jordan BF et al (2008) Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice. J Clin Invest 118:3930–3942

Sowter HM, Ferguson M, Pym C, Watson P, Fox SB, Han C, Harris AL (2003) Expression of the cell death genes BNip3 and NIX in ductal carcinoma in situ of the breast; correlation of BNip3 levels with necrosis and grade. J Pathol 201:573–580

Tan EY, Campo L, Han C, Turley H, Pezzella F, Gatter KC, Harris AL, Fox SB (2007) BNIP3 as a progression marker in primary human breast cancer; opposing functions in in situ versus invasive cancer. Clin Cancer Res 13:467–474

Tondera D, Grandemange S, Jourdain A, Karbowski M, Mattenberger Y, Herzig S, Da Cruz S, Clerc P, Raschke I, Merkwirth C et al (2009) SLP-2 is required for stress-induced mitochondrial hyperfusion. EMBO J 28:1589–1600

Trachootham D, Alexandre J, Huang P (2009) Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nat Rev Drug Discov 8:579–591

Tracy K, Dibling BC, Spike BT, Knabb JR, Schumacker P, Macleod KF (2007) BNIP3 is an RB/E2F target gene required for hypoxia-induced autophagy. Mol Cell Biol 27:6229–6242

Twig G, Elorza A, Molina AJ, Mohamed H, Wikstrom JD, Walzer G, Stiles L, Haigh SE, Katz S, Las G et al (2008) Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. EMBO J 27:433–446

Umeda S, Muta T, Ohsato T, Takamatsu C, Hamasaki N, Kang D (2000) The D-loop structure of human mtDNA is destabilized directly by 1-methyl-4-phenylpyridinium ion (MPP+), a parkinsonism-causing toxin. Eur J Biochem 267:200–206

Van den Bogert C, Muus P, Haanen C, Pennings A, Melis TE, Kroon AM (1988) Mitochondrial biogenesis and mitochondrial activity during the progression of the cell cycle of human leukemic cells. Exp Cell Res 178:143–153

Vander Heiden MG, Cantley LC, Thompson CB (2009) Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science 324:1029–1033

Vazquez F, Lim JH, Chim H, Bhalla K, Girnun G, Pierce K, Clish CB, Granter SR, Widlund HR, Spiegelman BM et al (2013) PGC1alpha expression defines a subset of human melanoma tumors with increased mitochondrial capacity and resistance to oxidative stress. Cancer Cell 23:287–301

Vousden KH, Prives C (2009) Blinded by the light: the growing complexity of p53. Cell 137:413–431

Wallace DC (2012) Mitochondria and cancer. Nat Rev Cancer 12:685–698

Warburg O (1956) On respiratory impairment in cancer cells. Science 124:269–270

Wenz T (2013) Regulation of mitochondrial biogenesis and PGC-1alpha under cellular stress. Mitochondrion 13:134–142

Wong LJ, Lueth M, Li XN, Lau CC, Vogel H (2003) Detection of mitochondrial DNA mutations in the tumor and cerebrospinal fluid of medulloblastoma patients. Cancer Res 63:3866–3871

Yarosh W, Monserrate J, Tong JJ, Tse S, Le PK, Nguyen K, Brachmann CB, Wallace DC, Huang T (2008) The molecular mechanisms of OPA1-mediated optic atrophy in Drosophila model and prospects for antioxidant treatment. PLoS Genet 4, e6

Youle RJ, Narendra DP (2011) Mechanisms of mitophagy. Nat Rev Mol Cell Biol 12:9–14

Youle RJ, Strasser A (2008) The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol 9:47–59

Yuan P, Ito K, Perez-Lorenzo R, Del Guzzo C, Lee JH, Shen CH, Bosenberg MW, McMahon M, Cantley LC, Zheng B (2013) Phenformin enhances the therapeutic benefit of BRAF(V600E) inhibition in melanoma. Proc Natl Acad Sci U S A 110:18226–18231

Zhang J, Ney PA (2009) Role of BNIP3 and NIX in cell death, autophagy, and mitophagy. Cell Death Differ 16:939–946

Zhang H, Gao P, Fukuda R, Kumar G, Krishnamachary B, Zeller KI, Dang CV, Semenza GL (2007) HIF-1 inhibits mitochondrial biogenesis and cellular respiration in VHL-deficient renal cell carcinoma by repression of C-MYC activity. Cancer Cell 11:407–420

Zhang C, Lin M, Wu R, Wang X, Yang B, Levine AJ, Hu W, Feng Z (2011) Parkin, a p53 target gene, mediates the role of p53 in glucose metabolism and the Warburg effect. Proc Natl Acad Sci U S A 108:16259–16264

Zhang X, Fryknas M, Hernlund E, Fayad W, De Milito A, Olofsson MH, Gogvadze V, Dang L, Pahlman S, Schughart LA et al (2014) Induction of mitochondrial dysfunction as a strategy for targeting tumour cells in metabolically compromised microenvironments. Nat Commun 5:3295

Zu XL, Guppy M (2004) Cancer metabolism: facts, fantasy, and fiction. Biochem Biophys Res Commun 313:459–465

The Emerging Role of Mitochondrial Targeting in Kidney Disease

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H. Singh, S.-S. Sheu (eds.), *Pharmacology of Mitochondria*,
Handbook of Experimental Pharmacology 240, DOI 10.1007/164_2016_6

Abstract

Renal disease affects millions of people worldwide, imposing an enormous financial burden for health-care systems. Recent evidence suggests that mitochondria play an important role in the pathogenesis of different forms of renal disease, including genetic defects, acute kidney injury, chronic kidney disease, aging, renal tumors, and transplant nephropathy. Renal mitochondrial abnormalities and dysfunction affect several cellular pathways, leading to increased oxidative stress, apoptosis, microvascular loss, and fibrosis, all of which compromise renal function. Over recent years, compounds that specifically target mitochondria have emerged as promising therapeutic options for patients with renal disease. Although the most compelling evidence is based on preclinical studies, several compounds are currently being tested in clinical trials. This chapter provides an overview of the involvement of mitochondrial dysfunction in renal disease and summarizes the current knowledge on mitochondria-targeted strategies to attenuate renal disease.

Keywords

ATP • Cardiolipin • Kidney • Mitochondria

1 Introduction

Renal disease encompasses acute or chronic conditions that damage the kidneys and reduce their function. It remains an increasing public health issue that affects a significant proportion of the world's population and is associated with tremendous medical costs (Jha et al. 2013). Furthermore, its short-term and long-term complications increase cardiovascular and all-cause morbidity and mortality rates. For example, acute kidney injury (AKI), characterized by a rapid loss of renal function, may result in fluid overload, electrolyte abnormalities, and coagulopathy, which might contribute to multi-organ failure. Likewise, gradual loss of kidney function, known as chronic kidney disease (CKD), is associated with grave complications, including cardiovascular disease, anemia, mineral and bone disorders, and cognitive decline. Moreover, congenital and inherited disorders, tumors, and aging may compromise kidney function and adversely impact several organ systems. Ultimately, all these conditions can progress toward end-stage renal disease (ESRD), requiring costly and renal replacement therapy. Alas, adequate strategies to prevent progressive renal dysfunction are in dire need.

The kidney receives 20% of the cardiac output and utilizes 10% of body oxygen consumption to accomplish its primary function, regulating the body fluid composition through filtering and reabsorbing materials. This process occurs at the level of the nephron, the functional unit of the kidney, by both energy-dependent and independent mechanisms. The most energy demanding process of the kidney is reabsorption of solutes, which occurs both passively and actively by the renal

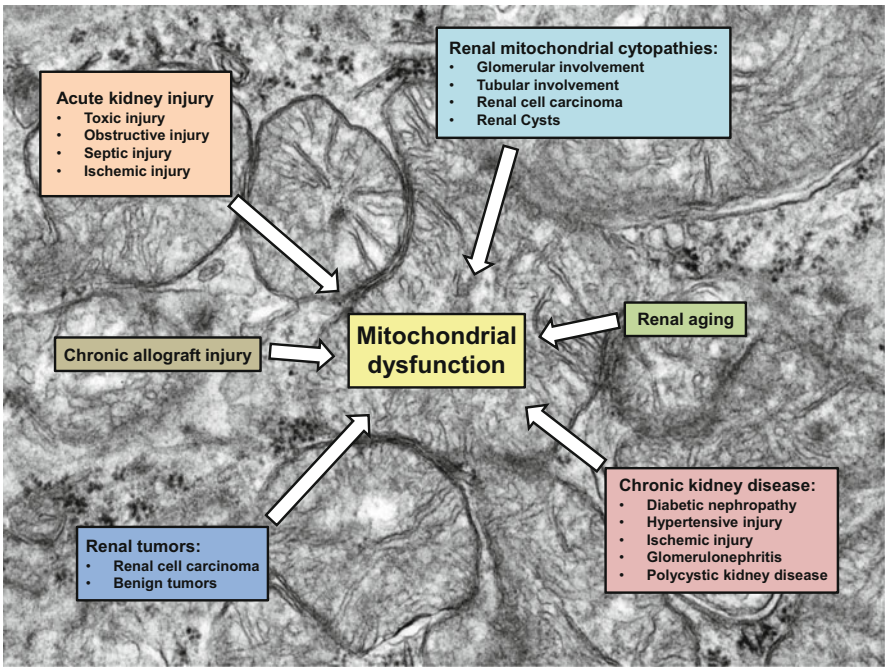

Fig. 1 Representative transmission electron microscopy showing swollen mitochondria with loss of cristae and matrix in swine renovascular disease. Mitochondrial damage and dysfunction have been implicated in several renal conditions

tubular cells, which consume adenosine triphosphate (ATP) generated exclusively by aerobic metabolism. Congruently, tubular cells are rich in mitochondria, and mitochondrial injury and dysfunction bear harmful consequences on multiple renal cell functions.

Accordingly, increasing evidence indicates that mitochondrial damage and dysfunction in renal disease (Fig. 1) may contribute to the multiple underlying pathological processes (Che et al. 2014). Acute or chronic insults might compromise mitochondrial structure, evoking mitochondrial DNA (mtDNA) damage, decreased matrix density, and/or impaired outer and inner membrane integrity. Furthermore, renal disease has been associated with changes in mitochondrial homeostasis, the molecular control of mitochondrial formation (biogenesis), morphology (fusion/fission), and degradation (mitophagy). Finally, mitochondrial abnormalities and impaired homeostasis lead to bioenergetic dysfunction (reduced ATP generation and calcium signaling), triggering oxidative stress and apoptosis. Therefore, over the past few years, mitochondria have emerged as novel therapeutic targets in renal diseases. Small molecules that specifically concentrate within mitochondria include mitochondrial permeability transition pore (mPTP) inhibitors, antioxidants, biogenesis activators, fission inhibitors, gene therapy, and cardiolipin protection (Tabara et al. 2014). The efficacy of these compounds has been tested in several in vitro and in vivo experimental studies, as well as in few

clinical trials. In this chapter, we evaluated and summarized evidence implicating mitochondrial dysfunction in the pathogenesis of renal disease, with particular attention to studies testing the potential of promising mitochondria-targeted therapies for ameliorating renal injury and dysfunction.

2 Evidence of Mitochondrial Injury in Renal Disease

2.1 Mitochondrial Genetic Defects

Mitochondrial cytopathies (MCs) encompass a group of disorders characterized by mitochondrial or nuclear DNA mutations in genes encoding for mitochondrial proteins. MCs can affect any organ, but have predilection for those dependent upon mitochondrial energy supply (Finsterer 2004). Mutations resulting in MCs could be either inherited (primary MCs) or imposed by exogenous factors (secondary MCs), such as drugs or increased oxidative stress. In the kidney, MCs commonly manifest as glomerular disease, tubular dysfunction, renal cysts, or neoplasia.

A point mutation in the mitochondrial tRNAleu (UUR) at position 3243 (A3243G) has been associated with focal and segmental glomerulosclerosis (FSGS) (Dinour et al. 2004), an important cause of nephrotic syndrome in children and adolescents that frequently progresses to ESRD. This mutation causes mitochondrial myopathy, encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) syndrome, as well as maternally inherited diabetes and deafness. Renal biopsies of patients with MELAS-associated FSGS reveal numerous abnormal mitochondria in tubular cells and podocytes, associated with severe effacement of foot processes (Gucer et al. 2005), implicating mitochondrial alterations in the pathogenesis of FSGS.

MCs have been also linked to renal tubular dysfunction, which mainly manifest as Bartter-like or Fanconi syndromes. The most common tubular defect associated with MCs is Toni-Debré-Fanconi syndrome, a rare disorder characterized by impaired tubular reabsorption. Mutations and large mtDNA deletions have been reported in patients with this syndrome (Lee et al. 2012), associated with mitochondrial respiratory complex defects and giant atypical mitochondria (Au et al. 2007). Kearns–Sayre syndrome, an MC caused by deletions of mtDNA and characterized by isolated involvement of the muscles controlling eyelid movement, may present with renal involvement resembling Barter syndrome (Emma et al. 2006). Patients with Barter syndrome suffer from electrolyte abnormalities due to mutations in ion transporters, which impair the ability to reabsorb potassium. Renal biopsies of patients with Kearns–Sayre and Bartter-like syndrome show ultrastructural changes in mitochondria in the thick ascending loop of Henle, associated with impaired cytochrome-c oxidase (COX) activity and fibrosis (Goto et al. 1990), implicating mitochondrial structural and functional impairment in the pathogenesis of tubular derangements.

Bilateral enlarged cystic kidneys have been also documented in patients with mitochondrial cytopathies including mutations of the mitochondrial tRNA genes (Guery et al. 2003). Furthermore, glomerulocystic kidneys have been reported in association with Leigh disease, a MC caused by mutations in the Surfeit locus protein-1 gene and COX assembly factors (Lake et al. 2015).

Lastly, MCs might coexist with renal tumors. Rare cases of patients with MELAS associated with renal cell carcinoma (RCC) have been previously reported. Mutations of components of the mitochondrial oxidative phosphorylation complex have been described in benign and malignant renal tumors (Housley et al. 2010; Ricketts et al. 2008). Furthermore, a high mutational rate of the mtDNA has been observed in benign renal tumors (Gasparre et al. 2008) and tumors arising in ESRD (Nagy et al. 2003), implicating MCs in renal tumorigenesis.

2.2 AKI

AKI has increased in incidence over the last decades and is currently responsible for 2% of hospitalized patients in the USA. AKI may result from prerenal (hypoperfusion), renal (intrinsic damage), or post-renal (urinary tract/venous obstruction) causes that trigger a rapid decline in GFR, associated with tubular necrosis, vascular changes, and interstitial inflammation. Evidence suggests that mitochondrial damage is associated with important events in the pathogenesis of several etiologies of AKI, including toxic, ischemic, septic, and hypertensive injury.

Toxic Injury Several studies have documented mitochondrial structural and functional changes in kidneys exposed to exogenous drugs or toxins. Cyclosporine (Yuan et al. 2005) and cisplatin (Zsengeller et al. 2012) nephrotoxicity is characterized by decreased mitochondrial mass, disruption of cristae, and extensive mitochondrial swelling, as shown in murine studies. Similarly, kidney mtDNA depletion and ultrastructural mitochondrial abnormalities were reported in human immunodeficiency virus-infected patients treated with antiretroviral therapy (Cote et al. 2006).

Renal mitochondrial structural abnormalities are often associated with impaired bioenergetics. Cisplatin-induced renal mitochondrial injury in mice is accompanied by reduced nicotinamide adenine dinucleotide dehydrogenase (NADH) and COX activity, indicating impaired mitochondrial function (Mukhopadhyay et al. 2012). Likewise, mtDNA depletion and loss of mitochondrial mass are associated with decreased COX efficiency in patients treated with the antiretroviral drug tenofovir (Lopez et al. 2006).

Nephrotoxic drugs can also compromise mitochondrial homeostasis. Renal mitochondrial biogenesis is suppressed in folic acid-induced AKI, disclosed by decreased expression of its master regulator, peroxisome proliferator gamma coactivator 1α (PGC-1α) (Stallons et al. 2014). In addition, expression of

dynamin-related protein (DRP)-1, which mediates outer mitochondrial membrane fission, is markedly upregulated in mice kidneys with glycerol-induced AKI, suggesting mitochondrial fragmentation (Funk and Schnellmann 2012). Moreover, expression of the autophagic marker microtubule-associated protein 1A-/1B-light chain 3 (LC3) is elevated in these animals, implying mitochondrial degradation.

Drug-induced changes in mitochondrial structure, function, and homeostasis may promote apoptosis. In rodent models of cisplatin-induced nephrotoxicity, mitochondrial outer membrane permeabilization triggers mitochondrial fragmentation, cytochrome-c release, and apoptosis (Brooks et al. 2009). Renal tubular epithelial cells of rats exposed to the organic compound ethylbenzene show damaged mitochondria with vacuolar structure, associated with increased numbers of apoptotic cells, and upregulated expression of the apoptogenic factor cytochrome-c, suggesting mitochondria-mediated renal tubular cell apoptosis (Zhang et al. 2010).

Obstructive Injury Obstruction in the urinary tract below the kidneys is a frequent problem, but accounts for only 5–10% of AKI. Tubular atrophy and nephron loss due to unilateral ureteral obstruction in rodents is associated with increased mitochondrial hydrogen peroxide production, autophagy, and apoptosis (Xu et al. 2013). These changes were confirmed by in vitro studies in renal tubular cells exposed to oxalate, a major component of kidney stones, demonstrating that obstructive AKI induce parallel autophagy and mitochondrial dysfunction-mediated apoptosis (Cao et al. 2004).

Septic Injury Sepsis is a frequent cause of AKI in critically ill patients. Mitochondrial damage is thought to play an important role in the pathogenesis of septic AKI (Parikh et al. 2015). For example, *Staphylococcus aureus*-induced sepsis damages renal mDNA in the mouse kidney, leading to induction of the nuclear program of mitochondrial biogenesis (Bartz et al. 2014). Similarly, administration of lipopolysaccharide in mice increases renal tubular cytochrome-c release into the cytosol and active caspase-3 expression, implying mitochondria-dependent apoptosis (Stoyanoff et al. 2014). Renal tubular cells from septic mice show mitochondrial ultrastructural changes and reduced expression of COX (Choi et al. 2013), which may contribute to renal tubular cell apoptosis and AKI in sepsis.

Ischemic Injury An abrupt interruption or decrease in renal oxygen supply and ischemia–reperfusion injury (IRI) are the most common causes of AKI and kidney allograft dysfunction. Several studies suggest that mitochondrial damage plays a pivotal role in ischemic AKI, contributing to renal dysfunction. Mitochondria respiratory capacity is significantly reduced in rats with uncontrolled hemorrhagic shock (Li et al. 2012b), associated with increased mitochondrial reactive oxygen species (ROS) and lipid peroxidation (Wang et al. 2015a).

Renal IRI in rats is characterized by rounded, swollen renal tubular cell mitochondria with disrupted cristae membranes and release of matrix materials into the cytosol (Szeto et al. 2011). Mitochondrial respiration and ATP production

decreases, whereas oxidative stress increases, suggesting mitochondrial structural and functional decline. Autophagy and mitophagy are activated in both in vivo and in vitro models of renal IRI (Ishihara et al. 2013). We have shown in swine renovascular disease that ongoing post-stenotic inflammatory and pro-fibrotic injury that renal revascularization fails to reverse is associated with impaired renal mitochondrial biogenesis, apoptosis, and oxidative stress, implicating mitochondrial homeostasis in the pathogenesis of renal IRI (Eirin et al. 2012). Finally, IRI in kidney transplants has been associated with increased renal tubular expression of proapoptotic molecules and diffuse cytosolic distribution of cytochrome-c, suggesting activation of mitochondria-dependent apoptosis (Castaneda et al. 2003).

2.3 CKD

The prevalence of CKD is estimated to be 8–16% worldwide and is associated with catastrophic health expenditures. Importantly, many uremic conditions are associated with changes in mitochondrial structure and dysfunction.

Diabetic Nephropathy Diabetic kidney disease is the leading cause of CKD, accounting for 42% of patients on ESRD. Importantly, severity of CKD predicts all-cause mortality in type-1 and type-2 diabetes mellitus. Studies have suggested that mitochondrial abnormalities and dysfunction might favor the development and progression of diabetic nephropathy.

Apoptotic tubular cells and dysmorphic mitochondria were observed in the kidneys of diabetic mice, associated with decreased mtDNA content and altered mitochondrial function (Sun et al. 2008). COX-III activity is significantly decreased and contributes to oxidant production in diabetic renal mitochondria (Munusamy et al. 2009). In agreement, diabetic mice show decreased renal mitochondrial ATP production and excess generation of superoxide (Tan et al. 2010), a ROS with the ability of exacerbating renal mitochondrial dysfunction in hyperglycemic rats (Munusamy and MacMillan-Crow 2009). In line with this finding, studies in two murine models of type-1 and type-2 diabetes showed that glucose-induced mitochondrial ROS production initiates podocyte apoptosis in vitro and in vivo (Susztak et al. 2006). Moreover, polymorphisms in the mitochondrial antioxidant superoxide dismutase (SOD)-2 are associated with progressive renal functional decline in patients with type-1 diabetes (Mohammedi et al. 2014), suggesting that mitochondrial oxidative stress constitutes a major pathway resulting in diabetic renal injury.

Mitochondrial homeostasis seems to play an important role in diabetic nephropathy. Overexpression of the fusion marker mitofusin-2 attenuates pathological changes in the kidneys of diabetic rats (Tang et al. 2012). Likewise, renal expression of PGC-1α is downregulated in patients with both diabetes and CKD. Urine metabolome in patients with diabetic kidney disease reveals metabolites linked to mitochondrial metabolism and reduced mitochondrial content of urinary exosomes,

suggesting suppression of mitochondrial activity in diabetic kidney disease (Sharma et al. 2013).

Hypertensive Injury Experimental studies directly implicate mitochondrial injury in the development and progression of renal hypertension (Eirin et al. 2015). In kidneys from spontaneously hypertensive rats, mitochondrial membrane potential, nitric oxide synthase, COX activity, and mitochondrial uncoupling protein-2- content were reduced, suggesting that hypertension occurs in concurrence with a decline of kidney mitochondrial function (de Cavanagh et al. 2006). Furthermore, expression of SOD-2 is blunted in hypertensive rats, and its deficiency is associated with activation of intrarenal inflammatory and ROS-generating pathways (Jin and Vaziri 2014). Finally, a proteomic analysis of mitochondria isolated from medullary thick ascending limb cells identified seven differentially expressed proteins between hypertensive and control rats involved in mitochondrial metabolism and oxygen utilization (Zheleznova et al. 2012). These observations highlight the critical role of renal mitochondrial injury in the pathogenesis of hypertensive CKD, although it remains unknown whether mitochondrial abnormalities are primary or secondary to hypertension.

Ischemic Injury Chronic underperfusion of the renal parenchyma secondary to renal artery stenosis (RAS) is an important cause of CKD in the elderly population and has been linked to mitochondrial structural alterations and dysfunction. In the rat RAS model, necrotic death of tubular epithelial cells in the clipped kidneys is dependent on upregulation and mitochondrial translocation of the pro-mitophagy protein BCL2/adenovirus E1B 19 kDa protein-interacting protein-3, associated with impaired mitochondrial biogenesis, mass, and mtDNA copy number (Fedorova et al. 2013). Furthermore, in swine atherosclerotic RAS, the post-stenotic kidney exhibits loss of cardiolipin, a phospholipid exclusively distributed in the inner mitochondrial membrane that regulates mitochondrial structure and function (Klingenberg 2009), associated with apoptosis, oxidative stress, microvascular loss, fibrosis, and renal dysfunction (Eirin et al. 2014). Taken together, these studies implicate mitochondrial structural and functional alterations in the pathogenesis of ischemic CKD.

Glomerulonephritis Chronic glomerulonephritis accounts for approximately 10% of all causes of CKD. Accumulation of abnormal-shaped mitochondria are commonly found in podocytes, distal tubules, and collecting ducts of patients with genetically proven mitochondrial disease and secondary FSGS (Kobayashi et al. 2010). Mutation of pro-autophagic genes in mice during nephrogenesis causes podocyte and tubular cell mitochondrial abnormalities that precede the appearance of FSGS (Kawakami et al. 2015), suggesting that impaired autophagic mitochondrial turnover is sufficient to recapitulate the characteristic features of FSGS in mice.

Polycystic Kidney Disease (PKD) Comparative proteomics analysis implicates mitochondria in autosomal recessive PKD, a genetic disorder characterized by cyst development. Abnormally expressed proteins in PKD include proteins involved in biological processes related to signal transduction, cell cycle regulation, and electron transport, which play key roles in the pathogenesis of PKD (Li et al. 2012a). Notably, 13 of these proteins, including SOD-2, COX subunit Va, and peroxiredoxin-3, are localized in mitochondria, implying that mitochondrial dysfunction partly contributes to renal injury in PKD.

2.4 Renal Tumors

Tumors can originate from different renal cell types, and their incidence has increased in the last two decades. Mitochondrial damage has been suggested to be causally linked to benign renal tumors and RCCs (Hervouet and Godinot 2006; Hervouet et al. 2007). A recent clinical trial indicates that low mitochondrial DNA copy number in peripheral blood leukocytes is associated with significantly increased risk of clear cell RCC (Melkonian et al. 2015). Furthermore, decreased renal tumor expression of cytochrome-c and human 8-oxoguanine DNA glycosylase-1, a DNA repair protein located in the mitochondria, has been reported, implicating mitochondrial loss and defective DNA repair in tumor development or progression (Mukunyadzi et al. 2003). Mitochondrial dysfunction in patients with RCC correlates with oxidative phosphorylation complexes content and ATPase activity rather than to the mtDNA content, suggesting that decreased mitochondrial capacity primarily favors tumor invasiveness (Simonnet et al. 2002). Contrarily, the number of enzymes involved in mitochondrial energy metabolism is reduced in RCC, but does correlate with tumor grade, metastasis, or proliferative activity, implying that low renal mitochondrial activity is an early event in RCC formation (Meierhofer et al. 2004).

Renal oncocytoma is a rare and almost invariably benign tumor. Interestingly, renal oncocytomas show mitochondria with piled lamellar cristae, whereas chromophobe RCCs exhibit mitochondria with tubulovesicular cristae (Barcena et al. 2010). Despite increased COX activity, complex-I activity is decreased in renal oncocytomas (Simonnet et al. 2003), associated with increased number of mitochondrial vacuoles, suggesting increased mitophagy (Koller et al. 2000).

2.5 Aging

Aging is associated with gradual loss of function in the kidney, accompanied by mesangial expansion, glomerulosclerosis, and interstitial fibrosis. Aged rats show aging-associated ultrastructural changes in kidney mitochondria, disclosed by ill-defined cristae and reduced density, associated with increased mitochondrial hydrogen peroxide production and impaired respiratory control, antioxidant

activity, and uncoupling protein-2 levels (de Cavanagh et al. 2003). Furthermore, reduction of age-associated renal damage in mice chronically treated with angiotensin-converting enzyme inhibitors is accompanied by increased number of mitochondria in the proximal tubules (Ferder et al. 2002), implicating mitochondria in the pathogenesis of age-related kidney disease.

Importantly, mitochondria regulate permanent cell growth, modulating cellular senescence, leading to a state of irreversible growth arrest (Ziegler et al. 2015). In line with this notion, oxidative stress and cell senescence promote tubular cell apoptosis and mitochondrial dysfunction in vitro, impairing the kidney's regenerative potential (Small et al. 2012). In old rats, increased expression of markers of senescence, such as p16 and senescence-associated-galactosidase, is accompanied by decreased expression of autophagosome and mitophagy markers (Cui et al. 2013). Interestingly, these changes are exacerbated in animals fed with a high-calorie diet, but ameliorated in those with calorie restriction, suggesting that diet modulates mitochondrial degradation and recycling that occur in the aging kidney. In aged diabetic rats, oxidative stress promotes mitochondrial oxidative dysfunction, reflected as increased lipid peroxidation and decreased glutathione activity (Perez-Gallardo et al. 2014). Notably, nonsteroid anti-inflammatory drugs do not aggravate aging-induced injury (Rocha-Rodrigues et al. 2013).

2.6 Chronic Allograft Injury

Chronic allograft nephropathy, characterized by a slow decline in renal function more than three months posttransplant, remains one of the most common causes of ESRD. Several immunological risk factors for chronic allograft dysfunction have been suggested, yet non-immunological mediators of this progressive injury largely remain unknown. A recent study that analyzed gene expression microarray of kidney transplant biopsies taken one year after transplantation revealed a unique molecular signature of impaired mitochondrial function, characterized by inadequate mitochondrial energy generation, biogenesis, and antioxidant response (Zepeda-Orozco et al. 2015). These observations support development of mitochondria-targeted treatments to slow the progression of chronic allograft dysfunction.

3 Renal Mitochondrial Targeting

In recent years, several mitochondria-targeted strategies have been designed to prevent or attenuate renal disease (Fig. 2). Although their efficacy in human renal disease needs to be explored, several studies demonstrated their ability to attenuate renal injury in experimental animal models.

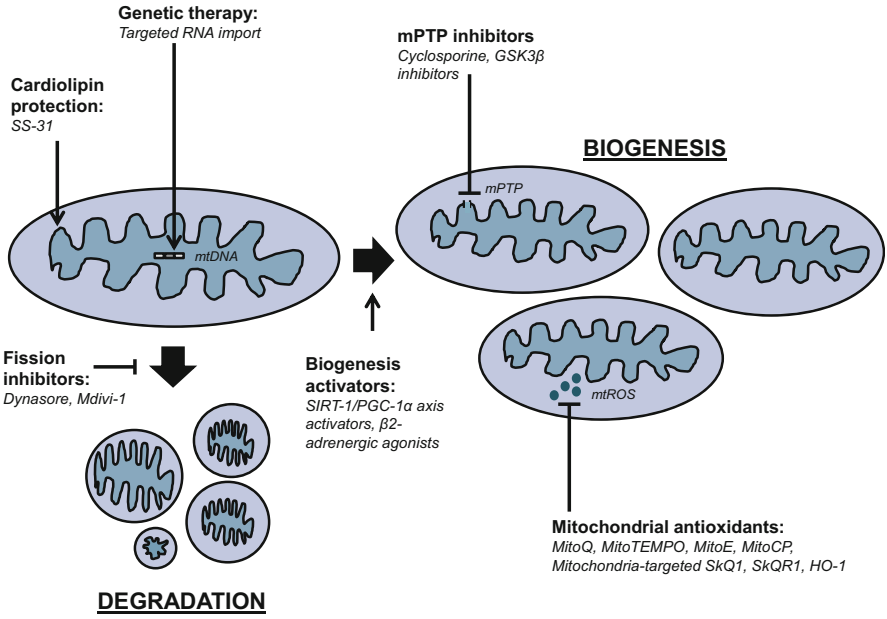

Fig. 2 Schematic of experimental therapeutic interventions that may ameliorate renal mitochondrial (mt) injury and dysfunction. *mPTP* mt permeability transition pore, *SS* Szeto–Schiller peptide, *PGC* peroxisome proliferator-activated receptor gamma coactivator, *GSK* glycogen synthase kinase, *Mdivi1* mt division inhibitor, *Mito* mitochondrial targeted, *Q* coenzyme-Q, *TEMPO* piperidine nitroxide, *E* α-tocopherol, *CP* nitroxide, *SkQ1* plastoquinonyl-decyl-triphenylphosphonium, *SkQR1* plastoquinonyl decylrhodamine 19, *HO* heme oxygenase

3.1 Genetic Therapy

Neutralizing deleterious mtDNA alterations using targeted mitochondrial RNA import is a novel and promising therapy for rescuing mitochondrial function in patients with MCs. Mitochondrial defects in cytoplasmic hybrid (cybrid) cells derived from patients with myoclonic epilepsy with ragged red fibers (MERRF) and MELAS can be partially rescued by targeted import of allotopically encoded wild-type tRNAs, an approach that specifically targets mRNA to the mitochondrial outer membrane (Wang et al. 2012). Notably, functional defects in mitochondrial RNA (mtRNA) translation and cell respiration were reversed in MERRF and MELAS cybrids cells. Similarly, mitochondrial targeting of recombinant tRNAs bearing the identity elements for human mitochondrial leucyl-tRNA synthetase rescues the phenotype caused by MELAS mutation in cultured transmitochondrial cybrid cells (Karicheva et al. 2011), whereas yeast tRNALys derivatives expressed in human immortalized cells and primary fibroblasts rescue mitochondrial functions in cultured cells from patients with the MERRF syndrome, underscoring the potential of these transcript engineering approaches to confer mitoprotection and mitigate renal injury in patients with MCs.

3.2 Biogenesis Activators

Synthesis and assembly of new mitochondria involve multiple coordinated processes tightly regulated by PGC-1α. Silent mating-type information regulation 2 homolog (SIRT)-1 is a NAD-dependent deacetylase that positively regulates PGC-1α activity and restores renal expression of PGC-1α, mitochondrial mass, ATP levels, and renal function in rats with ischemia–reperfusion injury (Funk and Schnellmann 2013; Khader et al. 2014). In line with this, treatment with the SIRT-1 activator resveratrol protects mice against aldosterone-induced podocyte injury by upregulating PGC-1α (Yuan et al. 2012). Resveratrol supplementation following hemorrhagic shock in rats also restores mitochondrial respiratory capacity and decreases mitochondrial ROS production and lipid peroxidation (Wang et al. 2015a), underscoring SIRT-1/PGC-1α axis activation as therapeutic approach.

Agonists for the β2-adrenoceptors induce mitochondrial biogenesis in both the renal proximal tubular cells and cardiomyocytes, disclosed by increased mtDNA copy numbers, oxygen consumption rate, and mRNA levels of PGC-1α and multiple genes involved in mitochondrial regulation (Wills et al. 2012). Moreover, the β2-adrenergic receptor agonist formoterol in mice with IRI-induced AKI restores renal function, rescues renal tubules from injury, and diminishes necrosis (Jesinkey et al. 2014). However, long-acting β2-adrenoceptor agonists, including formoterol, impair cardiac relaxation, mitochondrial protein synthesis, and oxidative capacity, limiting its clinical translation (Leger et al. 2011).

3.3 Mitochondrial Antioxidants

Mitochondrial ROS has been implicated in the pathogenesis of several types of renal disease, which often results from an imbalance between mitochondrial ROS production and antioxidant defenses. Thus, compounds that specifically target mitochondria may confer greater protection against renal injury due to increased mitochondrial ROS generation than untargeted cellular antioxidants such as vitamin E or N-acetylcysteine.

Several triphenylalkylphosphonium cation (TPP+)-conjugated antioxidants have been designed to reduce mitochondrial ROS. These positively charged compounds can cross the mitochondria-phospholipid bilayer and concentrate in their matrix in a membrane potential-dependent manner, where they exert potent antioxidant properties by sequestering ROS. Conjugating TPP+ to lipophilic antioxidants such as coenzyme-Q (MitoQ) attenuates renal dysfunction due to several types of AKI and CKD. For example, administration of MitoQ prior to bilateral renal ischemia in mice decreases mitochondrial oxidative damage and renal dysfunction (Dare et al. 2015). Furthermore, addition of MitoQ to cold storage solution (during kidney transplantation) preserves mitochondrial function by decreasing oxidative stress and tubular damage in isolated rat and porcine kidneys (Parajuli et al. 2012). In a genetic model of type-1 diabetes, increased proteinuria and tubulointerstitial fibrosis were also attenuated by MitoQ (Chacko et al. 2010).

Importantly, MitoQ has been shown to be safe for patients with Parkinson's disease (NCT00329056), fatty liver disease (NCT01167088), and hepatitis C (NCT00433108), encouraging future clinical studies in renal disease.

MitoTEMPO, a piperidine nitroxide conjugated to a TPP+ (Sims et al. 2014), scavenges ROS in the mitochondria, reverses renal mitochondrial dysfunction, and attenuates sepsis-induced AKI in mice (Patil et al. 2014). Treatment with either MitoTEMPO or conjugated TPP+ with α-tocopherol (MitoE) improves mitochondrial respiration and reduces oxidative stress and inflammation in septic rats kidneys (Lowes et al. 2013), whereas TPP+ conjugation with the SOD mimetic nitroxide (MitoCP) prevents mitochondrial damage and renal injury in mice with cisplatin-induced nephropathy (Mukhopadhyay et al. 2012).

In addition to TPP+-conjugated drugs, several antioxidants have been successfully delivered into renal mitochondria. Mitochondria-targeted antioxidants of the SkQ group such as plastoquinonyl-decyl-triphenylphosphonium (SkQ1) and plastoquinonyl decylrhodamine 19 (SkQR1) are positively charged compounds that prevent IRI-induced AKI (Plotnikov et al. 2012) and ameliorate gentamicin-induced damage of rat kidney (Jankauskas et al. 2012). Likewise, specific mitochondrially targeted heme oxygenase (HO)-1 protects against hypoxia-dependent renal epithelial cell death and loss of mitochondrial membrane potential (Bolisetty et al. 2013). HO-1 is a potent cytosolic antioxidant enzyme that translocates to the mitochondrion under conditions of oxidative stress and modulates their biogenesis (Piantadosi and Suliman 2012). Taken together, these results suggest that mitochondrially targeted antioxidants represent a novel approach to prevent or attenuate several forms of kidney injury.

3.4 mPTP Inhibitors

Opening of the mPTP, a channel formed in the inner membrane of the mitochondria in response to certain pathological conditions, plays a central role in several forms of AKI. Indeed, mPTP inhibitors have been shown to ameliorate renal IRI and shock-induced AKI.

In addition to its well-known immunosuppressive properties, cyclosporine-A (CSA) is a potent inhibitor of the mPTP, which acts by interacting with cyclophilin D, an essential structural component of the pore that regulates its calcium and ROS-mediated activation (Kim et al. 2014). In small clinical trials in patients with myocardial infarction undergoing reperfusion, CSA showed ability to reduce infarct size (Piot et al. 2008), but a recent randomized clinical trial failed to confirm its efficacy to improve clinical outcomes (Cung et al. 2015). Currently, two more clinical trials are testing safety and effectiveness of CSA in cardiac arrest (NCT01595958) and severe traumatic brain injury (NCT01825044). CSA improves renal function, histopathological damage, and antioxidant enzyme status in rats with renal IRI (Singh et al. 2005) and preserves rat kidneys subjected to traumatic hemorrhagic shock (Lei et al. 2015). Yet, high-dose CSA would shift mitochondrial dynamics toward fission (de Arriba et al. 2013); decrease activity of the

mitochondrial Krebs cycle, oxidative phosphorylation, and electron transfer (Puigmule et al. 2009); and result in nephrotoxicity, limiting their use in patients with renal disease (Issa et al. 2013).

Targeting glycogen synthase kinase (GSK) 3β, a ubiquitous serine–threonine protein kinase that phosphorylates cyclophilin D and promotes mPTP opening, has also shown promising therapeutic potential for preventing toxic AKI. The GSK3β inhibitor 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8) confers protection against podocyte injury in a murine model of adriamycin-induced AKI (Wang et al. 2015b). Likewise, TDZD-8 diminishes mitochondrial permeability transition, improves acute kidney dysfunction, and ameliorates tubular injury in mice with nonsteroidal anti-inflammatory drug-induced AKI (Bao et al. 2012), suggesting GSK3β inhibition as adjunct therapy in drug-induced AKI.

3.5 Cardiolipin Protection

Peroxidation and loss of cardiolipin has been shown to play a crucial role in the pathogenesis of several forms of AKI and CKD, leading to discovery and development of cardiolipin-targeted compounds. Szeto–Schiller (SS)-31 is a tetrapeptide that concentrates in the mitochondria and selectively binds to cardiolipin, preventing its peroxidation and loss, as well as the transformation of cytochrome-c into a peroxidase (Birk et al. 2013; Szeto 2014).

Administration of SS-31 in rats before onset of ischemia and at the onset of reperfusion prevents mitochondria swelling and protects cristae membranes in endothelial and tubular cells four weeks after bilateral renal ischemia, associated with increased number of peritubular capillaries and cortical arterioles and decreased interstitial inflammation and fibrosis (Liu et al. 2014). In rats, SS-31 reduces oxidative stress and inflammation, preventing AKI caused by warm IRI (Szeto et al. 2011) and unilateral ureteral obstruction (Mizuguchi et al. 2008). SS-31 pretreatment also serves a protective role against hypoxia-/reoxygenation-induced apoptosis of human renal tubular epithelial cells, partly by suppression of p66Shc (Zhao et al. 2013), a gene that encodes for an adaptor protein that regulates oxidative stress and apoptosis. Moreover, intraperitoneal injections of SS-31 in rats alleviate contrast-induced AKI, primarily due to an antioxidant action (Duan et al. 2013).

Similarly, in swine atherosclerotic RAS systemic infusion of SS-31 during renal revascularization promotes renal mitochondrial biogenesis and ameliorates renal injury four weeks later (Eirin et al. 2012). Furthermore, chronic subcutaneous injections of SS-31 attenuate swine stenotic-kidney microvascular loss and injury and improves renal oxygenation, hemodynamics, and function (Eiin et al. 2014), demonstrating the efficacy of cardiolipin-targeted therapies for preserving the ischemic kidney in chronic experimental renovascular disease. SS-31 has demonstrated to be safe in several clinical trials (NCT01754818, NCT01513200, NCT01518985, NCT01115920, NCT01786915), and its efficacy is currently being tested in patients with renovascular disease undergoing renal revascularization

(NCT01755858). Outcomes of this study will advance our understanding of the role of cardiolipin in renal disease as well as the efficacy of mitochondria-targeted therapies.

3.6 Fission Inhibitors

Mitochondrial fission is governed by dynamin-related protein (DRP)-1, a GTPase protein localized in the perinuclear region. Once recruited from the cytosol to the mitochondrion, DRP-1 interacts with mitochondrial fission-1 protein to induce outer mitochondrial membrane constriction and fragmentation (Qi et al. 2013). Furthermore, activation of DRP-1 triggers mitochondrial depolarization and subsequent mitophagy (Twig and Shirihai 2011). Therefore, targeting DRP-1 might be beneficial in the treatment of diseases associated with altered mitochondrial fission.

Dynasore is a cell-permeable small molecule that inhibits the GTP hydrolysis of DRP-1, interferes with endocytic functions, and inhibits cell spreading and migration (Macia et al. 2006), but its efficacy in preserving renal mitochondria has yet to be tested. Unlike dynasore, mitochondrial division inhibitor (Mdivi)-1 selectively inhibits DRP-1 activity by blocking its assembly, acting through the GTPase domain (Cassidy-Stone et al. 2008). Although treatment of porcine preimplantation embryos and fibroblast cells with mdivi-1 reduces mitochondrial membrane potential and blastocyst cell number, increasing ROS and apoptosis (Yeon et al. 2015), its delivery in vivo inhibits mPTP opening and protects cardiomyocytes exposed to renal (Sumida et al. 2015) and cardiac (Ong et al. 2010) IRI. Furthermore, intraperitoneal injections of mdiv-1 prevent mitochondrial fragmentation and tubular cell apoptosis in murine AKI (Tang et al. 2013). Nevertheless, no studies have addressed their renoprotective properties in humans.

4 Conclusions and Perspectives

Studies in various animal models have implicated mitochondrial damage in the pathogenesis of genetic defects, acute kidney injury, chronic kidney disease, aging, and renal tumors. Kidney mitochondrial injury may manifest as ultrastructural abnormalities, changes in homeostasis, dysfunction, and loss. These result in decreased cellular energy production, increased oxidative stress, and apoptosis, triggering microvascular loss, inflammation, fibrosis, and renal failure. Notwithstanding the evidence supporting mitochondrial damage in the pathogenesis of different types of renal disease, a cause–effect relationship remains to be established.

Mitochondrial targeting has been demonstrated as a potential intervention to preserve mitochondrial structure and function and ameliorate kidney injury in several animal models of renal disease. Although these compounds concentrate at the level of mitochondria, it is difficult to rule out non-mitochondrial effects that

could have been partly responsible for attenuating renal injury and dysfunction. Some of these compounds such as SS-31 and MitoQ are being evaluated in humans for various therapeutic indications (see ClinicalTrials.gov). Yet, further in vivo animal studies and clinical trials are needed to confirm the efficacy and safety of mitochondrial targeting.

Sources of Funding This work was supported by the NIH grants DK73608, DK102325, DK106427, and HL-123160 and the Mayo Clinic Center for Regenerative Medicine.

Disclosures Drs. A. Lerman and L.O. Lerman: Advisory Board, Stealth Biotherapeutics.

References

Au KM, Lau SC, Mak YF, Lai WM, Chow TC, Chen ML, Chiu MC, Chan AY (2007) Mitochondrial DNA deletion in a girl with Fanconi's syndrome. Pediatr Nephrol 22:136–140

Bao H, Ge Y, Zhuang S, Dworkin LD, Liu Z, Gong R (2012) Inhibition of glycogen synthase kinase-3beta prevents NSAID-induced acute kidney injury. Kidney Int 81:662–673

Barcena C, Martinez MA, Ortega MP, Munoz HG, Sarraga GU (2010) Mitochondria with tubulovesicular cristae in renal oncocytomas. Ultrastruct Pathol 34:315–320

Bartz RR, Fu P, Suliman HB, Crowley SD, MacGarvey NC, Welty-Wolf K, Piantadosi CA (2014) Staphylococcus aureus sepsis induces early renal mitochondrial DNA repair and mitochondrial biogenesis in mice. PLoS One 9:e100912

Birk AV, Liu S, Soong Y, Mills W, Singh P, Warren JD, Seshan SV, Pardee JD, Szeto HH (2013) The mitochondrial-targeted compound SS-31 re-energizes ischemic mitochondria by interacting with cardiolipin. J Am Soc Nephrol 24:1250–1261

Bolisetty S, Traylor A, Zarjou A, Johnson MS, Benavides GA, Ricart K, Boddu R, Moore RD, Landar A, Barnes S, Darley-Usmar V, Agarwal A (2013) Mitochondria-targeted heme oxygenase-1 decreases oxidative stress in renal epithelial cells. Am J Physiol Renal Physiol 305:F255–F264

Brooks C, Wei Q, Cho SG, Dong Z (2009) Regulation of mitochondrial dynamics in acute kidney injury in cell culture and rodent models. J Clin Invest 119:1275–1285

Cao LC, Honeyman TW, Cooney R, Kennington L, Scheid CR, Jonassen JA (2004) Mitochondrial dysfunction is a primary event in renal cell oxalate toxicity. Kidney Int 66:1890–1900

Cassidy-Stone A, Chipuk JE, Ingerman E, Song C, Yoo C, Kuwana T, Kurth MJ, Shaw JT, Hinshaw JE, Green DR, Nunnari J (2008) Chemical inhibition of the mitochondrial division dynamin reveals its role in Bax/Bak-dependent mitochondrial outer membrane permeabilization. Dev Cell 14:193–204

Castaneda MP, Swiatecka-Urban A, Mitsnefes MM, Feuerstein D, Kaskel FJ, Tellis V, Devarajan P (2003) Activation of mitochondrial apoptotic pathways in human renal allografts after ischemiareperfusion injury. Transplantation 76:50–54

Chacko BK, Reily C, Srivastava A, Johnson MS, Ye Y, Ulasova E, Agarwal A, Zinn KR, Murphy MP, Kalyanaraman B, Darley-Usmar V (2010) Prevention of diabetic nephropathy in Ins2(+/)(-)(AkitaJ) mice by the mitochondria-targeted therapy MitoQ. Biochem J 432:9–19

Che R, Yuan Y, Huang S, Zhang A (2014) Mitochondrial dysfunction in the pathophysiology of renal diseases. Am J Physiol Renal Physiol 306:F367–F378

Choi HM, Jo SK, Kim SH, Lee JW, Cho E, Hyun YY, Cha JJ, Kang YS, Cha DR, Cho WY, Kim HK (2013) Glucocorticoids attenuate septic acute kidney injury. Biochem Biophys Res Commun 435:678–684

Cote HC, Magil AB, Harris M, Scarth BJ, Gadawski I, Wang N, Yu E, Yip B, Zalunardo N, Werb R, Hogg R, Harrigan PR, Montaner JS (2006) Exploring mitochondrial nephrotoxicity as a potential mechanism of kidney dysfunction among HIV-infected patients on highly active antiretroviral therapy. Antivir Ther 11:79–86

Cui J, Shi S, Sun X, Cai G, Cui S, Hong Q, Chen X, Bai XY (2013) Mitochondrial autophagy involving renal injury and aging is modulated by caloric intake in aged rat kidneys. PLoS One 8:e69720

Cung TT, Morel O, Cayla G, Rioufol G, Garcia-Dorado D, Angoulvant D, Bonnefoy-Cudraz E, Guerin P, Elbaz M, Delarche N, Coste P, Vanzetto G, Metge M, Aupetit JF, Jouve B, Motreff P, Tron C, Labeque JN, Steg PG, Cottin Y, Range G, Clerc J, Claeys MJ, Coussement P, Prunier F, Moulin F, Roth O, Belle L, Dubois P, Barragan P, Gilard M, Piot C, Colin P, De Poli F, Morice MC, Ider O, Dubois-Rande JL, Unterseeh T, Le Breton H, Beard T, Blanchard D, Grollier G, Malquarti V, Staat P, Sudre A, Elmer E, Hansson MJ, Bergerot C, Boussaha I, Jossan C, Derumeaux G, Mewton N, Ovize M (2015) Cyclosporine before PCI in patients with acute myocardial infarction. N Engl J Med 373:1021–1031

Dare AJ, Bolton EA, Pettigrew GJ, Bradley JA, Saeb-Parsy K, Murphy MP (2015) Protection against renal ischemia-reperfusion injury in vivo by the mitochondria targeted antioxidant MitoQ. Redox Biol 5:163–168

de Arriba G, Calvino M, Benito S, Parra T (2013) Cyclosporine A-induced apoptosis in renal tubular cells is related to oxidative damage and mitochondrial fission. Toxicol Lett 218:30–38

de Cavanagh EM, Piotrkowski B, Basso N, Stella I, Inserra F, Ferder L, Fraga CG (2003) Enalapril and losartan attenuate mitochondrial dysfunction in aged rats. FASEB J 17:1096–1098

de Cavanagh EM, Toblli JE, Ferder L, Piotrkowski B, Stella I, Inserra F (2006) Renal mitochondrial dysfunction in spontaneously hypertensive rats is attenuated by losartan but not by amlodipine. Am J Physiol Regul Integr Comp Physiol 290:R1616–R1625

Dinour D, Mini S, Polak-Charcon S, Lotan D, Holtzman EJ (2004) Progressive nephropathy associated with mitochondrial tRNA gene mutation. Clin Nephrol 62:149–154

Duan SB, Yang SK, Zhou QY, Pan P, Zhang H, Liu F, Xu XQ (2013) Mitochondria-targeted peptides prevent on contrast-induced acute kidney injury in the rats with hypercholesterolemia. Ren Fail 35:1124–1129

Eiin A, Ebrahimi B, Zhang X, Zhu XY, Woollard JR, He Q, Textor SC, Lerman A, Lerman LO (2014) Mitochondrial protection restores renal function in swine atherosclerotic renovascular disease. Cardiovasc Res 103:461–472

Eirin A, Li Z, Zhang X, Krier JD, Woollard JR, Zhu XY, Tang H, Herrmann SM, Lerman A, Textor SC, Lerman LO (2012) A mitochondrial permeability transition pore inhibitor improves renal outcomes after revascularization in experimental atherosclerotic renal artery stenosis. Hypertension 60:1242–1249

Eirin A, Lerman A, Lerman LO (2015) Mitochondria: a pathogenic paradigm in hypertensive renal disease. Hypertension 65:264–270

Emma F, Pizzini C, Tessa A, Di Giandomenico S, Onetti-Muda A, Santorelli FM, Bertini E, Rizzoni G (2006) "Bartter-like" phenotype in Kearns-Sayre syndrome. Pediatr Nephrol 21:355–360

Fedorova LV, Sodhi K, Gatto-Weis C, Puri N, Hinds TD Jr, Shapiro JI, Malhotra D (2013) Peroxisome proliferator-activated receptor delta agonist, HPP593, prevents renal necrosis under chronic ischemia. PLoS One 8:e64436

Ferder LF, Inserra F, Basso N (2002) Advances in our understanding of aging: role of the renin-angiotensin system. Curr Opin Pharmacol 2:189–194

Finsterer J (2004) Mitochondriopathies. Eur J Neurol 11:163–186

Funk JA, Schnellmann RG (2012) Persistent disruption of mitochondrial homeostasis after acute kidney injury. Am J Physiol Renal Physiol 302:F853–F864

Funk JA, Schnellmann RG (2013) Accelerated recovery of renal mitochondrial and tubule homeostasis with SIRT1/PGC-1alpha activation following ischemia-reperfusion injury. Toxicol Appl Pharmacol 273:345–354

Gasparre G, Hervouet E, de Laplanche E, Demont J, Pennisi LF, Colombel M, Mege-Lechevallier-F, Scoazec JY, Bonora E, Smeets R, Smeitink J, Lazar V, Lespinasse J, Giraud S, Godinot C, Romeo G, Simonnet H (2008) Clonal expansion of mutated mitochondrial DNA is associated with tumor formation and complex I deficiency in the benign renal oncocytoma. Hum Mol Genet 17:986–995

Goto Y, Itami N, Kajii N, Tochimaru H, Endo M, Horai S (1990) Renal tubular involvement mimicking Bartter syndrome in a patient with Kearns-Sayre syndrome. J Pediatr 116:904–910

Gucer S, Talim B, Asan E, Korkusuz P, Ozen S, Unal S, Kalkanoglu SH, Kale G, Caglar M (2005) Focal segmental glomerulosclerosis associated with mitochondrial cytopathy: report of two cases with special emphasis on podocytes. Pediatr Dev Pathol 8:710–717

Guery B, Choukroun G, Noel LH, Clavel P, Rotig A, Lebon S, Rustin P, Bellane-Chantelot C, Mougenot B, Grunfeld JP, Chauveau D (2003) The spectrum of systemic involvement in adults presenting with renal lesion and mitochondrial tRNA(Leu) gene mutation. J Am Soc Nephrol 14:2099–2108

Hervouet E, Godinot C (2006) Mitochondrial disorders in renal tumors. Mitochondrion 6:105–117

Hervouet E, Simonnet H, Godinot C (2007) Mitochondria and reactive oxygen species in renal cancer. Biochimie 89:1080–1088

Housley SL, Lindsay RS, Young B, McConachie M, Mechan D, Baty D, Christie L, Rahilly M, Qureshi K, Fleming S (2010) Renal carcinoma with giant mitochondria associated with germline mutation and somatic loss of the succinate dehydrogenase B gene. Histopathology 56:405–408

Ishihara M, Urushido M, Hamada K, Matsumoto T, Shimamura Y, Ogata K, Inoue K, Taniguchi Y, Horino T, Fujieda M, Fujimoto S, Terada Y (2013) Sestrin-2 and BNIP3 regulate autophagy and mitophagy in renal tubular cells in acute kidney injury. Am J Physiol Renal Physiol 305:F495–F509

Issa N, Kukla A, Ibrahim HN (2013) Calcineurin inhibitor nephrotoxicity: a review and perspective of the evidence. Am J Nephrol 37:602–612

Jankauskas SS, Plotnikov EY, Morosanova MA, Pevzner IB, Zorova LD, Skulachev VP, Zorov DB (2012) Mitochondria-targeted antioxidant SkQR1 ameliorates gentamycin-induced renal failure and hearing loss. Biochemistry (Mosc) 77:666–670

Jesinkey SR, Funk JA, Stallons LJ, Wills LP, Megyesi JK, Beeson CC, Schnellmann RG (2014) Formoterol restores mitochondrial and renal function after ischemia-reperfusion injury. J Am Soc Nephrol 25:1157–1162

Jha V, Garcia-Garcia G, Iseki K, Li Z, Naicker S, Plattner B, Saran R, Wang AY, Yang CW (2013) Chronic kidney disease: global dimension and perspectives. Lancet 382:260–272

Jin K, Vaziri ND (2014) Salt-sensitive hypertension in mitochondrial superoxide dismutase deficiency is associated with intra-renal oxidative stress and inflammation. Clin Exp Nephrol 18:445–452

Karicheva OZ, Kolesnikova OA, Schirtz T, Vysokikh MY, Mager-Heckel AM, Lombes A, Boucheham A, Krasheninnikov IA, Martin RP, Entelis N, Tarassov I (2011) Correction of the consequences of mitochondrial 3243A>G mutation in the MT-TL1 gene causing the MELAS syndrome by tRNA import into mitochondria. Nucleic Acids Res 39:8173–8186

Kawakami T, Gomez IG, Ren S, Hudkins K, Roach A, Alpers CE, Shankland SJ, D'Agati VD, Duffield JS (2015) Deficient autophagy results in mitochondrial dysfunction and FSGS. J Am Soc Nephrol 26:1040–1052

Khader A, Yang WL, Kuncewitch M, Jacob A, Prince JM, Asirvatham JR, Nicastro J, Coppa GF, Wang P (2014) Sirtuin 1 activation stimulates mitochondrial biogenesis and attenuates renal injury after ischemia-reperfusion. Transplantation 98:148–156

Kim SY, Shim MS, Kim KY, Weinreb RN, Wheeler LA, Ju WK (2014) Inhibition of cyclophilin D by cyclosporin A promotes retinal ganglion cell survival by preventing mitochondrial alteration in ischemic injury. Cell Death Dis 5:e1105

Klingenberg M (2009) Cardiolipin and mitochondrial carriers. Biochim Biophys Acta 1788:2048–2058

Kobayashi A, Goto Y, Nagata M, Yamaguchi Y (2010) Granular swollen epithelial cells: a histologic and diagnostic marker for mitochondrial nephropathy. Am J Surg Pathol 34:262–270

Koller A, Kain R, Haitel A, Mazal PR, Asboth F, Susani M (2000) Renal oncocytoma with prominent intracytoplasmic vacuoles of mitochondrial origin. Histopathology 37:264–268

Lake NJ, Bird MJ, Isohanni P, Paetau A (2015) Leigh syndrome: neuropathology and pathogenesis. J Neuropathol Exp Neurol 74:482–492

Lee JJ, Tripi LM, Erbe RW, Garimella-Krovi S, Springate JE (2012) A mitochondrial DNA deletion presenting with corneal clouding and severe Fanconi syndrome. Pediatr Nephrol 27:869–872

Leger B, Koopman R, Walrand S, Gehrig SM, Murphy KT, Lynch GS (2011) Chronic formoterol administration reduces cardiac mitochondrial protein synthesis and oxidative capacity in mice. Int J Cardiol 146:270–272

Lei Y, Peng X, Liu L, Dong Z, Li T (2015) Beneficial effect of cyclosporine A on traumatic hemorrhagic shock. J Surg Res 195:529–540

Li QW, Lu XY, You Y, Sun H, Liu XY, Ai JZ, Tan RZ, Chen TL, Chen MZ, Wang HL, Wei YQ, Zhou Q (2012a) Comparative proteomic analysis suggests that mitochondria are involved in autosomal recessive polycystic kidney disease. Proteomics 12:2556–2570

Li T, Lin X, Zhu Y, Li L, Liu L (2012b) Short-term, mild hypothermia can increase the beneficial effect of permissive hypotension on uncontrolled hemorrhagic shock in rats. Anesthesiology 116:1288–1298

Liu S, Soong Y, Seshan SV, Szeto HH (2014) Novel cardiolipin therapeutic protects endothelial mitochondria during renal ischemia and mitigates microvascular rarefaction, inflammation, and fibrosis. Am J Physiol Renal Physiol 306:F970–F980

Lopez S, Negredo E, Garrabou G, Puig J, Ruiz L, Sanjurjo E, Ramos X, Infante AB, Casademont J, Cardellach F, Clotet B, Miro O (2006) Longitudinal study on mitochondrial effects of didanosine-tenofovir combination. AIDS Res Hum Retroviruses 22:33–39

Lowes DA, Webster NR, Murphy MP, Galley HF (2013) Antioxidants that protect mitochondria reduce interleukin-6 and oxidative stress, improve mitochondrial function, and reduce biochemical markers of organ dysfunction in a rat model of acute sepsis. Br J Anaesth 110:472–480

Macia E, Ehrlich M, Massol R, Boucrot E, Brunner C, Kirchhausen T (2006) Dynasore, a cell-permeable inhibitor of dynamin. Dev Cell 10:839–850

Meierhofer D, Mayr JA, Foetschl U, Berger A, Fink K, Schmeller N, Hacker GW, Hauser-Kronberger C, Kofler B, Sperl W (2004) Decrease of mitochondrial DNA content and energy metabolism in renal cell carcinoma. Carcinogenesis 25:1005–1010

Melkonian SC, Wang X, Gu J, Matin SF, Tannir NM, Wood CG, Wu X (2015) Mitochondrial DNA copy number in peripheral blood leukocytes and the risk of clear cell renal cell carcinoma. Carcinogenesis 36:249–255

Mizuguchi Y, Chen J, Seshan SV, Poppas DP, Szeto HH, Felsen D (2008) A novel cell-permeable antioxidant peptide decreases renal tubular apoptosis and damage in unilateral ureteral obstruction. Am J Physiol Renal Physiol 295:F1545–F1553

Mohammedi K, Bellili-Munoz N, Driss F, Roussel R, Seta N, Fumeron F, Hadjadj S, Marre M, Velho G (2014) Manganese superoxide dismutase (SOD2) polymorphisms, plasma advanced oxidation protein products (AOPP) concentration and risk of kidney complications in subjects with type 1 diabetes. PLoS One 9:e96916

Mukhopadhyay P, Horvath B, Zsengeller Z, Zielonka J, Tanchian G, Holovac E, Kechrid M, Patel V, Stillman IE, Parikh SM, Joseph J, Kalyanaraman B, Pacher P (2012) Mitochondrial-targeted antioxidants represent a promising approach for prevention of cisplatin-induced nephropathy. Free Radic Biol Med 52:497–506

Mukunyadzi P, Huang H, Liu K, Fan CY (2003) Concomitant loss of mitochondria and the DNA repair protein hOGG1 in clear cell carcinoma of the kidney. Appl Immunohistochem Mol Morphol 11:334–338

Munusamy S, MacMillan-Crow LA (2009) Mitochondrial superoxide plays a crucial role in the development of mitochondrial dysfunction during high glucose exposure in rat renal proximal tubular cells. Free Radic Biol Med 46:1149–1157

Munusamy S, Saba H, Mitchell T, Megyesi JK, Brock RW, Macmillan-Crow LA (2009) Alteration of renal respiratory Complex-III during experimental type-1 diabetes. BMC Endocr Disord 9:2

Nagy A, Wilhelm M, Kovacs G (2003) Mutations of mtDNA in renal cell tumours arising in end-stage renal disease. J Pathol 199:237–242

Ong SB, Subrayan S, Lim SY, Yellon DM, Davidson SM, Hausenloy DJ (2010) Inhibiting mitochondrial fission protects the heart against ischemia/reperfusion injury. Circulation 121:2012–2022

Parajuli N, Campbell LH, Marine A, Brockbank KG, Macmillan-Crow LA (2012) MitoQ blunts mitochondrial and renal damage during cold preservation of porcine kidneys. PLoS One 7: e48590

Parikh SM, Yang Y, He L, Tang C, Zhan M, Dong Z (2015) Mitochondrial function and disturbances in the septic kidney. Semin Nephrol 35:108–119

Patil NK, Parajuli N, MacMillan-Crow LA, Mayeux PR (2014) Inactivation of renal mitochondrial respiratory complexes and manganese superoxide dismutase during sepsis: mitochondria-targeted antioxidant mitigates injury. Am J Physiol Renal Physiol 306:F734–F743

Perez-Gallardo RV, Noriega-Cisneros R, Esquivel-Gutierrez E, Calderon-Cortes E, Cortes-Rojo C, Manzo-Avalos S, Campos-Garcia J, Salgado-Garciglia R, Montoya-Perez R, Boldogh I, Saavedra-Molina A (2014) Effects of diabetes on oxidative and nitrosative stress in kidney mitochondria from aged rats. J Bioenerg Biomembr 46:511–518

Piantadosi CA, Suliman HB (2012) Redox regulation of mitochondrial biogenesis. Free Radic Biol Med 53:2043–2053

Piot C, Croisille P, Staat P, Thibault H, Rioufol G, Mewton N, Elbelghiti R, Cung TT, Bonnefoy E, Angoulvant D, Macia C, Raczka F, Sportouch C, Gahide G, Finet G, Andre-Fouet X, Revel D, Kirkorian G, Monassier JP, Derumeaux G, Ovize M (2008) Effect of cyclosporine on reperfusion injury in acute myocardial infarction. N Engl J Med 359:473–481

Plotnikov EY, Silachev DN, Jankauskas SS, Rokitskaya TI, Chupyrkina AA, Pevzner IB, Zorova LD, Isaev NK, Antonenko YN, Skulachev VP, Zorov DB (2012) Mild uncoupling of respiration and phosphorylation as a mechanism providing nephro- and neuroprotective effects of penetrating cations of the SkQ family. Biochemistry (Mosc) 77:1029–1037

Puigmule M, Lopez-Hellin J, Sune G, Tornavaca O, Camano S, Tejedor A, Meseguer A (2009) Differential proteomic analysis of cyclosporine A-induced toxicity in renal proximal tubule cells. Nephrol Dial Transplant 24:2672–2686

Qi X, Qvit N, Su YC, Mochly-Rosen D (2013) A novel Drp1 inhibitor diminishes aberrant mitochondrial fission and neurotoxicity. J Cell Sci 126:789–802

Ricketts C, Woodward ER, Killick P, Morris MR, Astuti D, Latif F, Maher ER (2008) Germline SDHB mutations and familial renal cell carcinoma. J Natl Cancer Inst 100:1260–1262

Rocha-Rodrigues S, Santos-Alves E, Coxito PM, Marques-Aleixo I, Passos E, Guimaraes JT, Martins MJ, Oliveira PJ, Magalhaes J, Ascensao A (2013) Combined effects of aging and in vitro non-steroid anti-inflammatory drugs on kidney and liver mitochondrial physiology. Life Sci 93:329–337

Sharma K, Karl B, Mathew AV, Gangoiti JA, Wassel CL, Saito R, Pu M, Sharma S, You YH, Wang L, Diamond-Stanic M, Lindenmeyer MT, Forsblom C, Wu W, Ix JH, Ideker T, Kopp JB, Nigam SK, Cohen CD, Groop PH, Barshop BA, Natarajan L, Nyhan WL, Naviaux RK (2013) Metabolomics reveals signature of mitochondrial dysfunction in diabetic kidney disease. J Am Soc Nephrol 24:1901–1912

Simonnet H, Alazard N, Pfeiffer K, Gallou C, Beroud C, Demont J, Bouvier R, Schagger H, Godinot C (2002) Low mitochondrial respiratory chain content correlates with tumor aggressiveness in renal cell carcinoma. Carcinogenesis 23:759–768

Simonnet H, Demont J, Pfeiffer K, Guenaneche L, Bouvier R, Brandt U, Schagger H, Godinot C (2003) Mitochondrial complex I is deficient in renal oncocytomas. Carcinogenesis 24:1461–1466

Sims CR, MacMillan-Crow LA, Mayeux PR (2014) Targeting mitochondrial oxidants may facilitate recovery of renal function during infant sepsis. Clin Pharmacol Ther 96:662–664

Singh D, Chander V, Chopra K (2005) Cyclosporine protects against ischemia/reperfusion injury in rat kidneys. Toxicology 207:339–347

Small DM, Bennett NC, Roy S, Gabrielli BG, Johnson DW, Gobe GC (2012) Oxidative stress and cell senescence combine to cause maximal renal tubular epithelial cell dysfunction and loss in an in vitro model of kidney disease. Nephron Exp Nephrol 122:123–130

Stallons LJ, Whitaker RM, Schnellmann RG (2014) Suppressed mitochondrial biogenesis in folic acid-induced acute kidney injury and early fibrosis. Toxicol Lett 224:326–332

Stoyanoff TR, Todaro JS, Aguirre MV, Zimmermann MC, Brandan NC (2014) Amelioration of lipopolysaccharide-induced acute kidney injury by erythropoietin: involvement of mitochondria-regulated apoptosis. Toxicology 318:13–21

Sumida M, Doi K, Ogasawara E, Yamashita T, Hamasaki Y, Kariya T, Takimoto E, Yahagi N, Nangaku M, Noiri E (2015) Regulation of mitochondrial dynamics by dynamin-related protein-1 in acute cardiorenal syndrome. J Am Soc Nephrol 26:2378–2387

Sun L, Xie P, Wada J, Kashihara N, Liu FY, Zhao Y, Kumar D, Chugh SS, Danesh FR, Kanwar YS (2008) Rap1b GTPase ameliorates glucose-induced mitochondrial dysfunction. J Am Soc Nephrol 19:2293–2301

Susztak K, Raff AC, Schiffer M, Bottinger EP (2006) Glucose-induced reactive oxygen species cause apoptosis of podocytes and podocyte depletion at the onset of diabetic nephropathy. Diabetes 55:225–233

Szeto HH (2014) First-in-class cardiolipin-protective compound as a therapeutic agent to restore mitochondrial bioenergetics. Br J Pharmacol 171:2029–2050

Szeto HH, Liu S, Soong Y, Wu D, Darrah SF, Cheng FY, Zhao Z, Ganger M, Tow CY, Seshan SV (2011) Mitochondria-targeted peptide accelerates ATP recovery and reduces ischemic kidney injury. J Am Soc Nephrol 22:1041–1052

Tabara LC, Poveda J, Martin-Cleary C, Selgas R, Ortiz A, Sanchez-Nino MD (2014) Mitochondria-targeted therapies for acute kidney injury. Expert Rev Mol Med 16:e13

Tan AL, Sourris KC, Harcourt BE, Thallas-Bonke V, Penfold S, Andrikopoulos S, Thomas MC, O'Brien RC, Bierhaus A, Cooper ME, Forbes JM, Coughlan MT (2010) Disparate effects on renal and oxidative parameters following RAGE deletion, AGE accumulation inhibition, or dietary AGE control in experimental diabetic nephropathy. Am J Physiol Renal Physiol 298: F763–F770

Tang WX, Wu WH, Zeng XX, Bo H, Huang SM (2012) Early protective effect of mitofusion 2 overexpression in STZ-induced diabetic rat kidney. Endocrine 41:236–247

Tang WX, Wu WH, Qiu HY, Bo H, Huang SM (2013) Amelioration of rhabdomyolysis-induced renal mitochondrial injury and apoptosis through suppression of Drp-1 translocation. J Nephrol 26:1073–1082

Twig G, Shirihai OS (2011) The interplay between mitochondrial dynamics and mitophagy. Antioxid Redox Signal 14:1939–1951

Wang G, Shimada E, Zhang J, Hong JS, Smith GM, Teitell MA, Koehler CM (2012) Correcting human mitochondrial mutations with targeted RNA import. Proc Natl Acad Sci U S A 109:4840–4845

Wang H, Guan Y, Karamercan MA, Ye L, Bhatti T, Becker LB, Baur JA, Sims CA (2015a) Resveratrol rescues kidney mitochondrial function following hemorrhagic shock. Shock 44:173–180

Wang Z, Bao H, Ge Y, Zhuang S, Peng A, Gong R (2015b) Pharmacological targeting of GSK3beta confers protection against podocytopathy and proteinuria by desensitizing mitochondrial permeability transition. Br J Pharmacol 172:895–909

Wills LP, Trager RE, Beeson GC, Lindsey CC, Peterson YK, Beeson CC, Schnellmann RG (2012) The beta2-adrenoceptor agonist formoterol stimulates mitochondrial biogenesis. J Pharmacol Exp Ther 342:106–118

Xu Y, Ruan S, Wu X, Chen H, Zheng K, Fu B (2013) Autophagy and apoptosis in tubular cells following unilateral ureteral obstruction are associated with mitochondrial oxidative stress. Int J Mol Med 31:628–636

Yeon JY, Min SH, Park HJ, Kim JW, Lee YH, Park SY, Jeong PS, Park H, Lee DS, Kim SU, Chang KT, Koo DB (2015) Mdivi-1, mitochondrial fission inhibitor, impairs developmental competence and mitochondrial function of embryos and cells in pigs. J Reprod Dev 61:81–89

Yuan J, Zhou J, Chen BC, Zhang X, Zhou HM, Du DF, Chang S, Chen ZK (2005) Magnesium supplementation prevents chronic cyclosporine nephrotoxicity via adjusting nitric oxide synthase activity. Transplant Proc 37:1892–1895

Yuan Y, Huang S, Wang W, Wang Y, Zhang P, Zhu C, Ding G, Liu B, Yang T, Zhang A (2012) Activation of peroxisome proliferator-activated receptor-gamma coactivator 1alpha ameliorates mitochondrial dysfunction and protects podocytes from aldosterone-induced injury. Kidney Int 82:771–789

Zepeda-Orozco D, Kong M, Scheuermann RH (2015) Molecular profile of mitochondrial dysfunction in kidney transplant biopsies is associated with poor allograft outcome. Transplant Proc 47:1675–1682

Zhang M, Wang Y, Wang Q, Yang J, Yang D, Liu J, Li J (2010) Involvement of mitochondria-mediated apoptosis in ethylbenzene-induced renal toxicity in rat. Toxicol Sci 115:295–303

Zhao WY, Han S, Zhang L, Zhu YH, Wang LM, Zeng L (2013) Mitochondria-targeted antioxidant peptide SS31 prevents hypoxia/reoxygenation-induced apoptosis by down-regulating p66Shc in renal tubular epithelial cells. Cell Physiol Biochem 32:591–600

Zheleznova NN, Yang C, Ryan RP, Halligan BD, Liang M, Greene AS, Cowley AW Jr (2012) Mitochondrial proteomic analysis reveals deficiencies in oxygen utilization in medullary thick ascending limb of Henle in the Dahl salt-sensitive rat. Physiol Genomics 44:829–842

Ziegler DV, Wiley CD, Velarde MC (2015) Mitochondrial effectors of cellular senescence: beyond the free radical theory of aging. Aging Cell 14:1–7

Zsengeller ZK, Ellezian L, Brown D, Horvath B, Mukhopadhyay P, Kalyanaraman B, Parikh SM, Karumanchi SA, Stillman IE, Pacher P (2012) Cisplatin nephrotoxicity involves mitochondrial injury with impaired tubular mitochondrial enzyme activity. J Histochem Cytochem 60:521–529

Mitochondrial Dynamics as a Therapeutic Target for Treating Cardiac Diseases

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H. Singh, S.-S. Sheu (eds.), *Pharmacology of Mitochondria*,
Handbook of Experimental Pharmacology 240, DOI 10.1007/164_2016_7

Abstract

Mitochondria are dynamic in nature and are able to shift their morphology between elongated interconnected mitochondrial networks and a fragmented disconnected arrangement by the processes of mitochondrial fusion and fission, respectively. Changes in mitochondrial morphology are regulated by the mitochondrial fusion proteins – mitofusins 1 and 2 (Mfn1 and 2), and optic atrophy 1 (Opa1) as well as the mitochondrial fission proteins – dynamin-related peptide 1 (Drp1) and fission protein 1 (Fis1). Despite having a unique spatial arrangement, cardiac mitochondria have been implicated in a variety of disorders including ischemia–reperfusion injury (IRI), heart failure, diabetes, and pulmonary hypertension. In this chapter, we review the influence of mitochondrial dynamics in these cardiac disorders as well as their potential as therapeutic targets in tackling cardiovascular disease.

Keywords

Cardiac diseases • Cardiac disorders • Drp1 • Mfn1 • Mfn2 • Mitochondrial dynamics • Mitochondrial morphology • Opa1

Abbreviations

Akt	Protein kinase B (PKB)
ANT	Adenine nucleotide translocator
APOO	Apolipoprotein O
ATP	Adenosine triphosphate
Ca^{2+}	Calcium ions
CsA	Cyclosporine A
Cx-43	Connexin-43
CypD	Cyclophilin D
Drp1	Dynamin-related peptide 1
ER	Endoplasmic reticulum
ETC	Electron transport chain

FATP1	Fatty acid transport protein 1
Grp75	Glucose-regulated protein 75
GTPases	Guanosine triphosphatases
HKII	Hexokinase II
H_2O_2	Hydrogen peroxide
IF	Interfibrillar
IMM	Inner mitochondrial membrane
IP3R	Inositol 1,4,5-triphosphate receptor
IR	Ischemia–reperfusion
IRI	Ischemia–reperfusion injury
KCl	Potassium chloride
LV	Left ventricle
MARF	Mitochondrial assembly regulatory factor
Mdivi-1	Mitochondrial division inhibitor 1
Mff	Mitochondrial fission factor
Mfn1	Mitofusin 1
Mfn2	Mitofusin 2
MI	Myocardial infarct
MiD49/51	Mitochondrial dynamics proteins of 49 and 51 kDa
MLKL	Mixed lineage kinase domain like protein
MMP	Mitochondrial membrane potential
MOMP	Mitochondrial outer membrane permeabilization
mPTP	Mitochondrial permeability transition pore
mTOR	Mechanistic target of rapamycin
OMM	Outer mitochondrial membrane
Opa1	Optic atrophy 1
OPA1-KD	OPA1-knockdown
PAH	Pulmonary arterial hypertension
pAkt	Phospho-Akt
PASMC	Pulmonary artery smooth muscle cell
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PGC-1α	PPAR gamma coactivator-1
PiC	Inorganic phosphate carrier
PPAR	Peroxisome proliferator-activated receptor
RIP	Receptor-interacting protein
ROS	Reactive oxygen species
SENP3	SUMO1/sentrin/SMT3 specific peptidase 3
sI	Simulated ischemia
sIRI	Simulated ischemia–reperfusion injury
siRNA	Small interfering RNA
Smac/DIABLO	Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI
SSC	Subsarcolemmal
SUMOs	Small ubiquitin-like modifiers
TNF-α	Tumor necrosis factor alpha

UPR	Unfolded protein response
VDAC	Voltage-dependent anion channel

1 Introduction

The shapes of mitochondria vary between different cell types and continuously change through processes of fusion, fission, and motility, termed mitochondrial dynamics [as reviewed in Youle and van der Bliek (2012)]. Mitochondrial dynamics refer to the motility of mitochondria within the cell as well as the ability to fuse and divide. In the heart, this dynamic movement of the mitochondria is not easily detected as the mitochondria are arranged alongside myofibrils with their movement heavily restricted (Beraud et al. 2009; Hom and Sheu 2009). The fusion/fission cycle has been proposed to last 14–16 days in adult cardiomyocytes, further leading to the misimpression that mitochondrial dynamics may be irrelevant in the heart in short-term studies (Chen et al. 2011). Nevertheless, continuous biogenesis and turnover by autophagy/mitophagy as well as the presence of mitochondrial-shaping proteins have substantiated the role of mitochondrial dynamics in the heart (Diaz and Moraes 2008; Twig et al. 2008). The advent of imaging techniques further validates the relevance of mitochondrial dynamics in the heart, particularly in the setting of cardiovascular disorders (see Fig. 1).

Both fusion and fission of mitochondria are mediated by large guanosine triphosphatases (GTPases) in the dynamin family. These GTPases are conserved between yeast, flies, and mammals (as reviewed in Chan 2006; Hoppins et al. 2007; Zhang and Chan 2007; Chen and Chan 2010; Ong and Hausenloy 2010; Chen et al. 2011; Ong et al. 2012; Piquereau et al. 2013). Mitochondrial fusion consists of three distinct steps: (1) GTP-independent tethering of the outer membranes of two mitochondria by either (or both) of two other dynamin superfamily GTPases, the mitofusins (Mfn) (Koshiba et al. 2004), (2) GTP-dependent fusion of the outer membranes of the two tethered mitochondria by the same Mfns, followed by (3) inner mitochondrial membrane (IMM) fusion mediated by yet another dynamin superfamily GTPase, Optic Atrophy 1 (Opa1) (Cipolat et al. 2004; Song et al. 2009).

Fission of the mitochondria results from recruitment and directed multimerization of a dynamin superfamily GTPase – Dynamin-related protein 1 (Drp1) (Smirnova et al. 1998, 2001). The Drp1 protein oligomerizes in a chain-like fashion around the center of the mitochondria and constricts in a GTP-dependent manner, dissecting the parent organelle into two daughters. The exact details of the Drp1-recruitment signaling mechanism to the mitochondria are not fully elucidated, although ER-contact points/ER-associated proteins (Pinton et al. 2001; Germain et al. 2005; Alirol et al. 2006; Friedman et al. 2011), actin–myosin binding (Chhabra and Higgs 2006; Korobova et al. 2013, 2014; Hatch et al. 2014), and docking proteins (mitochondrial fission protein 1 – Fis1 (Mozdy et al. 2000; Yoon et al. 2003; James et al. 2003; Stojanovski et al. 2004);

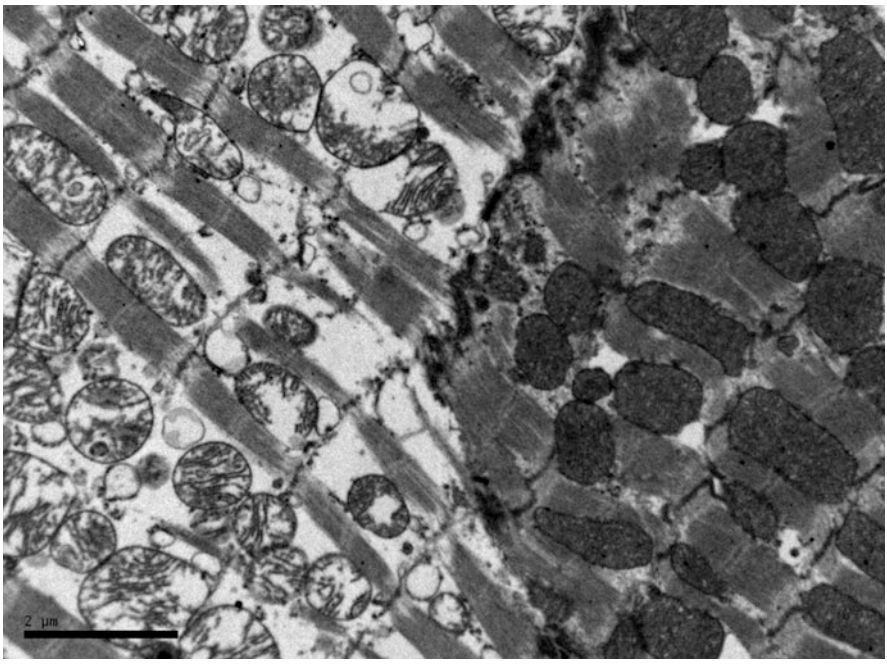

Fig. 1 Representative electron micrograph image showing the different shapes and sizes of mitochondria in the border zone of an infarcted tissue where the mitochondria with disrupted cristae and myofibril on the *left* constitute the infarcted area whereas the area on the *right* is the area-at-risk with normal mitochondria and myofibrils

Mitochondrial fission factor – Mff (Gandre-Babbe and van der Bliek 2008; Otera et al. 2010; Losón et al. 2013); mitochondrial dynamics proteins of 49 and 51 kDa – MiD49/51 (Palmer et al. 2011, 2013; Losón et al. 2013, 2014, 2015)) have been suggested.

2 Mitochondrial Dynamics and Cell Death

2.1 Pro-fission Proteins and Cell Death

The process of apoptosis, or cell death, initiates changes in mitochondrial morphology from long interconnected tubules to small puncta-like organelles (Frank et al. 2001; Yamaguchi and Perkins 2009). Interestingly, mitochondrial fragmentation by Drp1 has also been documented to contribute to apoptotic cell death, hence implicating a role for the mitochondrial-shaping proteins in regulating cell fate (Frank et al. 2001; Breckenridge et al. 2003; Sugioka et al. 2004; Germain et al. 2005; Parone et al. 2006). The posttranslational modification of Drp1, and its association with the OMM and Bax/Bak regulate its pro-fission and pro-apoptotic function (Wasiak et al. 2007; Chang and Blackstone 2007;

Cribbs and Strack 2007). The fragmentation of mitochondria coincides with the activation of the pro-apoptotic Bcl-2 family member Bax and permeabilization of the outer mitochondrial membrane (OMM). Bax colocalizes with Drp1 at defined foci on the OMM to initiate apoptosis (Frank et al. 2001; Karbowski et al. 2002). Nevertheless, inhibition of Drp1 only delays but does not inhibit cell death, thus demonstrating that mitochondrial fragmentation is not a requisite for Bax/Bak-dependent apoptosis (Sugioka et al. 2004; Parone et al. 2006). Furthermore, the dominant-negative form of Drp1 – $Drp1_{K38A}$ – did not reduce translocation of the pro-apoptotic protein Bax to mitochondria, thus placing Drp1 downstream of Bax in apoptosis. Preventing mitochondrial fission only partially prevented the release of cytochrome *c* from the mitochondria whereas the release of second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO) ensues (Scorrano et al. 2002; Germain et al. 2005; Arnoult et al. 2005; Parone et al. 2006). It is noteworthy to point that cells with fragmented mitochondria were sensitized to apoptotic stimuli and more predisposed to cell death (Sugioka et al. 2004). The predisposal to cell death may be due to impaired mitochondrial bioenergetic function as fragmented mitochondria may be less efficient in adenosine triphosphate (ATP) production (Gomes et al. 2011). In addition, there may be increased reactive oxygen species (ROS) production in the fragmented mitochondria (Yu et al. 2006; Shenouda et al. 2011). This may be due to spatial modification of the electron transport chain (ETC) subunits in the IMM or distribution of antioxidant enzymes [as reviewed in Ong and Gustafsson (2012)]. Downregulation of Fis1 has previously been shown to inhibit mitochondrial fission and cell death (Lee et al. 2004). Nevertheless, mitochondrial outer membrane permeabilization (MOMP), mitochondrial fragmentation, and release of cytochrome *c* do not always lead to cell death (Alirol et al. 2006).

2.2 Pro-fusion Proteins and Cell Death

A reduction in OPA1 levels and increased mitochondrial fragmentation were also detected in the ligated adult rat heart – a model of heart failure where a left lateral thoracotomy was performed in the fourth intercostal space, and a ligature (6.0 nylon suture) was placed around the left anterior descending (LAD) coronary artery 2 mm below its origin following intubation and placement on a respirator (Lin et al. 2007; Chen et al. 2009). Simulated ischemia by placing the H9c2 rat cardiomyoblast cell line in serum- and glucose-free medium in an anaerobic chamber for a certain duration of time also induces loss of OPA1 and promotes mitochondrial fragmentation, while overexpression of OPA1 preserved mitochondrial morphology during ischemia (Chen et al. 2009).

Renault et al. have recently demonstrated that Mfn1 establishes a mitochondrial size that is permissive for pro-apoptotic Bcl-2 family function (Renault et al. 2014). Cells with hyperfragmented mitochondria, along with size-restricted OMM model systems however, fail to support BAX-dependent membrane association and

permeabilization due to an inability to stabilize BAXα9·membrane interactions (Renault et al. 2014).

Mfn2 has been shown to colocalize with Drp1 and Bax in the mitochondrial foci (Karbowski et al. 2002). Bax functions to repress the Mfn2 activity (Karbowski et al. 2002, 2006) and promotes mitochondrial fragmentation following MOMP (Brooks et al. 2007). Overexpression of Mfn2 or inhibiting the colocalization at the mitochondrial foci prevents Bax translocation and delays MOMP (Neuspiel et al. 2005; Karbowski et al. 2006). Similarly reduced levels of Opa1 or Opa1-knockdown (Opa1-KD) by small interfering RNA (siRNA) decreases mitochondrial fusion leading to mitochondrial fragmentation, decreased mitochondrial membrane potential (MMP), and cell death (Olichon et al. 2003; Lee et al. 2004).

Defective mitochondrial fusion leads to erratic exchange of inner membrane and matrix components. Fibroblasts lacking Mfn1 and Mfn2 have been shown to demonstrate a more heterogenous distribution of OMM proteins (Weaver et al. 2014). The varying ways the proteins associate in the OMM affect the heterogeneity in the Mfn1/2 (−/−) cells as well as their transfer kinetics during mitochondrial fusion (Weaver et al. 2014). Bak is upstream of Bid-induced MOMP and release of cytochrome *c*. The lack of Mfn1/2 causes dysregulation of Bid-dependent apoptotic signaling and this is restored upon expression of Mfn2 or upon fusion with Bak-containing mitochondria (Weaver et al. 2014).

In a study conducted by Papanicolaou et al., conditional ablation of Mfn2 in cardiac myocytes causes mild cardiac hypertrophy and systolic dysfunction in murine hearts (Papanicolaou et al. 2011). Nevertheless, there was reduced ROS- and mitochondrial permeability transition pore (mPTP)-mediated cell death in these Mfn2−/− hearts, raising the possibility that Mfn2 may also regulate mPTP opening (Papanicolaou et al. 2011). This may implicate the role of Mfn2 in tethering of endoplasmic reticulum (ER) to mitochondria and calcium (Ca^{2+}) signaling between the two organelles (de Brito and Scorrano 2008). The pleiotropic effects of Mfn2 may provide a plausible explanation as to why cell fate may differ in varying conditions in the presence or absence of Mfn2.

Overexpression of a dominant-active form of Mfn2 reduced Bax-mediated cytochrome *c* release (Neuspiel et al. 2005), while downregulation of Mfn2 exacerbated ceramide-induced mitochondrial dysfunction and release of cytochrome *c* (Papanicolaou et al. 2011). Elevated levels of Mfn2 in the HL-1 cardiac cell line which is derived from the AT-1 mouse atrial cardiomyocyte tumor lineage also protect against IR-mediated mPTP opening and cell death (Ong et al. 2010). Nevertheless, there have also been reports of elevated Mfn2 in myocytes undergoing apoptosis following hydrogen peroxide (H_2O_2) treatment, myocardial infarction, or ischemia–reperfusion injury (IRI) (Shen et al. 2007).

2.3 Mitochondrial Dynamics in Necroptosis

More recently, a pathway of programmed cell necrosis has been described. The cytokine tumor necrosis factor alpha (TNF-α) activates the receptor-interacting

serine–threonine kinases RIP1 and RIP3 which will interact with the mixed lineage kinase domain like protein (MLKL), leading to ROS generation, Ca^{2+} overload, and the opening of the mPTP (Vanlangenakker et al. 2008; Cho et al. 2009b; Chen et al. 2013). RIP3 forms a complex with the mitochondrial protein phosphatase PGAM5, which then leads to mitochondrial fragmentation via dephosphorylation of Drp1 at its Ser-637 site and subsequent recruitment to the OMM (Wang et al. 2012), although this was debated in a more recent study (Moujalled et al. 2014; Remijsen et al. 2014). The RIP3-RIP1 kinase complex-dependent necroptosis is also suppressed by caspase-8 via proteolytic cleavage (He et al. 2009). Caspase-8 deficient embryos die between embryonic days 10.5 and 11.5 due to activation of RIP3 (He et al. 2009). Conversely, maturation into adulthood is possible for double KOs of RIP3 and caspase-8 (Kaiser et al. 2011). Pharmacological inhibition of this pathway, using Necrostatin-1 to target RIP1, has been reported to limit myocardial infarct (MI) size and prevent post-left ventricular (LV) remodeling in cell lines as well as animal models of acute IRI (Lim et al. 2007; Oerlemans et al. 2012; Zhang et al. 2013a). Repression of RIP3 or MLKL inhibits necroptosis but activates RIP1-dependent apoptosis, although this phenomenon may be species-specific (mice versus humans) (Remijsen et al. 2014). In addition, the different identities of cell death initiators may govern the pathway of necroptosis (Obitsu et al. 2013). The signaling events underlying necroptosis has been shown to involve Akt/mechanistic target of rapamycin (mTOR) where cell death was preceded by RIP1-RIP3-phospho Akt (pAkt) assembly in the neuronal model (Liu et al. 2014). Pretreatment with Akt inhibitor viii [a cell-permeable and reversible quinoxaline compound that potently and selectively inhibits Akt1/Akt2 activity by recognizing the pleckstrin homology (PH) domain] and rapamycin inhibited Akt and S6-phosphorylation events, mitochondrial ROS production, and necroptosis by over 50% without affecting RIP1-RIP3 complex assembly (Liu et al. 2014). Similar results were seen using small inhibitory ribonucleic acid-mediated knockdown of AKT1/2 and mTOR (Liu et al. 2014). Whether this is similar in the heart remains to be investigated.

2.4 Summary

Collectively, these studies imply the presence of crosstalk between proteins governing mitochondrial morphology and proteins of the apoptotic response. Pharmacological modulation of mitochondrial morphology in various studies has been demonstrated to affect cell fate. Nevertheless, the efficacy and specificity of these drugs, particularly in the heart, remains to be further investigated. The following sections in this chapter will depict the role of mitochondrial dynamics in different cardiovascular diseases and their potential as therapeutic targets.

3 Mitochondrial Dynamics and Ischemia–Reperfusion Injury

3.1 Perturbations in Mitochondrial Dynamics in Ischemia–Reperfusion Injury

Disruption of blood flow to the myocardium by a sudden and sustained thrombotic occlusion of a coronary artery leads to myocardial infarction. For patients presenting with an acute myocardial infarction, the most effective treatment for limiting myocardial infarct (MI) size is timely reperfusion. Nevertheless, reperfusion itself, in addition to the injury incurred during acute myocardial ischemia, induces further myocardial injury and cardiomyocyte death, the combination of which can be referred to as acute IRI (Yellon and Hausenloy 2007). The changes in cardiac mitochondrial dynamics following ischemia–reperfusion (IR) were first demonstrated by Brady et al. who showed that fragmentation of the mitochondria occurs following 2 h of simulated ischemia (sI) in HL-1 cells (Brady et al. 2006). The changes persisted for 5 h into simulated reperfusion. Treatment with the pharmacological p38MAPK inhibitor, SB203580, reverted mitochondria into an elongated phenotype, suggesting that p38MAPK may have contributed to the fragmented morphology induced by simulated ischemia (sI) (Brady et al. 2006). Decreased levels of OPA1 were also found to be associated with sI-induced mitochondrial fragmentation in H9c2 cells (Chen et al. 2009). We have also demonstrated that sI induces mitochondrial fragmentation in HL-1 cells and genetically or pharmacologically inhibiting Drp1 reverses this process and reduces cardiac cell death (Ong et al. 2010). The change in mitochondrial dynamics in the adult heart has also been shown to occur, where in vivo acute coronary occlusion via ligation of the LAD coronary artery at ~2 mm below the tip of the left atrium using an 8/0 prolene monofilament polypropylene suture for 20 min promotes fragmentation of the mitochondria (Ong et al. 2010).

Formation of unique "donut"-shaped mitochondria is also induced following sI in H9c2 cells (Liu and Hajnóczky 2011). The mitochondrial "donuts" were formed upon mitochondrial swelling from mPTP opening, where the mitochondria were detached from the cytoskeleton and anomalous fusion events (Liu and Hajnóczky 2011). These mitochondrial "donuts" serve to inhibit further cell swelling and allow the offspring to regain MMP and protect against further cellular stress (Liu and Hajnóczky 2011). Nevertheless, whether these "donut"-shaped mitochondria form in adult cardiomyocytes remains to be investigated.

3.2 Causes of Changes in Mitochondrial Dynamics in Ischemia–Reperfusion Injury

Various factors have been postulated to induce the change in mitochondrial morphology following ischemia, including hypoxia-induced inhibition of oxidative phosphorylation, collapse in MMP, Ca^{2+} overloading, or generation of ROS. Ca^{2+} overloading has been demonstrated to induce mitochondrial fragmentation in both

neonatal and adult rat cardiomyocytes (Hom et al. 2010). The downstream effects of Ca^{2+} overloading include activation of calcineurin followed by dephosphorylation and activation of Drp1 (Cereghetti et al. 2008). In addition to Ca^{2+}, calcineurin is also activated by the decreased levels of mIR-499 (Wang et al. 2011), while Drp1 itself can be modulated by posttranslational modifications such as phosphorylation (Chang and Blackstone 2007; Cribbs and Strack 2007), SUMOylation (Wasiak et al. 2007), and nitrosylation (Cho et al. 2009a; Nakamura et al. 2010).

Cytosolic calcium overloading which is responsible for activating calcineurin is induced by using either arachidonic acid (an mPTP opener) or a protonophore which causes depolarization of the MMP (Cereghetti et al. 2008), although this was initially observed in HeLa cells. In addition, calcium influx through the voltage-gated calcium channels (activated by extracellular potassium) can also induce the translocation of Drp1 to the mitochondria via the activation of Ca^{2+}/CaMKIalpha and subsequent phosphorylation at Ser600 (Han et al. 2008).

Calcium overloading in neonatal rat ventricular cardiomyocytes induced by either thapsigargin (a pharmacological inhibitor of calcium into the sarcoplasmic reticulum) or potassium chloride (KCl) (which opens L-type calcium channels via membrane depolarization) fragments the mitochondria, while genetically inhibiting Drp1 by the dominant-negative mutant $Drp1_{K38A}$ prevents the fragmentation (Hom et al. 2010). Similar fragmentation of the mitochondria was also observed in adult rat ventricular cardiomyocytes, using electron microscopy (Hom et al. 2010). Nevertheless, the potential of cardiac ischemia to induce activation of calcineurin and subsequent dephosphorylation and translocation of Drp1 remains to be clarified.

mPTP opening following ischemia has also been postulated to be caused by hexokinase II (HKII) dissociation from the mitochondrial contact sites at the OMM and IMM, although the interplay between HKII and Drp1 in maintenance of the contact sites remains to be determined (Pasdois et al. 2013; Halestrap et al. 2014). Ischemia also degrades the SUMO-2/3-specific protease SENP3, via a pathway involving the unfolded protein response (UPR) kinase PERK and the lysosomal enzyme cathepsin B. Ischemia-induced cell death however is suppressed during sustained Drp1 SUMOylation by depletion of SENP3. Restoration of SENP3 levels following reoxygenation allows deSUMOylation of Drp1 and subsequent Drp1 localization at mitochondria leading to fragmentation and cytochrome *c* release (Guo et al. 2013).

3.3 Genetic Inhibition of Ischemia-Induced Mitochondrial Fission

sI-induced mitochondrial fragmentation in the H9c2 cell line was associated with reduced OPA1 and blocking OPA1 with siRNA exacerbated the deleterious effects of mitochondrial fragmentation (Chen et al. 2009). Transfection of HL-1 cells with $Drp1_{K38A}$, Mfn1, or Mfn2, promoted mitochondrial elongation and reduced cell death following simulated IRI (SIRI) indicating that inhibition of mitochondrial fission confers cardioprotection (Ong et al. 2010). Inducing mitochondrial fragmentation by transfection with hFis1 conversely promoted cell death following sIRI

(Ong et al. 2010). Nevertheless, conflicting findings where siRNA knockdown of Mfn2 delayed mPTP opening and rendered adult cardiomyocytes less susceptible to oxidative stress have been found (Papanicolaou et al. 2011; Chen et al. 2012). Overexpression of OPA1 also failed to protect the cells against ischemia-induced apoptosis (Chen et al. 2009). More interestingly, the neonatal myocytes demonstrated opposite findings to that found in the adult cardiomyocytes, suggesting that the developmental status of the cardiac cells may also affect the effects of changes in mitochondrial morphology (Papanicolaou et al. 2011). Pretreatment of the cells with cyclosporine A (CsA) prevented OPA1 reduction, suggesting that the OPA1 levels may be influenced by the opening of the mPTP (Chen et al. 2009). Nevertheless, it is noteworthy to point out that CsA can also inhibit calcineurin (Cereghetti et al. 2008), thus likely to prevent Drp1-mediated mitochondrial fragmentation.

3.4 Pharmacological Inhibition of Ischemia-Induced Mitochondrial Fission

Pharmacologically inhibiting Drp1 by the use of *mdivi*-1 (a recently described small molecule Drp1 inhibitor – mitochondrial division inhibitor 1) also prevented mitochondrial fragmentation and cell death, in cardiac cell lines and the adult murine heart (Ong et al. 2010). The proportion of elongated interfibrillar (IF) mitochondria (defined by mitochondria >2 μm in length, the average length of sarcomeres) was also significantly increased by *mdivi*-1, as assessed by electron microscopy (Ong et al. 2010). The usage of *mdivi*-1 in the heart reduced mitochondrial ROS, reduced mitochondrial metabolism during IR, improved LV-developed pressure, and lowered LV end diastolic pressure following IR – effects mirrored using the calcineurin inhibitor, FK506 (Zepeda et al. 2014; Sharp et al. 2014). Inhibiting Drp1 via upstream pathways, e.g., overexpression of Pim1 kinase (Din et al. 2013) and usage of the nonspecific dynamin inhibitor, Dynasore (Gao et al. 2013) also proved to be cardioprotective. Similar findings in conferring protection via inhibition of mitochondrial fission have also been demonstrated in the kidney and brain (Brooks et al. 2009; Zhang et al. 2013b).

More recently, a specific peptide inhibitor of Drp1 – P110 – applied at reperfusion reduced myocardial infarct size and prevented adverse left ventricular remodeling post-MI in the adult rat heart (Disatnik et al. 2013) as well as in the brain (Guo et al. 2014). Furthermore, the discovery of other components of the mitochondrial fission machinery such as Mff and mitochondrial dynamics proteins of 49 and 51 kDa (MiD49/51) may serve as additional targets to mediate cardioprotection (Long et al. 2013). Nevertheless, these therapeutic strategies via transient pharmacological inhibition of mitochondrial fission may only be protective in the short-term. Chronic inhibition of mitochondrial fission would be detrimental to the heart and other organs as fission of the mitochondria is critical to maintaining a healthy mitochondrial network (Ikeda et al. 2014).

Pretreating the primary renotubular cells with an antioxidant or insulin could also inhibit ischemia-induced mitochondrial fragmentation although the underlying mechanism was not determined at that time (Plotnikov et al. 2008). More recently, activation of the protein kinase B (Akt) by using either a transgenic approach or pharmacological approach with the cytokine, erythropoietin, promotes mitochondrial elongation, inhibits mPTP opening, and reduces cardiac cell death following IRI (Ong et al. 2014). The effect of Akt on mitochondrial elongation was shown to be associated with Mfn1 (Ong et al. 2014) although further studies will be needed to clarify the exact mechanism.

3.5 Summary

Mitochondrial fission is a key element in acute IRI and inhibition of mitochondrial fission either by drugs or genetic methods protect against acute IRI injury. Whether prolonged elongation of mitochondria is beneficial remains to be investigated. The effects of IRI in expression levels of the mitochondrial-shaping proteins are also unclear and whether the changes in these proteins directly affect cell fate following IRI remains to be elucidated. Furthermore, hurdles exist in the investigation of the role of these proteins as these proteins have their characteristic posttranslational modification, varying expression levels, and subcellular localization. The specificity and efficacy of pharmacological modulators of mitochondrial dynamics in the heart warrants detailed investigation prior to clinical trials.

4 Mitochondrial Dynamics and Heart Failure

4.1 Pro-fusion Proteins in Heart Failure

Heart failure is characterized by fragmentation of cardiac mitochondria, e.g., an increase in the number of mitochondria with a reduction in size and mass as well as compromised structural integrity (Schaper et al. 1991; Sabbah et al. 1992; Beutner et al. 2001; Joubert et al. 2008). Severity of heart failure is tightly associated with the disruption of mitochondrial function (Sabbah et al. 1992). Investigations into the possibility of manipulating mitochondrial dynamics to reduce the severity of heart failure have been initiated. Changes in levels of mitochondrial-shaping proteins such as OPA1 downregulation have been postulated to play a role in heart failure (Chen et al. 2009; Chen and Knowlton 2010). Nevertheless, Javadov et al. showed no change in OPA1 in rat hearts 12–18 weeks after MI, albeit Mfn2 decreases and Fis1 increases (Javadov et al. 2011). The discrepancies may be due to the different models and duration of stress employed. Furthermore it has been suggested that the changes in levels of mitochondrial-shaping proteins could be linked to alteration in mitochondrial biogenesis governed by the PPAR gamma coactivator-1 (PGC-1α) (Garnier et al. 2005; Ventura-Clapier et al. 2011). The potential role of posttranslational modification particularly in the aspect of changes

in Drp1 as well as changes in levels of GTP/GDP also cannot be excluded [as reviewed in Piquereau et al. (2013)].

Cardiac cell death is also a hallmark feature of heart failure. Cardiac mitochondria are a determinant of cardiac cell fate and mitochondrial dynamics may regulate cardiac cell death in heart failure. Abnormalities in rates of fusion and fission may lead to cell death and loss of cardiomyocytes in heart failure (Olivetti et al. 1997; Narula et al. 1999). Heart failure is associated with impaired mitochondrial oxidative phosphorylation and increased ROS production from the mitochondria. Whether changes in mitochondrial dynamics lead to impairment of mitochondrial function during heart failure is unknown. In dilated cardiomyopathy (Schaper et al. 1991) and myocardial hibernation (Kalra and Zoghbi 2002), disorganized small mitochondria have been observed. In addition, Chen et al. have also demonstrated disorganized small fragmented mitochondria in an adult rat model of post-MI heart failure (Chen et al. 2009). The authors found an increased number of mitochondria per area, while the individual mitochondrial cross-sectional areas were significantly decreased in the failing adult rat heart. The fragmentation of the mitochondria was due to decreased OPA1, while there were no reported changes to the levels of other mitochondrial-shaping proteins (Chen et al. 2009). Interestingly, the mRNA level of OPA1 was unchanged suggesting that the reduction in OPA1 levels was probably due to posttranscriptional modification (Chen et al. 2009). In human hearts with ischemic cardiomyopathy, Mfn1, Mfn2, and Drp1 were increased, while OPA1 was reduced with no change in level of hFis1 (Chen et al. 2009).

In hearts with dilated cardiomyopathy however, there was no change in levels of OPA1 and hFis1 but Mfn1, Mfn2, and Drp1 were increased (Chen et al. 2009). Similarly during initial compensatory cardiac hypertrophy, an increase in OPA1 and decrease in Drp1 occur in concert with decreased Parkin and Sirt1/AMPK/PGC-1α signaling, signifying a compromised mitochondrial remodeling system (Tang et al. 2014). The significance of these changes in levels of mitochondrial-shaping proteins in development of heart failure is currently unknown and unraveling the mystery of this will benefit improvement of mitochondrial and cardiac function.

Dorn et al. have investigated the role of the mitochondrial fusion proteins MARF (mitochondrial assembly regulatory factor) and OPA1 in the *Drosophila* fly heart tube (Dorn et al. 2011). MARF (ortholog of mammalian Mfn) is the sole OMM fusion protein in the *Drosophila* fly and silencing cardiac-specific MARF and OPA1 causes a shift in mitochondrial morphology (increased heterogeneity and 30% reduction in mean mitochondrial size) as well as cardiomyopathy (characterized by dilatation of the heart tube and severe contractile impairment) (Dorn et al. 2011). Overexpression of either the mitofusins Mfn1 or Mfn2 or superoxide dismutase 1 reverses MARF-deficient cardiomyopathy, thus showing that mitochondrial fragmentation and oxidative stress plays a role in the pathogenesis of the MARF-deficient cardiomyopathy. The authors also did not detect a change in arrangement of endoplasmic reticulum (ER), sarcoplasmic reticulum,

or T-tubules, thus excluding the tethering role of MARF between the mitochondria and the ER (Dorn et al. 2011).

The formation of giant mitochondria has also been described in a variety of cardiomyopathy, including mitochondrial cardiomyopathy (Kanzaki et al. 2010). These giant mitochondria are formed by end-to-end fusion of adjacent mitochondria and may help to compensate for the functional impairment of mutations in mitochondrial DNA [as reviewed in Hoppel et al. (2009)].

Ablation of both Mfn1 and Mfn2 results in embryonic lethality (Chen et al. 2011). Conditional cardiac ablation of Mfn1 and Mfn2 promotes fragmentation of the mitochondria, defective mitochondrial respiration, and a severe dilated cardiomyopathy (Chen et al. 2011). Cardiac ablation of Mfn2 leads to impaired Parkin-mediated mitophagy causing an accumulation of damaged ROS-producing organelles and progressive heart failure. In the Mfn2-null hearts, mitochondrial ROS, suppressed at an optimal level, prevented mitochondrial depolarization, respiratory impairment, and structural degeneration. Over-suppressing mitochondrial ROS however leads to disrupted secondary autophagic pathways and impairment in mitochondrial health, delineating the importance of mitochondrial ROS alongside mitochondrial dynamics in mediating mitophagy and minimizing cardiac failure (Song et al. 2014). The deficiency in Parkin and the resulting mitophagic disruption promotes the formation of enlarged hollow donut mitochondria with dilated cardiomyopathy (Bhandari et al. 2014). Completely inhibiting mitochondrial fusion prevented the cardiomyopathy and corrected mitochondrial dysfunction, demonstrating the link between improper mitochondrial fusion, defective mitophagy, and organ dysfunction (Bhandari et al. 2014).

4.2 Pro-fission Proteins in Heart Failure

A novel mutation in the Drp1 gene (C452F) causing an autosomal dominant form of dilated cardiomyopathy in the python mouse has also been described (Ashrafian et al. 2010). Although the homozygous mutation is embryonically lethal, the heterozygous form survives until adulthood. Nevertheless, severe dilated cardiomyopathy associated with reduced levels of the ETC enzymes and impairment of ATP production develops after 11 weeks (Ashrafian et al. 2010).

Although the association between Drp1 and heart failure has been established, a decrease in SUMOylated Drp1 has been detected in SENP5-Tg hearts. In the early developmental stage of the SENP5-Tg hearts, mitochondria have been detected to be significantly larger which would account for the pathological change observed (Kim et al. 2015). Increased mitochondrial oxidative stress and aberrant mitophagy promotes abnormal activation of matrix metalloproteinase-9 (MMP-9), leading to degradation of the gap junction protein connexin-43 (Cx-43) in the ventricular myocardium (Givvimani et al. 2010; Jansen et al. 2012). The reduction in Cx-43 levels is associated with increased fibrosis and ventricular dysfunction in heart failure (Jansen et al. 2012). Treatment with *mdivi*-1 normalized the decreased ratio of Mfn2 to Drp1 (Gharanei et al. 2013) as well as the expression levels of MMP-9

and Cx-43, leading to improved cardiac function (Givvimani et al. 2010, 2012, 2014; Sharp et al. 2014).

4.3 Mitochondrial Dynamics in Left Ventricular Hypertrophy

Hypertrophy or enlargement of the left ventricle (LV) can occur due to physiological cues (in response to exercise) or pathological causes (in response to an increased load). LV hypertrophy can lead to an increased risk of arrhythmias and heart failure (Frey and Olson 2003; Frey et al. 2004). In neonatal rat cardiomyocytes, usage of the hypertrophic agonist norepinephrine induces mitochondrial fission and impaired mitochondrial function (Pennanen et al. 2014). The increase in mitochondrial number and decrease in mitochondrial mean volume was due to elevated cytoplasmic Ca^{2+} and activation of calcineurin via action of norepinephrine on α1-adrenergic receptors (Pennanen et al. 2014). Interestingly, the dominant-negative $Drp1_{K38A}$ prevented mitochondrial fission, and also blocked hypertrophic growth, while an antisense adenovirus against the fusion protein Mfn2 (AsMfn2) was sufficient to increase mitochondrial fission and stimulate a hypertrophic response without agonist treatment (Pennanen et al. 2014). The inhibition of Mfn2 in this case may also impair increased Ca^{2+} signaling and transfer from the ER to the mitochondria, thus reducing translocation of Drp1 to sever the mitochondria.

4.4 Summary

The pharmacological modulators of mitochondrial dynamics have yet to be widely used in the settings of heart failure. This may be due to the multifaceted nature of heart failure as well as the lack of proven specificity of the drugs. Future studies should investigate the application of pharmacological approach to modulating mitochondrial dynamics in tackling heart failure.

5 Mitochondrial Dynamics and Diabetes

5.1 Mitochondrial-Shaping Proteins in Diabetes

The presence of diabetes mellitus causes cardiovascular disease with a worse prognosis in the patients. The increased risk of cardiovascular disease is due to an exacerbation of cellular injury and dysfunction from hyperglycemia-induced mitochondrial oxidative stress (Nishikawa et al. 2000; Green et al. 2004). More recently, mitochondrial fragmentation has been observed in hyperglycemic conditions (Yu et al. 2006). Yu et al. and Watanabe et al. have demonstrated fragmentation of the mitochondria and enhanced ROS production in conditions of hyperglycemia in the H9c2 rat heart myoblast cell line (Yu et al. 2008; Watanabe et al. 2014). The shift in mitochondrial dynamics and ROS production causes mPTP opening and

apoptosis which could be reversed by transfecting the cells with $Drp1_{K38A}$, suggesting that Drp1 is responsible for the hyperglycemia-induced mitochondrial fragmentation (Yu et al. 2008; Watanabe et al. 2014).

Mitochondrial fragmentation has also been observed in coronary endothelial cells isolated from the diabetic murine heart (Type I model of diabetes), while this was absent in the nondiabetic mice (Makino et al. 2010). The fragmentation was due to reduced OPA1 and increased levels of Drp1, while the levels of other mitochondrial-shaping proteins remain unchanged. Four weeks of pretreatment with an antioxidant prevented the fragmentation of the mitochondria, albeit the levels of the mitochondrial-shaping proteins (OPA1 and Drp1) remain unchanged (Makino et al. 2010). This suggests that oxidative stress is the mediator of mitochondrial fragmentation in the hyperglycemic state (Makino et al. 2010). Similarly in the right atrial myocardium of humans, diabetes mellitus was associated with cardiac mitochondrial network fragmentation and decreased Mfn1 which was inversely proportional to hemoglobin A1C (Montaigne et al. 2014). In the streptozocin-induced diabetic adult mouse heart, interfibrillar (IF) mitochondria have been demonstrated to be smaller in size with a lower MMP and more predisposed to mPTP opening and apoptosis, compared to the subsarcolemmal (SSC) mitochondria (Williamson et al. 2010). This may be related to the gradual decrease in expression of cardiac Mfn2 over time as shown by a recent study by Gao et al. (2012).

Mice fed with a high-fat diet, independent of obesity-induced comorbidities, were also found to be more vulnerable to reperfusion injury (Littlejohns et al. 2014). The high-fat diet causes elevated intracellular calcium, oxidative stress, HKII dissociation from the mitochondria, and mitochondrial fragmentation, with an increase in Mfn2 and Drp1 and decreased OPA1 (Littlejohns et al. 2014). The elevated Mfn2 has been postulated to increase mitochondrial tethering to ER which will increase calcium concentrations and recruitment of Drp1. Furthermore, there was a significant decrease in the expression of voltage-dependent anion channel (VDAC), an increase in mitochondrial inorganic phosphate carrier (PiC) but no change in cyclophilin D (CypD) and adenine nucleotide translocator (ANT) (Littlejohns et al. 2014). Obesity and excess of fatty acids may lead to metabolic heart disease with impairment of mitochondrial function. Mice with cardiomyocyte-specific overexpression of the fatty acid transport protein FATP1 experienced an increase in phosphorylation of PKCα and PKCδ, and a decrease in phosphorylation of AKT and expression of CREB, PGC1α, and PPARα (Elezaby et al. 2014). The excessive delivery of FAs to the cardiac myocyte leads to remodeling of mitochondrial structure by fragmentation via decreased MFN1, MFN2, and OPA1 (Elezaby et al. 2014). Complex-II activity and expression was also markedly reduced (Elezaby et al. 2014).

5.2 Novel Mitochondrial Proteins in Diabetes

Mitofilin, which is also known as heart muscle protein, is located in the IMM and is crucial for maintaining cristae morphology and structure. Type 1 diabetes (T1D) mellitus has been reported to cause a downregulation in IFM mitofilin. Genetically, overexpression of mitofilin in mice restored mitochondrial cristae structure, cardiac contractile function as well as respiratory chain functions following streptozotocin (STZ)-induced diabetes (Thapa et al. 2014).

Aberrant neuronal mitochondrial morphology, reduced ATP production, and impaired complex I activity in neurons caused by imbalanced mitochondrial fusion and fission have been linked to Type II diabetes via a glycogen synthase kinase-3β (GSK3β)/Drp1-dependent mechanism (Huang et al. 2014). Whether this holds true in the heart remains to be investigated. More recently, the diabetes susceptibility gene Clec16a has been identified as a novel regulator of mitophagy in beta cells through upstream regulation of the Nrdp1/Parkin pathway (Soleimanpour et al. 2014). The E3 ubiquitin ligase Nrdp1 is a major interacting partner of Clec16a, and Clec16a appears to protect Nrdp1 from proteosomal degradation (Soleimanpour et al. 2014). Islets from mice with pancreas-specific deletion of Clec16a have an accumulation of abnormal mitochondria with reduced oxygen consumption and ATP concentration, signifying an impairment of proper mitophagy (Soleimanpour et al. 2014). The relevance of the association of cardiac mitophagy with diabetes remains to be investigated.

Mitochondria-associated endoplasmic reticulum (ER) membranes (MAMs), consisting of the VDAC-1/glucose-regulated protein 75 (Grp75)/inositol 1,4,5-triphosphate receptor (IP3R)-1 complex, are required for proper insulin signaling (Tubbs et al. 2014). Disruption of MAM integrity altered insulin signaling in mouse and human primary hepatocytes, while treatment of CypD-knockout mice with metformin improved both insulin sensitivity and MAM integrity (Tubbs et al. 2014). There is also a possibility that the MAM contacts may be affected in the heart and targeting this may render the heart more resistant against cardiac pathological stresses.

A more recent study has cloned the gene encoding apolipoprotein O (APOO), which is overexpressed in hearts from diabetic patients and generated APOO-Tg mice, which expresses physiological levels of human APOO in heart tissue (Turkieh et al. 2014). Localized to the mitochondria, APOO enhanced mitochondrial uncoupling and respiration leading to fatty acid metabolism and ROS production, increased AMPK phosphorylation, and Ppara and Pgc1a expression (Turkieh et al. 2014). These changes generate a mitochondrial metabolic sink whereby accumulation of lipotoxic byproducts leads to lipoapoptosis, loss of cardiac cells, and cardiomyopathy (Turkieh et al. 2014). It remains to be investigated whether APOO regulates mitochondrial morphology as a missing link between impaired mitochondrial function and onset of cardiomyopathy.

The induced in high glucose-1 (IHG-1) is a conserved mitochondrial protein found in the kidney which amplifies profibrotic transforming growth factor (TGF)-β 1 signaling and increases mitochondrial biogenesis (Hickey et al. 2014).

Overexpression of IHG-1 leads to increased mitochondrial fusion by forming complexes with the Mfns and enhancing the GTP-binding capacity of Mfn2 (Hickey et al. 2014). IHG-1 also protects renal cells from ROS-induced apoptosis (Hickey et al. 2014), raising the speculation that if found in the heart, similar protective responses may be observed.

5.3 Summary

In summary, mitochondrial fragmentation seems to be a key element associated with the deleterious effects of diabetes. Whether pharmacological modulation of mitochondrial dynamics can successfully control diabetes will require further investigation. Insulin treatment, although tested in STZ-induced T1D rats, has successfully normalized alterations in neuronal mitochondrial dynamics, biogenesis, and autophagy (Santos et al. 2014). Future therapeutics using insulin should be tested in the heart in the hope of demonstrating similar positive results.

6 Mitochondrial Dynamics and Pulmonary Hypertension

6.1 Mitochondrial-Shaping Proteins in Pulmonary Hypertension

Pulmonary arterial hypertension (PAH) is defined as a condition where the pulmonary arteries become obstructed, caused, in part, by pulmonary artery smooth muscle cell (PASMC) hyperproliferation, resulting in right ventricular hypertrophy and heart failure [as reviewed in Archer et al. (2010)]. In this regard, recent experimental data has implicated the mitochondrial fusion and fission proteins as potential causative factors for induction of PAH.

Early endoplasmic reticulum (ER) stress, which is associated with clinical triggers of PAH including hypoxia may cause abnormalities of mitochondria and contribute to vascular remodeling. Nogo-B, a regulator of ER structure, has been shown to be induced by hypoxia in SMCs of the pulmonary arteries through activation of the ER stress-sensitive transcription factor ATF6 (Sutendra et al. 2011). The activation of Nogo-B increased the distance between the ER and mitochondria and decreased ER-to-mitochondria phospholipid transfer and intramitochondrial calcium (Sutendra et al. 2011). Inhibition of calcium-sensitive mitochondrial enzymes increased MMP, decreased mitochondrial ROS, and decreased mitochondria-dependent apoptosis ensue, thus enabling the development of PAH through overproliferation (Sutendra et al. 2011). The role of Mfn2, which is known for mitochondrial-ER tethering, was not investigated in this study though the decrease in Mfn2 may enhance this distance between ER–mitochondria. In the skeletal muscle however, diminished AKT and p70S6 kinase phosphorylation were evident alongside decreased Mfn2 and increased phosphorylation of ryanodine receptor 1 receptors, providing a link between impaired excitation–contraction coupling and altered sarcoplasmic reticulum Ca^{2+} sequestration (Batt et al. 2014).

Hyperproliferation of the PASMCs constitutes an essential part of the pathophysiology underlying PAH, where proper division and distribution of mitochondria is crucial. In this regard, the upregulation of Drp1 and downregulation of Mfn2, coupled with cyclin B1/CDK1 phosphorylation of Drp1 at Ser616 facilitates the mitochondrial fragmentation required (Marsboom et al. 2012). Interestingly, treatment with the small molecule Drp1 inhibitor, *mdivi*-1, was shown to prevent the progression of PAH in three different experimental models of PAH: (1) 2 mg $CoCl_2$ i.p./day for 4 weeks, (2) chronic hypoxia (10% oxygen), and (3) monocrotaline (60 mg/kg i.p.) (Marsboom et al. 2012). Similar results were also observed by genetically inhibiting Drp1 (Wang et al. 2015). Mfn2 was also found to be downregulated in PASMCs from idiopathic PAH patients (Marsboom et al. 2012) although this has been controversial as Mfn2 has been indicated to mediate PASMC proliferation in hypoxic pulmonary hypertension via the PI3K/Akt signaling pathway (Zhang et al. 2012). siRNA against Mfn2 suppressed effects of hypoxia including regulation of cells at G(2)/M + S phase, increased proliferating cell nuclear antigen and cyclin A expression (Zhang et al. 2012). A similar reduction was also detected for PGC1α, while overexpression of Mfn2 using adenovirus (Ad-Mfn2) in an early preclinical study rescued the deleterious phenotype (Ryan et al. 2013). This study utilizes rats with experimental PAH: either (1) the monocrotaline model or (2) a rat model involving a combination of chronic hypoxia plus the VEGF receptor antagonist, SU5416. The vector – 0.1 ml of Ad-Mfn2 or Ad-GFP virus (in saline, 2 × 109 plaque-forming units) – was administered intravenously and/or via tracheal nebulization, Ad-Mfn2 therapy resulted in positive vascular remodeling, improved functional capacity, and reduced pulmonary vascular resistance in these rat models of PAH (Ryan et al. 2013).

6.2 Summary

Together, these findings implicate the mitochondrial-shaping proteins as a novel therapeutic target for PAH. Although preventing PAH by using *mdivi*-1 is beneficial short-term, the feasibility remains to be determined as chronic inhibition of mitochondrial fission may be detrimental. In addition, the role of Mfn2 in mediating effects of PASMC proliferation in hypoxic pulmonary hypertension warrants further clarification for development of targeted therapeutics against PAH.

7 Conclusion

Mitochondria are organelles that are highly dynamic. The plasticity of mitochondria is influenced by a myriad of biological cues within the cells which in turn affect cellular physiological and pathophysiological conditions. In the heart, changes in mitochondrial dynamics have been shown to play a crucial role in manifestation of cardiac pathology and modulation of the mitochondrial-shaping

Table 1 List of mitochondrial-shaping proteins in the heart

	Mammal	Location	Function		Known changes during cardiac diseases	Potential therapeutic approaches	References
Mitochondrial fusion proteins	Opa1	IMM	IMM fusion	Pleiotropic effects include cristae remodeling	Ablation or downregulation leads to cardiac diseases	Activate or upregulate fusion proteins	Fang et al. (2007), Yu et al. (2011), Chen et al. (2011), Ngoh et al. (2012), Gao et al. (2012), Chen and Dorn (2013), Givvimani et al. (2014), and Ong et al. (2014)
	Mfn1	OMM	OMM fusion				
	Mfn2	OMM	OMM fusion	Pleiotropic effects on metabolism, apoptosis, proliferation, and ER tethering			
Mitochondrial fission proteins	Drp1 or DNM1L	Cytosol and OMM	OM fission		Activation or upregulation leads to cardiac diseases	Inhibit mitochondrial fission by pharmacological agents such as mdivi-1, Dynasore, and P110	Ong et al. (2010), Nakamura et al. (2010), Gao et al. (2013), Gharanei et al. (2013), Disatnik et al. (2013), and Kageyama et al. (2014)
	Fis1	OMM	OMM fission				
	Mff	OMM	OMM fission				
	MiD49/51	OMM	OMM fission				

proteins may constitute a novel therapy in targeting cardiovascular diseases (see Table 1). Nevertheless, it is noteworthy to point that it is the delicate balance between the fission and fusion which is more critical rather than the fission or fusion processes alone and this balance should be kept in targeting mitochondrial dynamics in pathological conditions. Understanding the association between mitochondrial dynamics and the inducers of cardiac diseases will facilitate the development of novel therapeutic agents in reducing mortality and morbidity from heart diseases.

Acknowledgements Sang-Bing Ong is supported by the Singapore Ministry of Health's National Medical Research Council under its Open Fund—Young Individual Research Grant (OF-YIRG) scheme and a Khoo Postdoctoral Fellowship Award (KPFA) from the Estate of Tan Sri Khoo Teck Puat, Singapore. Derek John Hausenloy was supported by the British Heart Foundation (FS/10/039/28270), the Rosetrees Trust, and the National Institute for Health Research University College London Hospitals Biomedical Research Centre.

References

Alirol E, James D, Huber D, et al. (2006) The mitochondrial fission protein hFis1 requires the endoplasmic reticulum gateway to induce apoptosis. Mol Biol Cell 17:4593–4605. doi:10.1091/mbc.E06-05-0377

Archer SL, Weir EK, Wilkins MR (2010) Basic science of pulmonary arterial hypertension for clinicians: new concepts and experimental therapies. Circulation 121:2045–2066. doi:10.1161/CIRCULATIONAHA.108.847707

Arnoult D, Rismanchi N, Grodet A, et al. (2005) Bax/Bak-dependent release of DDP/TIMM8a promotes Drp1-mediated mitochondrial fission and mitoptosis during programmed cell death. Curr Biol 15:2112–2118. doi:10.1016/j.cub.2005.10.041

Ashrafian H, Docherty L, Leo V, et al. (2010) A mutation in the mitochondrial fission gene Dnm1l leads to cardiomyopathy. PLoS Genet 6:e1001000. doi:10.1371/journal.pgen.1001000

Batt J, Ahmed SS, Correa J, et al. (2014) Skeletal muscle dysfunction in idiopathic pulmonary arterial hypertension. Am J Respir Cell Mol Biol 50:74–86. doi:10.1165/rcmb.2012-0506OC

Beraud N, Pelloux S, Usson Y, et al. (2009) Mitochondrial dynamics in heart cells: very low amplitude high frequency fluctuations in adult cardiomyocytes and flow motion in non beating Hl-1 cells. J Bioenerg Biomembr 41:195–214. doi:10.1007/s10863-009-9214-x

Beutner G, Sharma VK, Giovannucci DR, et al. (2001) Identification of a ryanodine receptor in rat heart mitochondria. J Biol Chem 276:21482–21488. doi:10.1074/jbc.M101486200

Bhandari P, Song M, Chen Y, et al. (2014) Mitochondrial contagion induced by Parkin deficiency in *Drosophila* hearts and its containment by suppressing mitofusin. Circ Res 114:257–265. doi:10.1161/CIRCRESAHA.114.302734

Brady NR, Hamacher-Brady A, Gottlieb RA (2006) Proapoptotic BCL-2 family members and mitochondrial dysfunction during ischemia/reperfusion injury, a study employing cardiac HL-1 cells and GFP biosensors. Biochim Biophys Acta 1757:667–678. doi:10.1016/j.bbabio.2006.04.011

Breckenridge DG, Stojanovic M, Marcellus RC, Shore GC (2003) Caspase cleavage product of BAP31 induces mitochondrial fission through endoplasmic reticulum calcium signals, enhancing cytochrome c release to the cytosol. J Cell Biol 160:1115–1127. doi:10.1083/jcb.200212059

Brooks C, Wei Q, Feng L, et al. (2007) Bak regulates mitochondrial morphology and pathology during apoptosis by interacting with mitofusins. Proc Natl Acad Sci U S A 104:11649–11654. doi:10.1073/pnas.0703976104

Brooks C, Wei Q, Cho S-G, Dong Z (2009) Regulation of mitochondrial dynamics in acute kidney injury in cell culture and rodent models. J Clin Invest 119:1275–1285. doi:10.1172/JCI37829

Cereghetti GM, Stangherlin A, Martins de Brito O, et al. (2008) Dephosphorylation by calcineurin regulates translocation of Drp1 to mitochondria. Proc Natl Acad Sci U S A 105:15803–15808. doi:10.1073/pnas.0808249105

Chan DC (2006) Mitochondrial fusion and fission in mammals. Annu Rev Cell Dev Biol 22:79–99. doi:10.1146/annurev.cellbio.22.010305.104638

Chang C-R, Blackstone C (2007) Cyclic AMP-dependent protein kinase phosphorylation of Drp1 regulates its GTPase activity and mitochondrial morphology. J Biol Chem 282:21583–21587. doi:10.1074/jbc.C700083200

Chen H, Chan DC (2010) Physiological functions of mitochondrial fusion. Ann N Y Acad Sci 1201:21–25. doi:10.1111/j.1749-6632.2010.05615.x

Chen Y, Dorn GW (2013) PINK1-phosphorylated mitofusin 2 is a Parkin receptor for culling damaged mitochondria. Science 340:471–475. doi:10.1126/science.1231031

Chen L, Knowlton AA (2010) Mitochondria and heart failure: new insights into an energetic problem. Minerva Cardioangiol 58:213–229

Chen L, Gong Q, Stice JP, Knowlton AA (2009) Mitochondrial OPA1, apoptosis, and heart failure. Cardiovasc Res 84:91–99. doi:10.1093/cvr/cvp181

Chen Y, Liu Y, Dorn GW (2011) Mitochondrial fusion is essential for organelle function and cardiac homeostasis. Circ Res 109:1327–1331. doi:10.1161/CIRCRESAHA.111.258723

Chen Y, Csordás G, Jowdy C, et al. (2012) Mitofusin 2-containing mitochondrial-reticular microdomains direct rapid cardiomyocyte bioenergetic responses via interorganelle Ca(2+) crosstalk. Circ Res 111:863–875. doi:10.1161/CIRCRESAHA.112.266585

Chen W, Zhou Z, Li L, et al. (2013) Diverse sequence determinants control human and mouse receptor interacting protein 3 (RIP3) and mixed lineage kinase domain-like (MLKL) interaction in necroptotic signaling. J Biol Chem 288:16247–16261. doi:10.1074/jbc.M112.435545

Chhabra ES, Higgs HN (2006) INF2 Is a WASP homology 2 motif-containing formin that severs actin filaments and accelerates both polymerization and depolymerization. J Biol Chem 281:26754–26767. doi:10.1074/jbc.M604666200

Cho D-H, Nakamura T, Fang J, et al. (2009a) S-nitrosylation of Drp1 mediates beta-amyloid-related mitochondrial fission and neuronal injury. Science 324:102–105. doi:10.1126/science.1171091

Cho YS, Challa S, Moquin D, et al. (2009b) Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell 137:1112–1123. doi:10.1016/j.cell.2009.05.037

Cipolat S, Martins de Brito O, Dal Zilio B, Scorrano L (2004) OPA1 requires mitofusin 1 to promote mitochondrial fusion. Proc Natl Acad Sci U S A 101:15927–15932. doi:10.1073/pnas.0407043101

Cribbs JT, Strack S (2007) Reversible phosphorylation of Drp1 by cyclic AMP-dependent protein kinase and calcineurin regulates mitochondrial fission and cell death. EMBO Rep 8:939–944. doi:10.1038/sj.embor.7401062

De Brito OM, Scorrano L (2008) Mitofusin 2 tethers endoplasmic reticulum to mitochondria. Nature 456:605–610. doi:10.1038/nature07534

Diaz F, Moraes CT (2008) Mitochondrial biogenesis and turnover. Cell Calcium 44:24–35. doi:10.1016/j.ceca.2007.12.004

Din S, Mason M, Völkers M, et al. (2013) Pim-1 preserves mitochondrial morphology by inhibiting dynamin-related protein 1 translocation. Proc Natl Acad Sci U S A 110:5969–5974. doi:10.1073/pnas.1213294110

Disatnik M-H, Ferreira JCB, Campos JC, et al. (2013) Acute inhibition of excessive mitochondrial fission after myocardial infarction prevents long-term cardiac dysfunction. J Am Heart Assoc 2:e000461. doi:10.1161/JAHA.113.000461

Dorn GW, Clark CF, Eschenbacher WH, et al. (2011) MARF and Opa1 control mitochondrial and cardiac function in *Drosophila*. Circ Res 108:12–17. doi:10.1161/CIRCRESAHA.110.236745

Elezaby A, Sverdlov AL, Tu VH, et al. (2014) Mitochondrial remodeling in mice with cardiomyocyte-specific lipid overload. J Mol Cell Cardiol 79C:275–283. doi:10.1016/j.yjmcc.2014.12.001

Fang L, Moore X-L, Gao X-M, et al. (2007) Down-regulation of mitofusin-2 expression in cardiac hypertrophy in vitro and in vivo. Life Sci 80:2154–2160. doi:10.1016/j.lfs.2007.04.003

Frank S, Gaume B, Bergmann-Leitner ES, et al. (2001) The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. Dev Cell 1:515–525

Frey N, Olson EN (2003) Cardiac hypertrophy: the good, the bad, and the ugly. Annu Rev Physiol 65:45–79. doi:10.1146/annurev.physiol.65.092101.142243

Frey N, Katus HA, Olson EN, Hill JA (2004) Hypertrophy of the heart: a new therapeutic target? Circulation 109:1580–1589. doi:10.1161/01.CIR.0000120390.68287.BB

Friedman JR, Lackner LL, West M, et al. (2011) ER tubules mark sites of mitochondrial division. Science 334:358–362. doi:10.1126/science.1207385

Gandre-Babbe S, van der Bliek AM (2008) The novel tail-anchored membrane protein Mff controls mitochondrial and peroxisomal fission in mammalian cells. Mol Biol Cell 19:2402–2412. doi:10.1091/mbc.E07-12-1287

Gao Q, Wang X-M, Ye H-W, et al. (2012) Changes in the expression of cardiac mitofusin-2 in different stages of diabetes in rats. Mol Med Rep 6:811–814. doi:10.3892/mmr.2012.1002

Gao D, Zhang L, Dhillon R, et al. (2013) Dynasore protects mitochondria and improves cardiac lusitropy in Langendorff perfused mouse heart. PLoS One 8:e60967. doi:10.1371/journal.pone.0060967

Garnier A, Fortin D, Zoll J, et al. (2005) Coordinated changes in mitochondrial function and biogenesis in healthy and diseased human skeletal muscle. FASEB J 19:43–52. doi:10.1096/fj.04-2173com

Germain M, Mathai JP, McBride HM, Shore GC (2005) Endoplasmic reticulum BIK initiates DRP1-regulated remodelling of mitochondrial cristae during apoptosis. EMBO J 24:1546–1556. doi:10.1038/sj.emboj.7600592

Gharanei M, Hussain A, Janneh O, Maddock H (2013) Attenuation of doxorubicin-induced cardiotoxicity by mdivi-1: a mitochondrial division/mitophagy inhibitor. PLoS One 8:e77713. doi:10.1371/journal.pone.0077713

Givvimani S, Tyagi N, Sen U, et al. (2010) MMP-2/TIMP-2/TIMP-4 versus MMP-9/TIMP-3 in transition from compensatory hypertrophy and angiogenesis to decompensatory heart failure. Arch Physiol Biochem 116:63–72. doi:10.3109/13813451003652997

Givvimani S, Munjal C, Tyagi N, et al. (2012) Mitochondrial division/mitophagy inhibitor (Mdivi) ameliorates pressure overload induced heart failure. PLoS One 7:e32388. doi:10.1371/journal.pone.0032388

Givvimani S, Pushpakumar S, Veeranki S, Tyagi SC (2014) Dysregulation of Mfn2 and Drp-1 proteins in heart failure. Can J Physiol Pharmacol 92:583–591. doi:10.1139/cjpp-2014-0060

Gomes LC, Di Benedetto G, Scorrano L (2011) During autophagy mitochondria elongate, are spared from degradation and sustain cell viability. Nat Cell Biol 13:589–598. doi:10.1038/ncb2220

Green K, Brand MD, Murphy MP (2004) Prevention of mitochondrial oxidative damage as a therapeutic strategy in diabetes. Diabetes 53(Suppl 1):S110–S118

Guo C, Hildick KL, Luo J, et al. (2013) SENP3-mediated deSUMOylation of dynamin-related protein 1 promotes cell death following ischaemia. EMBO J 32:1514–1528. doi:10.1038/emboj.2013.65

Guo X, Sesaki H, Qi X (2014) Drp1 stabilizes p53 on the mitochondria to trigger necrosis under oxidative stress conditions in vitro and in vivo. Biochem J 461:137–146. doi:10.1042/BJ20131438

Halestrap AP, Pereira GC, Pasdois P (2014) The role of hexokinase in cardioprotection - mechanism and potential for translation. Br J Pharmacol. doi:10.1111/bph.12899

Han X-J, Lu Y-F, Li S-A, et al. (2008) CaM kinase I alpha-induced phosphorylation of Drp1 regulates mitochondrial morphology. J Cell Biol 182:573–585. doi:10.1083/jcb.200802164

Hatch AL, Gurel PS, Higgs HN (2014) Novel roles for actin in mitochondrial fission. J Cell Sci 127:4549–4560. doi:10.1242/jcs.153791

He S, Wang L, Miao L, et al. (2009) Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell 137:1100–1111. doi:10.1016/j.cell.2009.05.021

Hickey FB, Corcoran JB, Griffin B, et al. (2014) IHG-1 increases mitochondrial fusion and bioenergetic function. Diabetes 63:4314–4325. doi:10.2337/db13-1256

Hom J, Sheu S-S (2009) Morphological dynamics of mitochondria – a special emphasis on cardiac muscle cells. J Mol Cell Cardiol 46:811–820. doi:10.1016/j.yjmcc.2009.02.023

Hom J, Yu T, Yoon Y, et al. (2010) Regulation of mitochondrial fission by intracellular Ca2+ in rat ventricular myocytes. Biochim Biophys Acta 1797:913–921. doi:10.1016/j.bbabio.2010.03.018

Hoppel CL, Tandler B, Fujioka H, Riva A (2009) Dynamic organization of mitochondria in human heart and in myocardial disease. Int J Biochem Cell Biol 41:1949–1956. doi:10.1016/j.biocel.2009.05.004

Hoppins S, Lackner L, Nunnari J (2007) The machines that divide and fuse mitochondria. Annu Rev Biochem 76:751–780. doi:10.1146/annurev.biochem.76.071905.090048

Huang S, Wang Y, Gan X, et al. (2014) Drp1-mediated mitochondrial abnormalities link to synaptic injury in diabetes model. Diabetes. doi:10.2337/db14-0758

Ikeda Y, Shirakabe A, Maejima Y, et al. (2014) Endogenous Drp1 mediates mitochondrial autophagy and protects the heart against energy stress. Circ Res. doi:10.1161/CIRCRESAHA.116.303356

James DI, Parone PA, Mattenberger Y, Martinou J-C (2003) hFis1, a novel component of the mammalian mitochondrial fission machinery. J Biol Chem 278:36373–36379. doi:10.1074/jbc.M303758200

Jansen JA, van Veen TAB, de Jong S, et al. (2012) Reduced Cx43 expression triggers increased fibrosis due to enhanced fibroblast activity. Circ Arrhythm Electrophysiol 5:380–390. doi:10.1161/CIRCEP.111.966580

Javadov S, Rajapurohitam V, Kilić A, et al. (2011) Expression of mitochondrial fusion-fission proteins during post-infarction remodeling: the effect of NHE-1 inhibition. Basic Res Cardiol 106:99–109. doi:10.1007/s00395-010-0122-3

Joubert F, Wilding JR, Fortin D, et al. (2008) Local energetic regulation of sarcoplasmic and myosin ATPase is differently impaired in rats with heart failure. J Physiol 586:5181–5192. doi:10.1113/jphysiol.2008.157677

Kageyama Y, Hoshijima M, Seo K, et al. (2014) Parkin-independent mitophagy requires Drp1 and maintains the integrity of mammalian heart and brain. EMBO J 33:2798–2813. doi:10.15252/embj.201488658

Kaiser WJ, Upton JW, Long AB, et al. (2011) RIP3 mediates the embryonic lethality of caspase-8-deficient mice. Nature 471:368–372. doi:10.1038/nature09857

Kalra DK, Zoghbi WA (2002) Myocardial hibernation in coronary artery disease. Curr Atheroscler Rep 4:149–155

Kanzaki Y, Terasaki F, Okabe M, et al. (2010) Giant mitochondria in the myocardium of a patient with mitochondrial cardiomyopathy: transmission and 3-dimensional scanning electron microscopy. Circulation 121:831–832. doi:10.1161/CIR.0b013e3181d22e2d

Karbowski M, Lee Y-J, Gaume B, et al. (2002) Spatial and temporal association of Bax with mitochondrial fission sites, Drp1, and Mfn2 during apoptosis. J Cell Biol 159:931–938. doi:10.1083/jcb.200209124

Karbowski M, Norris KL, Cleland MM, et al. (2006) Role of Bax and Bak in mitochondrial morphogenesis. Nature 443:658–662. doi:10.1038/nature05111

Kim EY, Zhang Y, Beketaev I, et al. (2015) SENP5, a SUMO isopeptidase, induces apoptosis and cardiomyopathy. J Mol Cell Cardiol 78C:154–164. doi:10.1016/j.yjmcc.2014.08.003

Korobova F, Ramabhadran V, Higgs HN (2013) An actin-dependent step in mitochondrial fission mediated by the ER-associated formin INF2. Science 339:464–467. doi:10.1126/science.1228360

Korobova F, Gauvin TJ, Higgs HN (2014) A role for myosin II in mammalian mitochondrial fission. Curr Biol 24:409–414. doi:10.1016/j.cub.2013.12.032

Koshiba T, Detmer SA, Kaiser JT, et al. (2004) Structural basis of mitochondrial tethering by mitofusin complexes. Science 305:858–862. doi:10.1126/science.1099793

Lee Y, Jeong S-Y, Karbowski M, et al. (2004) Roles of the mammalian mitochondrial fission and fusion mediators Fis1, Drp1, and Opa1 in apoptosis. Mol Biol Cell 15:5001–5011. doi:10.1091/mbc.E04-04-0294

Lim SY, Davidson SM, Hausenloy DJ, Yellon DM (2007) Preconditioning and postconditioning: the essential role of the mitochondrial permeability transition pore. Cardiovasc Res 75:530–535. doi:10.1016/j.cardiores.2007.04.022

Lin L, Kim SC, Wang Y, et al. (2007) HSP60 in heart failure: abnormal distribution and role in cardiac myocyte apoptosis. Am J Physiol Heart Circ Physiol 293:H2238–H2247. doi:10.1152/ajpheart.00740.2007

Littlejohns B, Pasdois P, Duggan S, et al. (2014) Hearts from mice fed a non-obesogenic high-fat diet exhibit changes in their oxidative state, calcium and mitochondria in parallel with increased susceptibility to reperfusion injury. PLoS One 9:e100579. doi:10.1371/journal.pone.0100579

Liu X, Hajnóczky G (2011) Altered fusion dynamics underlie unique morphological changes in mitochondria during hypoxia-reoxygenation stress. Cell Death Differ 18:1561–1572. doi:10.1038/cdd.2011.13

Liu Q, Qiu J, Liang M, et al. (2014) Akt and mTOR mediate programmed necrosis in neurons. Cell Death Dis 5:e1084. doi:10.1038/cddis.2014.69

Long B, Wang K, Li N, et al. (2013) miR-761 regulates the mitochondrial network by targeting mitochondrial fission factor. Free Radic Biol Med 65:371–379. doi:10.1016/j.freeradbiomed.2013.07.009

Losón OC, Song Z, Chen H, Chan DC (2013) Fis1, Mff, MiD49, and MiD51 mediate Drp1 recruitment in mitochondrial fission. Mol Biol Cell 24:659–667. doi:10.1091/mbc.E12-10-0721

Losón OC, Liu R, Rome ME, et al. (2014) The mitochondrial fission receptor MiD51 requires ADP as a cofactor. Structure 22:367–377. doi:10.1016/j.str.2014.01.001

Losón OC, Meng S, Ngo H, et al. (2015) Crystal structure and functional analysis of MiD49, a receptor for the mitochondrial fission protein Drp1. Protein Sci. doi:10.1002/pro.2629

Makino A, Scott BT, Dillmann WH (2010) Mitochondrial fragmentation and superoxide anion production in coronary endothelial cells from a mouse model of type 1 diabetes. Diabetologia 53:1783–1794. doi:10.1007/s00125-010-1770-4

Marsboom G, Toth PT, Ryan JJ, et al. (2012) Dynamin-related protein 1-mediated mitochondrial mitotic fission permits hyperproliferation of vascular smooth muscle cells and offers a novel therapeutic target in pulmonary hypertension. Circ Res 110:1484–1497. doi:10.1161/CIRCRESAHA.111.263848

Montaigne D, Marechal X, Coisne A, et al. (2014) Myocardial contractile dysfunction is associated with impaired mitochondrial function and dynamics in type 2 diabetic but not in obese patients. Circulation 130:554–564. doi:10.1161/CIRCULATIONAHA.113.008476

Moujalled DM, Cook WD, Murphy JM, Vaux DL (2014) Necroptosis induced by RIPK3 requires MLKL but not Drp1. Cell Death Dis 5:e1086. doi:10.1038/cddis.2014.18

Mozdy AD, McCaffery JM, Shaw JM (2000) Dnm1p GTPase-mediated mitochondrial fission is a multi-step process requiring the novel integral membrane component Fis1p. J Cell Biol 151:367–380

Nakamura T, Cieplak P, Cho D-H, et al. (2010) S-nitrosylation of Drp1 links excessive mitochondrial fission to neuronal injury in neurodegeneration. Mitochondrion 10:573–578. doi:10.1016/j.mito.2010.04.007

Narula J, Pandey P, Arbustini E, et al. (1999) Apoptosis in heart failure: release of cytochrome c from mitochondria and activation of caspase-3 in human cardiomyopathy. Proc Natl Acad Sci U S A 96:8144–8149

Neuspiel M, Zunino R, Gangaraju S, et al. (2005) Activated mitofusin 2 signals mitochondrial fusion, interferes with Bax activation, and reduces susceptibility to radical induced depolarization. J Biol Chem 280:25060–25070. doi:10.1074/jbc.M501599200

Ngoh GA, Papanicolaou KN, Walsh K (2012) Loss of mitofusin 2 promotes endoplasmic reticulum stress. J Biol Chem 287:20321–20332. doi:10.1074/jbc.M112.359174

Nishikawa T, Edelstein D, Du XL, et al. (2000) Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. Nature 404:787–790. doi:10.1038/35008121

Obitsu S, Sakata K, Teshima R, Kondo K (2013) Eleostearic acid induces RIP1-mediated atypical apoptosis in a kinase-independent manner via ERK phosphorylation, ROS generation and mitochondrial dysfunction. Cell Death Dis 4:e674. doi:10.1038/cddis.2013.188

Oerlemans MIFJ, Liu J, Arslan F, et al. (2012) Inhibition of RIP1-dependent necrosis prevents adverse cardiac remodeling after myocardial ischemia-reperfusion in vivo. Basic Res Cardiol 107:270. doi:10.1007/s00395-012-0270-8

Olichon A, Baricault L, Gas N, et al. (2003) Loss of OPA1 perturbates the mitochondrial inner membrane structure and integrity, leading to cytochrome c release and apoptosis. J Biol Chem 278:7743–7746. doi:10.1074/jbc.C200677200

Olivetti G, Abbi R, Quaini F, et al. (1997) Apoptosis in the failing human heart. N Engl J Med 336:1131–1141. doi:10.1056/NEJM199704173361603

Ong S-B, Gustafsson AB (2012) New roles for mitochondria in cell death in the reperfused myocardium. Cardiovasc Res 94:190–196. doi:10.1093/cvr/cvr312

Ong S-B, Hausenloy DJ (2010) Mitochondrial morphology and cardiovascular disease. Cardiovasc Res 88:16–29. doi:10.1093/cvr/cvq237

Ong S-B, Subrayan S, Lim SY, et al. (2010) Inhibiting mitochondrial fission protects the heart against ischemia/reperfusion injury. Circulation 121:2012–2022. doi:10.1161/CIRCULATIONAHA.109.906610

Ong S-B, Hall AR, Hausenloy DJ (2012) Mitochondrial dynamics in cardiovascular health and disease. Antioxid Redox Signal. doi:10.1089/ars.2012.4777

Ong S-B, Hall AR, Dongworth RK, et al. (2014) Akt protects the heart against ischaemia-reperfusion injury by modulating mitochondrial morphology. Thromb Haemost. doi:10.1160/TH14-07-0592

Otera H, Wang C, Cleland MM, et al. (2010) Mff is an essential factor for mitochondrial recruitment of Drp1 during mitochondrial fission in mammalian cells. J Cell Biol 191:1141–1158. doi:10.1083/jcb.201007152

Palmer CS, Osellame LD, Laine D, et al. (2011) MiD49 and MiD51, new components of the mitochondrial fission machinery. EMBO Rep 12:565–573. doi:10.1038/embor.2011.54

Palmer CS, Elgass KD, Parton RG, et al. (2013) Adaptor proteins MiD49 and MiD51 can act independently of Mff and Fis1 in Drp1 recruitment and are specific for mitochondrial fission. J Biol Chem 288:27584–27593. doi:10.1074/jbc.M113.479873

Papanicolaou KN, Khairallah RJ, Ngoh GA, et al. (2011) Mitofusin-2 maintains mitochondrial structure and contributes to stress-induced permeability transition in cardiac myocytes. Mol Cell Biol 31:1309–1328. doi:10.1128/MCB.00911-10

Parone PA, James DI, Da Cruz S, et al. (2006) Inhibiting the mitochondrial fission machinery does not prevent Bax/Bak-dependent apoptosis. Mol Cell Biol 26:7397–7408. doi:10.1128/MCB.02282-05

Pasdois P, Parker JE, Halestrap AP (2013) Extent of mitochondrial hexokinase II dissociation during ischemia correlates with mitochondrial cytochrome c release, reactive oxygen species production, and infarct size on reperfusion. J Am Heart Assoc 2:e005645. doi:10.1161/JAHA.112.005645

Pennanen C, Parra V, López-Crisosto C, et al. (2014) Mitochondrial fission is required for cardiomyocyte hypertrophy mediated by a Ca2+-calcineurin signaling pathway. J Cell Sci 127:2659–2671. doi:10.1242/jcs.139394

Pinton P, Ferrari D, Rapizzi E, et al. (2001) The Ca2+ concentration of the endoplasmic reticulum is a key determinant of ceramide-induced apoptosis: significance for the molecular mechanism of Bcl-2 action. EMBO J 20:2690–2701. doi:10.1093/emboj/20.11.2690

Piquereau J, Caffin F, Novotova M, et al. (2013) Mitochondrial dynamics in the adult cardiomyocytes: which roles for a highly specialized cell? Front Physiol 4:102. doi:10.3389/fphys.2013.00102

Plotnikov EY, Vasileva AK, Arkhangelskaya AA, et al. (2008) Interrelations of mitochondrial fragmentation and cell death under ischemia/reoxygenation and UV-irradiation: protective effects of SkQ1, lithium ions and insulin. FEBS Lett 582:3117–3124. doi:10.1016/j.febslet.2008.08.002

Remijsen Q, Goossens V, Grootjans S, et al. (2014) Depletion of RIPK3 or MLKL blocks TNF-driven necroptosis and switches towards a delayed RIPK1 kinase-dependent apoptosis. Cell Death Dis 5:e1004. doi:10.1038/cddis.2013.531

Renault TT, Floros KV, Elkholi R, et al. (2014) Mitochondrial shape governs BAX-induced membrane permeabilization and apoptosis. Mol Cell 57:69–82. doi:10.1016/j.molcel.2014.10.028

Ryan JJ, Marsboom G, Fang Y-H, et al. (2013) PGC1α-mediated mitofusin-2 deficiency in female rats and humans with pulmonary arterial hypertension. Am J Respir Crit Care Med 187:865–878. doi:10.1164/rccm.201209-1687OC

Sabbah HN, Sharov V, Riddle JM, et al. (1992) Mitochondrial abnormalities in myocardium of dogs with chronic heart failure. J Mol Cell Cardiol 24:1333–1347

Santos RX, Correia SC, Alves MG, et al. (2014) Insulin therapy modulates mitochondrial dynamics and biogenesis, autophagy and tau protein phosphorylation in the brain of type 1 diabetic rats. Biochim Biophys Acta 1842:1154–1166. doi:10.1016/j.bbadis.2014.04.011

Schaper J, Froede R, Hein S, et al. (1991) Impairment of the myocardial ultrastructure and changes of the cytoskeleton in dilated cardiomyopathy. Circulation 83:504–514

Scorrano L, Ashiya M, Buttle K, et al. (2002) A distinct pathway remodels mitochondrial cristae and mobilizes cytochrome c during apoptosis. Dev Cell 2:55–67

Sharp WW, Fang YH, Han M, et al. (2014) Dynamin-related protein 1 (Drp1)-mediated diastolic dysfunction in myocardial ischemia-reperfusion injury: therapeutic benefits of Drp1 inhibition to reduce mitochondrial fission. FASEB J. doi:10.1096/fj.12-226225

Shen T, Zheng M, Cao C, et al. (2007) Mitofusin-2 is a major determinant of oxidative stress-mediated heart muscle cell apoptosis. J Biol Chem 282:23354–23361. doi:10.1074/jbc.M702657200

Shenouda SM, Widlansky ME, Chen K, et al. (2011) Altered mitochondrial dynamics contributes to endothelial dysfunction in diabetes mellitus. Circulation 124:444–453. doi:10.1161/CIRCULATIONAHA.110.014506

Smirnova E, Shurland DL, Ryazantsev SN, van der Bliek AM (1998) A human dynamin-related protein controls the distribution of mitochondria. J Cell Biol 143:351–358

Smirnova E, Griparic L, Shurland DL, van der Bliek AM (2001) Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells. Mol Biol Cell 12:2245–2256

Soleimanpour SA, Gupta A, Bakay M, et al. (2014) The diabetes susceptibility gene Clec16a regulates mitophagy. Cell 157:1577–1590. doi:10.1016/j.cell.2014.05.016

Song Z, Ghochani M, McCaffery JM, et al. (2009) Mitofusins and OPA1 mediate sequential steps in mitochondrial membrane fusion. Mol Biol Cell 20:3525–3532. doi:10.1091/mbc.E09-03-0252

Song M, Chen Y, Gong G, et al. (2014) Super-suppression of mitochondrial reactive oxygen species signaling impairs compensatory autophagy in primary mitophagic cardiomyopathy. Circ Res 115:348–353. doi:10.1161/CIRCRESAHA.115.304384

Stojanovski D, Koutsopoulos OS, Okamoto K, Ryan MT (2004) Levels of human Fis1 at the mitochondrial outer membrane regulate mitochondrial morphology. J Cell Sci 117:1201–1210. doi:10.1242/jcs.01058

Sugioka R, Shimizu S, Tsujimoto Y (2004) Fzo1, a protein involved in mitochondrial fusion, inhibits apoptosis. J Biol Chem 279:52726–52734. doi:10.1074/jbc.M408910200

Sutendra G, Dromparis P, Wright P, et al. (2011) The role of Nogo and the mitochondria-endoplasmic reticulum unit in pulmonary hypertension. Sci Transl Med 3:88ra55. doi:10.1126/scitranslmed.3002194

Tang Y, Mi C, Liu J, et al. (2014) Compromised mitochondrial remodeling in compensatory hypertrophied myocardium of spontaneously hypertensive rat. Cardiovasc Pathol 23:101–106. doi:10.1016/j.carpath.2013.11.002

Thapa D, Nichols CE, Lewis SE, et al. (2014) Transgenic overexpression of mitofilin attenuates diabetes mellitus-associated cardiac and mitochondria dysfunction. J Mol Cell Cardiol 79C:212–223. doi:10.1016/j.yjmcc.2014.11.008

Tubbs E, Theurey P, Vial G, et al. (2014) Mitochondria-associated endoplasmic reticulum membrane (MAM) integrity is required for insulin signaling and is implicated in hepatic insulin resistance. Diabetes 63:3279–3294. doi:10.2337/db13-1751

Turkieh A, Caubère C, Barutaut M, et al. (2014) Apolipoprotein O is mitochondrial and promotes lipotoxicity in heart. J Clin Invest 124:2277–2286. doi:10.1172/JCI74668

Twig G, Hyde B, Shirihai OS (2008) Mitochondrial fusion, fission and autophagy as a quality control axis: the bioenergetic view. Biochim Biophys Acta 1777:1092–1097. doi:10.1016/j.bbabio.2008.05.001

Vanlangenakker N, Vanden Berghe T, Krysko DV, et al. (2008) Molecular mechanisms and pathophysiology of necrotic cell death. Curr Mol Med 8:207–220

Ventura-Clapier R, Garnier A, Veksler V, Joubert F (2011) Bioenergetics of the failing heart. Biochim Biophys Acta 1813:1360–1372. doi:10.1016/j.bbamcr.2010.09.006

Wang J-X, Jiao J-Q, Li Q, et al. (2011) miR-499 regulates mitochondrial dynamics by targeting calcineurin and dynamin-related protein-1. Nat Med 17:71–78. doi:10.1038/nm.2282

Wang Z, Jiang H, Chen S, et al. (2012) The mitochondrial phosphatase PGAM5 functions at the convergence point of multiple necrotic death pathways. Cell 148:228–243. doi:10.1016/j.cell.2011.11.030

Wang L, Yu T, Lee H, et al. (2015) Decreasing mitochondrial fission diminishes vascular smooth muscle cell migration and ameliorates intimal hyperplasia. Cardiovasc Res. doi:10.1093/cvr/cvv005

Wasiak S, Zunino R, McBride HM (2007) Bax/Bak promote sumoylation of DRP1 and its stable association with mitochondria during apoptotic cell death. J Cell Biol 177:439–450. doi:10.1083/jcb.200610042

Watanabe T, Saotome M, Nobuhara M, et al. (2014) Roles of mitochondrial fragmentation and reactive oxygen species in mitochondrial dysfunction and myocardial insulin resistance. Exp Cell Res 323:314–325. doi:10.1016/j.yexcr.2014.02.027

Weaver D, Eisner V, Liu X, et al. (2014) Distribution and apoptotic function of outer membrane proteins depend on mitochondrial fusion. Mol Cell. doi:10.1016/j.molcel.2014.03.048

Williamson CL, Dabkowski ER, Baseler WA, et al. (2010) Enhanced apoptotic propensity in diabetic cardiac mitochondria: influence of subcellular spatial location. Am J Physiol Heart Circ Physiol 298:H633–H642. doi:10.1152/ajpheart.00668.2009

Yamaguchi R, Perkins G (2009) Dynamics of mitochondrial structure during apoptosis and the enigma of Opa1. Biochim Biophys Acta 1787:963–972. doi:10.1016/j.bbabio.2009.02.005

Yellon DM, Hausenloy DJ (2007) Myocardial reperfusion injury. N Engl J Med 357:1121–1135. doi:10.1056/NEJMra071667

Yoon Y, Krueger EW, Oswald BJ, McNiven MA (2003) The mitochondrial protein hFis1 regulates mitochondrial fission in mammalian cells through an interaction with the dynamin-like protein DLP1. Mol Cell Biol 23:5409–5420

Youle RJ, van der Bliek AM (2012) Mitochondrial fission, fusion, and stress. Science 337:1062–1065. doi:10.1126/science.1219855

Yu T, Robotham JL, Yoon Y (2006) Increased production of reactive oxygen species in hyperglycemic conditions requires dynamic change of mitochondrial morphology. Proc Natl Acad Sci U S A 103:2653–2658. doi:10.1073/pnas.0511154103

Yu T, Sheu S-S, Robotham JL, Yoon Y (2008) Mitochondrial fission mediates high glucose-induced cell death through elevated production of reactive oxygen species. Cardiovasc Res 79:341–351. doi:10.1093/cvr/cvn104

Yu H, Guo Y, Mi L, et al. (2011) Mitofusin 2 inhibits angiotensin II-induced myocardial hypertrophy. J Cardiovasc Pharmacol Ther 16:205–211. doi:10.1177/1074248410385683

Zepeda R, Kuzmicic J, Parra V, et al. (2014) Drp1 loss-of-function reduces cardiomyocyte oxygen dependence protecting the heart from ischemia-reperfusion injury. J Cardiovasc Pharmacol 63:477–487. doi:10.1097/FJC.0000000000000071

Zhang Y, Chan DC (2007) New insights into mitochondrial fusion. FEBS Lett 581:2168–2173. doi:10.1016/j.febslet.2007.01.095

Zhang D, Ma C, Li S, et al. (2012) Effect of Mitofusin 2 on smooth muscle cells proliferation in hypoxic pulmonary hypertension. Microvasc Res 84:286–296. doi:10.1016/j.mvr.2012.06.010

Zhang L, Jiang F, Chen Y, et al. (2013a) Necrostatin-1 attenuates ischemia injury induced cell death in rat tubular cell line NRK-52E through decreased Drp1 expression. Int J Mol Sci 14:24742–24754. doi:10.3390/ijms141224742

Zhang N, Wang S, Li Y, et al. (2013b) A selective inhibitor of Drp1, mdivi-1, acts against cerebral ischemia/reperfusion injury via an anti-apoptotic pathway in rats. Neurosci Lett 535:104–109. doi:10.1016/j.neulet.2012.12.049

Mitochondria in Alzheimer's Disease and Diabetes-Associated Neurodegeneration: License to Heal!

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Contents

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H. Singh, S.-S. Sheu (eds.), *Pharmacology of Mitochondria*,
Handbook of Experimental Pharmacology 240, DOI 10.1007/164_2017_3

Abstract

Alzheimer's disease (AD) is a difficult puzzle to solve, in part because the etiology of this devastating neurodegenerative disorder remains murky. However, diabetes has been pinpointed as a major risk factor for the sporadic forms of AD. Several overlapping neurodegenerative mechanisms have been identified between AD and diabetes, including mitochondrial malfunction. This is not surprising taking into account that neurons are cells with a complex morphology, long lifespan, and high energetic requirements which make them particularly reliant on a properly organized and dynamic mitochondrial network to sustain neuronal function and integrity. In this sense, this chapter provides an overview on the role of mitochondrial bioenergetics and dynamics to the neurodegenerative events that occur in AD and diabetes, and how these organelles may represent a mechanistic link between these two pathologies. From a therapeutic perspective, it will be discussed how mitochondria can be targeted in order to efficaciously counteract neurodegeneration associated with AD and diabetes.

Keywords

Alzheimer's disease • Diabetes • Mitochondrial bioenergetics • Mitochondrial dynamics • Neurodegeneration • Oxidative stress • Therapeutics

1 Introduction

Perhaps one of the most important revolutionary occurrences was the development of mitochondria, the evolutionary remains of aerobic bacteria that infected the anaerobic protoeukaryotic cell about a billion years ago, and which nowadays are designated "powerhouse of the cell" and "ATP reservoir" (Lane 2006; Breuer et al. 2012; Hagberg et al. 2014). For many years, the classical appraisal of mitochondrial function was based on energy-producing capacity. Such traditional vision of mitochondria as powerhouses quietly lingering around in the cytosol of cells is now becoming replaced by the perspective of a dynamic mitochondrial network that not only physically connects distant neuronal compartments but is also intrinsically involved in major neuronal life and death decisions (Otera and Mihara 2011; Karbowski and Neutzner 2012; Santos et al. 2012).

Since Rolf Luft and collaborators described for the first time a rare hypermetabolic disorder due to mitochondrial dysfunction (Luft et al. 1962), the medicine has advanced in understanding what role do mitochondria play in health and disease. At present, Alzheimer's disease (AD) and diabetes mellitus, often referred to simply as diabetes, are considered among the greatest threats to public health. Importantly, an interrelation between AD and diabetes has been extensively described during the last decades. The incidence of diabetes is accompanied by neurodegeneration and increases the risk for developing AD and vice versa, which suggests that these disorders share common pathological mechanisms (Correia et al. 2012b; Moreira 2012). In this sense, this chapter is mainly devoted to highlight the role of mitochondrial dysfunction as a point of intersection between AD and diabetes.

Therefore, the first part of this chapter succinctly describes the physiological role of mitochondrial bioenergetics and dynamics to neuronal populations. Thereafter, the importance of mitochondrial (dys)function in AD and diabetes-related neurodegeneration and the most promising mitochondrial-targeted therapeutic strategies aimed to confer neuronal protection in the abovementioned pathologies will be critically discussed.

2 What is the Physiological Relevance of Mitochondria in Neurons?

Due to a complex architecture, energetic demands that fluctuate in time and space, and long lifespan, neuronal fate is dictated by the befitting interplay between mitochondrial bioenergetics and dynamics (Kann and Kovacs 2007; Sheng and Cai 2012; Sheng 2014). Therefore, during neuronal lifetime, mitochondrial network is continually remodeled and rebuilt in order to maintain a healthy mitochondrial pool. The dynamic properties that modulate mitochondrial network comprise mitochondrial biogenesis, fusion and fission, transport, and degradation by autophagy (mitophagy) (Fig. 1). Within this scenario, this section is devoted to provide a brief overview of mitochondrial bioenergetics, dynamics, and quality control with the purpose to form a comprehensive picture on the mitochondrial contribution to normal synaptic and neuronal functioning.

2.1 Mitochondrial Bioenergetics

Owing to their limited glycolytic capacity, neurons are heavily reliant on mitochondrial oxidative phosphorylation (OXPHOS) for ATP generation. In this sense, the primary function of mitochondria in neurons is to supply ATP for energy-consuming processes such as axonal transport of macromolecules and organelles, ion pumps activity and synaptic transmission and plasticity (Sheng and Cai 2012; Sheng 2014). Of note, electron transport chain (ETC) is also a source of reactive oxygen species (ROS) (Finkel and Holbrook 2000). ROS are known to function as a double-edged sword. At low levels, ROS act as important intracellular signaling molecules. On the flipside, exacerbated ROS levels may directly "attack" mitochondrial components triggering neuronal oxidative damage.

However, mitochondria are more than a power station to neurons. These organelles are also major short-term reservoirs of Ca^{2+}, which in turn critically partakes in neuronal communication by controlling synaptic vesicles mobilization and recycling and neurotransmitter release (Vos et al. 2010). Mechanistically, mitochondrial Ca^{2+} uptake depends on a mitochondrial calcium uniporter (MCU) that resides in the inner mitochondrial membrane, while mitochondrial Ca^{2+} release occurs through the mitochondrial permeability transition pore (MPTP) (Vos et al. 2010). Importantly, under pathological conditions, mitochondrial Ca^{2+} overload compromises the integrity of the mitochondrial inner membrane, thereby triggering MPTP opening, a process when sustained results in cessation of ATP production,

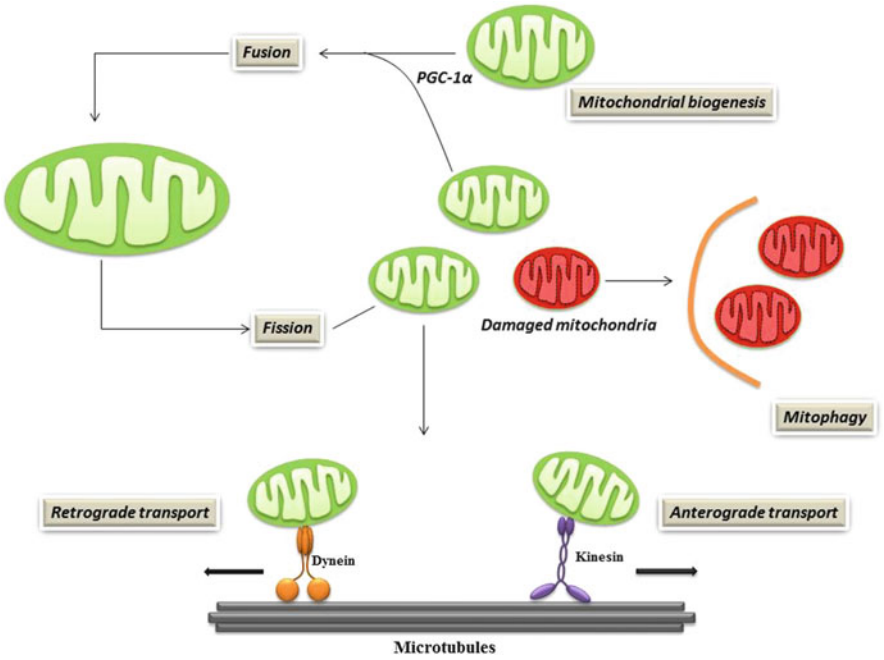

Fig. 1 Mitochondrial dynamics in neurons. Mitochondrial network is continually reshaped and renewed by the action of four major dynamic processes: mitochondrial fusion–fission, biogenesis, transport, and selective degradation by autophagy (mitophagy). Briefly, functional mitochondria are generated in the soma through mitochondrial biogenesis and transported in the anterograde direction to structures with high metabolic requirements such as synapses, where these organelles sustain synaptic function by providing ATP and buffering Ca^{2+}. On the other hand, damaged mitochondria return back to the soma by retrograde transport and degraded by the autophagy-lysosome machinery. In-between, mitochondria experience successive cycles of fusion and fission

exacerbated ROS generation, permeability of the outer mitochondrial membrane, cytochrome *c* leakage, and subsequent neuronal cell death (Pivovarova and Andrews 2010).

Overall, mitochondrial bioenergetics comprises several distinct but interconnected pathways necessary for neuronal survival, activity, connectivity, and plasticity.

2.2 Mitochondrial Fusion and Fission

Traditionally depicted as solitary and static entities, mitochondria are in reality dynamic organelles that continuously fuse and divide through the action of two opposing forces, mitochondrial fusion and fission (Chen and Chan 2009). Unopposed mitochondrial fusion favors the generation of an elongated and interconnected mitochondrial network, whereas unopposed mitochondrial fission induces a fragmented and disconnected mitochondrial phenotype (Chen and Chan 2009).

Mitochondrial fission is governed by a large cytosolic GTPase dynamin-like protein 1 (DRP-1) that is recruited from the cytosol to the outer mitochondrial membrane and homo-oligomerizes, forming large ring complexes that constrict and ultimately cause mitochondrial scission. Among the recruiters of DRP-1 to the outer mitochondrial membrane are mitochondrial fission protein 1 (Fis1), mitochondrial fission factor (Mff), and mitochondrial dynamics proteins 49 and 51 kDa (MiD49, MiD51). The core components of the mitochondrial fusion machinery are mitofusin 1 and 2 (Mfn1 and Mfn2) and optic atrophy protein 1 (OPA1). Briefly, mitofusins are responsible for the linkage of the outer mitochondrial membranes of two separated mitochondria, and OPA1, which resides in the inner mitochondrial membrane, facilitates the lengthening and tethering of adjacent mitochondria to form a connected tubular network being involved in maintaining/remodeling cristae structure (Burte et al. 2015; Ni et al. 2015).

But, what is the physiological relevance of mitochondrial fusion and fission in neurons? First and foremost, successive cycles of fusion and fission allow the generation of a heterogeneous mitochondrial population. The fine-tuning of these opposing forces allows proper neuronal functioning. While fusion events are crucial for the exchange of contents between mitochondria and enable damaged mitochondria to acquire components from healthy mitochondria, fission is essential for mitochondrial trafficking along axons and a prerequisite for mitophagy (Westermann 2010; Sheng and Cai 2012).

2.3 Mitochondrial Biogenesis and Mitophagy

Mitochondria are continuously renewed by the physiological equilibrium between generation and degradation. Mitochondria cannot be synthesized de novo; so, new mitochondria arise from the division of pre-existing organelles in the soma, a process denominated by mitochondrial biogenesis. Mitochondrial biogenesis is coordinated by a complex network of nuclear and mitochondrial transcription factors, where the peroxisome proliferator-activated receptor gamma (PPARγ) co-activator (PGC-1α) plays master regulatory roles. PGC-1α co-activates nuclear respiratory factors (NRFs) and mitochondrial transcription factor A (TFAM), a nuclear-encoded transcription factor crucial for replication, transcription, and maintenance of mitochondrial DNA (mtDNA) (Scarpulla 2002).

Mitophagy is a type of cargo-specific autophagy that mediates the selective removal of damaged mitochondria. Mitophagy represents an important node of mitochondrial quality control in post-mitotic cells as neurons due to their limited regenerative capacity. Briefly, the mitophagy machinery engulfs and digests small fusion-deficient mitochondria exhibiting sustained depolarization (Ni et al. 2015). The specific labeling of damaged mitochondria and their subsequent degradation is mediated by a PTEN-induced putative kinase protein 1 (PINK1)/parkin-dependent pathway (Wild and Dikic 2010). Upon mitochondria depolarization, PINK1 acts as a bioenergetic sensor that recruits the E3 ubiquitin ligase parkin from the cytosol to

the damaged mitochondria, with the subsequent mobilization of p62 to the mitochondria via ubiquitination and co-recruitment of LC3 (Wild and Dikic 2010).

2.4 Mitochondrial Transport

Neurons are highly polarized cells composed by subcellular compartments with distinct metabolic requirements and a non-uniform mitochondrial distribution. Therefore, neurons heavily depend on the intracellular transport machinery to distribute "new" and functional mitochondria from the soma to distant and metabolically demanding sites such as synapses, where these organelles remain stationary and meticulously regulate local ATP levels and Ca^{2+} homeostasis. Furthermore, mitochondrial transport also integrates a set of mitochondrial quality control mechanisms, in which aged or dysfunctional mitochondria are actively retrotransported to the soma in order to fuse with locally resident lysosomes and degraded by autophagy (mitophagy) (Sheng and Cai 2012; Sheng 2014; Lin and Sheng 2015).

The overwhelming majority of mitochondrial trafficking uses microtubules as rails and is mediated by two classes of motor proteins, kinesin and dynein, requiring ATP hydrolysis. The anterograde transport of mitochondria towards the synaptic terminals is mediated by the kinesin-1 family (KIF-5), whereas the retrograde transport of mitochondria back to soma is driven by dynein. Of note, Miro and Milton have been pinpointed as facilitators of the anterograde transport of mitochondria by tethering mitochondria to kinesin motors. Concerning mitochondrial docking, recent breakthroughs cast a spotlight on syntaphilin, which acts as a "static anchor" and arrests mitochondria at structures with high energetic demands (Sheng and Cai 2012; Sheng 2014; Lin and Sheng 2015).

3 Mitochondrial (Dys)Function in Alzheimer's Disease

AD is the most common form of dementia, affecting more than 30 million people worldwide. AD is usually categorized into two types: familial early-onset AD (fAD) and sporadic AD (sAD). In a small proportion (fAD), the disease has a genetic origin and is associated with mutations in the genes amyloid-β precursor protein (AβPP), presenilin-1, and presenilin-2. The vast majority of AD cases are sporadic and have aging and age-related diseases (e.g., diabetes) as main risk factors. However, familial and sporadic forms of AD share common clinical and neuropathological features. Clinically, this devastating neurodegenerative disease typically begins with a subtle decline in memory that progresses to global deterioration in cognitive functioning (Querfurth and LaFerla 2010; Correia et al. 2012a). From a neuropathological point of view, AD is mainly characterized by two distinctive lesions: the extracellular deposition of amyloid-β (Aβ) peptide in senile plaques and the intracellular accumulation of neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein (Querfurth and LaFerla 2010; Correia

et al. 2012a). Of note, AD-related changes in brain structure and function precede the clinical manifestations of the disease by 20–30 years (Braak and Braak 1995). Thus, it is imperative to identify the initial pathological events involved in AD in order to prevent AD symptomatology and neuropathology in a timely manner.

Considerable debate and controversy surround the "beginning" of AD pathology. Among the several hypotheses that have emerged during the last decades, the "mitochondrial cascade hypothesis" proposed by Swerdlow and Khan has gained some notoriety (Swerdlow and Khan 2004; Swerdlow et al. 2014). According to this hypothesis: (1) inheritance determines mitochondrial baseline function and robustness; (2) mitochondrial robustness determines how mitochondria change with age; and (3) when mitochondrial alterations reach a threshold, AD histopathology and symptoms ensue. In this sense, this section will highlight the recent breakthroughs regarding the contribution of mitochondrial abnormalities to AD pathology.

3.1 Mitochondrial Metabolism and Oxidative Stress in Alzheimer's Disease

Lessons from clinical and experimental studies revealed that altered mitochondrial metabolism and oxidative damage occurs prior to the occurrence of senile plaques and NFTs in both forms of AD (Nunomura et al. 2001; Resende et al. 2008; Yao et al. 2009; Chou et al. 2011). The first sign that metabolic defects are involved in the disease process derived from positron-emission tomography (PET) imaging with the tracer 2-[^{18}F] fluoro-2-deoxy-D-glucose in which reductions in cerebral glucose transport and utilization were detected in brain areas affected by AD pathology (Friedland et al. 1989; Jagust et al. 1991). Consistently, the AD brain is characterized by a reduction in the expression of glucose transporter-1 and -3, especially in the cerebral cortex. Importantly, this reduction has been associated with the aggravation of AD-related neuropathological phenotype by facilitating tau hyperphosphorylation and Aβ generation and compromising Aβ clearance (Shah et al. 2012). In terms of glucose metabolism, evidence from postmortem AD brain tissue and fibroblasts showed an impaired activity of pyruvate, isocitrate, and α-ketoglutarate dehydrogenases, three key enzymes involved in Krebs cycle (Correia et al. 2012a). Notably, these changes in the activity of Krebs cycle enzymes (especially pyruvate dehydrogenase) are correlated with the clinical state of AD, suggesting a progressive deterioration of mitochondrial functioning with the severity of the disease (Bubber et al. 2005). However, the most consistent mitochondrial abnormality documented in AD is the reduction in cytochrome c oxidase (COX) activity, which in turn compromise ETC activity and ATP synthesis (Mutisya et al. 1994). An exhaustive genome-wide transcriptome analysis also demonstrated that cerebral metabolic decline in AD is associated with reduced neuronal expression of nuclear genes encoding subunits of the mitochondrial electron transport complexes, including COX subunits (Liang et al. 2008). Importantly, the levels of mitochondrial encoded COX were shown to be correlated with

the amount of hyperphosphorylated tau protein accumulated in certain areas of the hippocampus in AD pathology.

At this point a major question arises: how mitochondrial metabolic defects integrate the AD scenario for the familial forms of the disease? Aβ, the major driving force underlying familial AD, was shown to directly perturb mitochondrial function. For instance, Aβ was found to be imported into mitochondria via the translocase of the outer membrane (TOM) import machinery and then transported through the inner mitochondrial membrane by the translocase of the inner membrane (TIM) (Hansson Petersen et al. 2008). In line with this, a progressive accumulation of Aβ monomers and oligomers within mitochondria has been reported in postmortem brain tissue from AD individuals and transgenic mouse models of the disease (Caspersen et al. 2005, Crouch et al. 2005, Devi et al. 2006 and Manczak et al. 2006). Once inside of mitochondria, Aβ has the ability to bind heme groups, which constitute critical redox centers of COX, and interact with the mitochondrial protein Aβ-binding alcohol dehydrogenase (ABAD), culminating in hampered COX activity, ROS overproduction, and ATP depletion (Atamna and Frey 2004; Atamna 2006). Additionally, Aβ can also block TOM40 and TIM23 from importing COX subunits into the mitochondria, further disturbing COX activity (Devi et al. 2006). To further understand the mechanisms underpinning mitochondrial bioenergetic defects in fAD, Rhein and collaborators dissected the close interrelationship between these organelles and Aβ and tau pathologies using the triple transgenic mouse model of AD (3xTg-AD) (Rhein et al. 2009). These authors found that disturbances in mitochondrial metabolism are due to a convergence of Aβ and tau on mitochondria, accelerating defects in respiratory capacity, and a main impairment in COX activity. Moreover, an age-related oxidative stress was found to exacerbate the disruption of mitochondrial metabolism in a vicious circle, finally culminating in neuronal loss. Overall, these findings consolidate the idea that a synergistic effect of Aβ and tau augments the pathological deterioration of mitochondria in familial AD.

Oxidative stress is another pathogenic step underlying defective mitochondrial metabolism in AD. As aforementioned, mitochondria can be a “Pandora box” since these organelles are one of the major hubs of ROS and the prime targets of oxidative damage. In this sense, oxidative damage has been shown to be coupled to a progressive decline of the mitochondrial function during the pathological course of AD (Moreira et al. 2010). Mitochondrial components are known to be highly susceptible to oxidative modification and are altered by exposure to a range of pro-oxidants (Tretter and Adam-Vizi 2000). In the AD brain, oxidative modification of mitochondrial proteins, such as α-ketoglutarate dehydrogenase and pyruvate dehydrogenase complexes and COX, has been demonstrated by elevated levels of protein carbonyl and nitration of tyrosine residues and indicate impaired metabolic activity (Sultana and Butterfield 2013). Importantly, impaired COX activity also exacerbated mitochondrial-derived ROS production, supporting the idea that defective mitochondrial bioenergetics potentiates oxidative stress and vice versa (Mutisya et al. 1994).

3.2 Mitochondrial Dynamics in Alzheimer's Disease

Besides hampered mitochondrial bioenergetics and oxidative stress, the AD brain also exhibits ultrastructural alterations in mitochondrial morphology such as reduced size and broken internal membrane cristae (Hirai et al. 2001; Baloyannis 2006). In face of this evidence a major question invades our minds: why mitochondrial morphology is altered in this devastating neurodegenerative disease? One plausible explanation is that mitochondrial fusion–fission balance is shifted towards fission. As a matter of fact, reduced levels of mitochondrial fusion proteins OPA1 and mitofusins were documented in hippocampal tissue from AD (Wang et al. 2009). Consistently, disruption of mitochondrial network was also reported in AD cybrids as denoted by the presence of fragmented, misshaped, and bleb-like mitochondria (Gan et al. 2014). Mechanistically, mitochondrial fragmented network in AD cybrids resulted from the combination of increased translocation of DRP-1 to mitochondria and reduced Mnf2 levels. In a step further, Cho and collaborators also found that the AD brain displays increased levels of S-nitrosylation of DRP-1(Cho et al. 2009). By modulating the GTPase activity of DRP-1, this post-translation modification favors the occurrence of mitochondrial fission phenotype and hampers mitochondrial bioenergetics, thus leading to synaptic damage and neuronal demise (Cho et al. 2009).

Other aspects of mitochondrial dynamics also integrate the scenario of mitochondrial abnormalities that occur in AD. For instance, reduced mitochondrial content was observed in susceptible hippocampal neurons from AD subjects, which points to a faulty mitochondrial turnover (Hirai et al. 2001; Baloyannis 2006). Recent scientific advances reported a drastic reduction in PGC-1α and NRFs proteins levels and mtDNA-to-nuclear DNA ratio in human AD brain tissue and experimental models of AD, indicating that the refurbishment of an "old" mitochondrial pool via mitochondrial biogenesis is compromised (Qin et al. 2009; Sheng et al. 2012). However, it is tempting to interrogate if this reduction in mitochondrial mass is also due to an overactivation of mitophagy. The first clue that mitophagy plays a role in AD derived from a study performed in postmortem AD brain tissue revealing the presence of mitochondrial DNA and COX in the neuronal cytoplasm, with mitochondrial DNA being presented in lipofuscin-containing vacuoles (Hirai et al. 2001). Consistently, elevated levels of mitochondrial components, namely COX and lipoic acid, within autophagosomes were detected in human postmortem brain tissue, suggesting an increase in the rate of mitochondrial degradation by autophagy (Moreira et al. 2007b, c). More recently, it was proposed that during the initial phase of AD pathology the induction of mitophagy may constitute a compensatory mechanism to "escape" from a neuronal energetic crisis (Shaerzadeh et al. 2014). However, a progressive decline in the cytosolic levels of Parkin was reported in AD, indicating that defects in the molecular machinery behind mitophagy contribute to abnormal accumulative dysfunctional mitochondria during the pathologic course of the disease (Ye et al. 2015).

Lastly, mitochondrial trafficking is also disrupted in AD pathology as denoted by mitochondrial overcrowding and mislocalization and impaired mitochondrial function in metabolic demanding structures like synaptic terminals (Trimmer and Borland 2005; Correia et al. 2012a). Particularly, recent scientific advances outlined that Aβ and tau can act as major "roadblocks" hampering the mitochondrial movement in the anterograde direction and compromising the delivery of healthy mitochondria to the synaptic terminals, and thus causing synaptic "starvation" (Correia et al. 2016). On the flipside, the AD brain exhibits an aberrant accumulation of defective mitochondria in the distal regions of neurons, in part due to defective retrograde mitochondrial transport that hinders damaged mitochondria from being shuttled to the soma for lysosomal degradation and leads to inadequate mitophagic clearance (Correia et al. 2015). In some ways the abovementioned mitochondrial traffic jams in AD is not surprising taking into account that mitochondrial travelling along the axons is an ATP-dependent process and mitochondrial fusion–fission and trafficking are closely interconnected (Hirokawa et al. 2010). Of note, loss of mitofusins during AD progression may underlie defective mitochondrial trafficking since these mitochondrial fusion proteins interact with the adaptor complex Miro-Milton, which is responsible for tethering mitochondria to kinesin motors (Misko et al. 2010).

4 Mitochondrial (Dys)Function in Diabetes

Diabetes is reported by the World Health Organization as one of the most common public health problems, estimated to affect a total population of 366 million worldwide by the year 2030 (Lam and LeRoith 2012). Initially known to be a disease predominant in developed countries, diabetes is now defined as the epidemic of the twenty-first century, mostly due to the economic improvement of underdeveloped countries, associated with the adoption of a sedentary lifestyle and alteration of diet habits to energy-rich diets (Tuomi 2005). Based on its etiology, diabetes can be divided into two main forms: type 1 diabetes mellitus (T1D) and type 2 diabetes mellitus (T2D). T1D is an autoimmune disease resulting from destruction of the insulin secreting pancreatic β cells leading to a complete absence of insulin in the body (Sparre et al. 2005), and accounts for 5–10% of all diabetic cases. T2D affects over 90% of diabetic patients and is characterized by relative insulin deficiency due to decreased insulin secretion by the β-cells and/or the decreased effect of insulin in the target tissues, also known as insulin resistance (Zimmet et al. 2001).

Due to the escalating rise in the number of positive diagnoses, increase in the diabetes-related deaths, and consequent enormous economic burden in the society, diabetes is attracting the world's attention. Besides the commonly associated chronic complications such as nephropathy, angiopathy, retinopathy, and peripheral neuropathy (Chen et al. 2012), it was also observed that people with diabetes performed poorly on cognitive tasks examining memory and attention (Biessels et al. 1994). The long-term effects of diabetes on the brain are manifested at

structural, neurophysiological, and neuropsychological level. Importantly, T1D and T2D have a different impact on brain functioning being the severity of cognitive deterioration strongly reliant on the age of onset of diabetes, degree of glycemic control, and duration of diabetes (Sims-Robinson et al. 2010). Multiple pathogenic factors have been implicated in the pathogenesis of cerebral dysfunction in diabetes, including defective brain energy metabolism and insulin signaling, hypoglycemic episodes, and hyperglycemia-mediated deleterious events (Muriach et al. 2014; Moheet et al. 2015). In this context, the next subsections will shed light on the differential role of mitochondria to T1D and T2D-related neurodegeneration and cognitive decline.

4.1 Mitochondrial Metabolism and Oxidative Stress in Diabetes

Disruption of mitochondrial metabolism and oxidative damage emerged as major culprits in the development of both T1D and T2D-related neurodegenerative processes. A causal relation between mitochondrial dysfunction and T1D pathology derived from evidence demonstrating that inherited defects in mtDNA causes an insulin-deficient form of diabetes mellitus that resembles T1D (Sims-Robinson et al. 2010). Additionally, the involvement of mitochondria in T2D is emphasized by the finding that mtDNA mutations in humans, as well as pancreatic β-cell-specific deletion of mitochondrial genes in animal models, reduces oxidative phosphorylation capacity and causes diabetes (Silva et al. 2000). However, what are the mitochondrial events that contribute to neurodegeneration in diabetes? Studies performed in dorsal ganglion root (DGR) neurons demonstrate that hyperglycemic conditions are associated with increased mitochondrial oxidative stress as denoted by increased ROS production (Nishikawa et al. 2000; Russell et al. 2002), lipid peroxidation (Obrosova et al. 2002), protein nitrosylation (Obrosova 2005), and diminished levels of glutathione and ascorbate (Obrosova et al. 2002). Using the streptozotocin (STZ)-rat model of T1D, Moreira and collaborators found lower content of coenzyme Q_9 (CoQ_9), indicator of a deficit in antioxidant defenses in diabetic animals and, consequently, of an increased probability for the occurrence of oxidative stress (Moreira et al. 2005), as well as a lower ATP content and ability to accumulate Ca^{2+} (Moreira et al. 2006). Similarly, Katyare and Patel observed that the rate of respiration/oxygen consumption is lower in brain mitochondria isolated from male STZ-diabetic rats when compared with the control group (Katyare and Patel 2006). In agreement, Mastrocola and collaborators demonstrated that the neuronal injury often described in the brain of STZ-treated rats is likely due to oxidative and nitrosative stress and associated mitochondrial dysfunction characterized by an impairment of the respiratory chain (Mastrocola et al. 2005).

Besides hyperglycemia, gathering data demonstrate that hypoglycemia, the unwanted side effect of intensive insulin therapy in T1D, also causes cognitive dysfunction (Strachan et al. 2000; Schultes et al. 2005) in part by affecting brain mitochondrial function. In that sense, mitochondria have occupied a central place in studies focusing on mechanisms of ROS production under hypoglycemia, widely considered an early event in the development of hypoglycemic damage of the brain

(Ballesteros et al. 2003). Earlier studies confirmed that the induction of a chronic (1 week) moderate hypoglycemia in STZ-diabetic rats resulted in lower state 3 respiration and RCR (Pelligrino et al. 1989). Later on, McGowan and collaborators demonstrated that a mitochondrial substrate limitation following hypoglycemia increases mitochondrial free radical production in brain cortex mitochondria from newborn pigs (McGowan et al. 2006). Previous data from our laboratory also revealed that insulin-induced acute episode of hypoglycemia potentiates lipid peroxidation and the imbalance of the antioxidant defenses occurring in brain cortical mitochondria isolated from STZ-induced diabetic rats (Cardoso et al. 2010). Furthermore, knowing that mitochondrial function in the brain is also intimately related to neurotransmitter synthesis and removal, an increased capacity of insulin-induced hypoglycemic cortical synaptosomes to release excitatory cytoplasmatic amino acids was also observed (Cardoso et al. 2011). Such observations prompted us to suggest that mitochondrial dysfunction, oxidative stress, and excitatory neurotransmitters release are interconnected factors that may underlie the cognitive impairment observed in T1D patients under insulin therapy. Going further, we also demonstrated that the induction of recurrent (2 weeks) hypoglycemia, as well as long-term hyperglycemia promotes an increase in oxidative stress alongside with a decrease in antioxidant defenses in brain cortical and hippocampal mitochondria (Cardoso et al. 2013b). Noteworthy, both mitochondrial populations presented a distinct behavior in response to the metabolic insults; only hippocampal mitochondria showed compromised bioenergetics and ATP production (Cardoso et al. 2013b). In the same line, Dave and collaborators verified that recurrent hypoglycemia exacerbates cerebral ischemic damage in T1D rats via increased production of mitochondrial-derived ROS that have been suggested as the possible cause of the ischemic damage exacerbation (Dave et al. 2011).

Besides disposing of an effective antioxidant defense system to cope with exacerbated ROS production, mitochondria have evolved mechanisms that regulate the rate of production of ROS through an intrinsic feedback loop involving the expression of uncoupling proteins (UCPs). UCPs-mediated dissociation of cellular ATP production from ROS generation has been stressed as being a major player in protective mechanisms involved in physiological and pathological adaptations of the brain (Bechmann et al. 2002; Cardoso et al. 2015). UCP2 gene variants have been associated with a reduced risk for diabetic neuropathy in T1D patients, suggesting that an increased expression of UCPs related to specific gene polymorphisms can limit neuronal death (Rudofsky et al. 2006). In this sense, previous data from our laboratory revealed that blood glucose fluctuations promote dissimilar susceptibilities of brain cortical mitochondria to mitochondrial proton-leak process (Cardoso et al. 2013a). Importantly, the occurrence of recurrent episodes of hypoglycemia rendered brain cortical mitochondria more susceptible to UCP-mediated uncoupling as compared with a situation of hyperglycemia (Cardoso et al. 2013a).

Concerning T2D, studies performed in isolated brain mitochondria from Goto-Kakizaki (GK) rats revealed a higher susceptibility to oxidative damage compared to control non-diabetic rats, this increased susceptibility being inversely correlated

with the antioxidant content (Santos et al. 2001). Using the same animal model, an age-related decline of the respiratory chain efficiency and an uncoupling of OXPHOS in brain mitochondria were observed (Moreira et al. 2003). Additionally, a significant downregulation of UCP2 gene expression was found in the hippocampus of a T2D animal model (Abdul-Rahman et al. 2012), which may suggest a vulnerability to neurodegenerative events.

4.2 Mitochondrial Dynamics in Diabetes

Perturbed mitochondrial biogenesis and autophagy have been believed to be the cause for reduced mitochondrial number as well as for reduced capacity for OXPHOS often described to occur in T1D and T2D (Mootha et al. 2003; Palmeira et al. 2007; Sivitz and Yorek 2010). In that sense, a previous work from our laboratory showed that cerebral cortices of T1D rats have an increased mitochondrial biogenesis, through increased expression levels of NRF2 and TFAM, and also mtDNA copy number, these alterations being considered a compensatory mechanism to maintain a healthy mitochondrial pool (Santos et al. 2014a). Furthermore, Hoffman and collaborators previously reported that autophagy is increased in the brains of young T1D patients with chronic poor metabolic control and increased oxidative stress (Hoffman et al. 2012). In the same line, the impairment of mitochondrial function described to occur in the brain of T2D mice was shown to result from an imbalance in mitochondrial biogenesis and autophagy, which in turn potentiates loss of synaptic integrity (Carvalho et al. 2015).

Mitochondrial fragmentation triggered by excessive mitochondrial fission seems to be also an important component for hyperglycemia-induced ROS overproduction (Yu et al. 2008). Even though the precise mechanisms underlying mitochondrial dynamics-mediated ROS overproduction are not clear, it has been proposed that the balance of mitochondrial fission/fusion can be sloped by oxidative stress and its abnormality could further enhance ROS generation (Yan et al. 2013). Interestingly, Santos and collaborators found that during the early stages of T2D, brain mitochondrial bioenergetics function is spared through an adaptive metabolic strategy involving a delicate balance between mitochondrial fusion–fission and biogenesis and autophagy (Santos et al. 2014b). Particularly, this study revealed that mitochondrial fission prevails, mitochondrial biogenesis is maintained, and autophagy decreases during the early stage of T2D (Santos et al. 2014b). According to authors, mitochondrial fission could be involved in the recruitment and transport of mitochondria to critical subcellular sites such as synaptic terminals, where these organelles remain stationary and preserve synaptic and neuronal function and integrity, in part by supplying ATP (Santos et al. 2014b). Consistently, in vitro findings revealed that early mitochondrial fission promoted by the up-regulation of DRP-1 represents a protective or metabolic fission in order to cope with hyperglycemia; in a later stage, excessive mitochondrial fission is associated with the activation of the pro-apoptotic proteins Bim and Bax, culminating in apoptosis (Leinninger et al. 2006). More recently, it was also described an increase in the

levels of the mitochondrial fission protein DRP-1 in cultured DRG neurons made insulin resistant by chronic exposure to hyperinsulinemic conditions (Um et al. 2011). In agreement, Edwards and collaborators found that diabetic DRG neurons have an increased mitochondrial fission and biogenesis when compared to control DRG neurons (Edwards et al. 2010). Ultimately, using a T2D animal model, Huang and collaborators pinpointed that the imbalanced mitochondrial fusion and fission processes via a glycogen synthase kinase-3β (GSK-3β)/DRP-1-dependent mechanism is an underlying cause of T2D-induced synaptic impairment (Huang et al. 2015).

Overall, despite the fact that diabetes is a heterogeneous, multifactorial, and chronic disease, disruption of mitochondrial bioenergetics and dynamics is considered one of the main culprits in the development of T1D and T2D-related neurodegeneration and cognitive decline.

5 Mitochondria as Mechanistic Bridge Between Alzheimer's Disease and Diabetes

During the last decades epidemiological, clinical, and basic studies established a close relation between AD and diabetes. Longitudinal studies have reported that both T1D and T2D are associated with the deterioration of cognitive function and increased risk of developing AD (Zhao and Townsend 2009; Crane et al. 2013). Interestingly, basic research showed the occurrence of the key histopathological hallmarks of AD in the brain cortex and hippocampus of both T1D and T2D animal models (Jolivalt et al. 2008; Kim et al. 2009). On the flipside, AD subjects are also more prone to develop T2D (Janson et al. 2004), fostering the notion that these disorders are intimately linked. Furthermore, AD and diabetes share several pathological features being proposed that AD represents a neuroendocrine disorder that resembles a brain-specific form of T2D, which is sometimes termed type 3 diabetes (T3D) (Kandimalla et al. 2016). However, whether and how these pathological conditions are casually linked is still an active area of investigation. As a common denominator in AD and diabetes, mitochondrial dysfunction has also emerged as a possible mechanistic bridge between these two pathologies. A pioneer study from Moreira and collaborators found that brain mitochondria isolated from GK diabetic rats are more susceptible to the neurotoxic effects of the amyloidogenic peptides $A\beta_{25-35}$ and $A\beta_{1-40}$ (Moreira et al. 2003). Consistently, an in vitro study revealed that brain endothelial cells under chronic hyperglycemia are more susceptible to $A\beta_{1-40}$ toxicity, an effect mediated by mitochondrial ROS (Carvalho et al. 2014). Subsequently, it was found that wild-type mice treated with sucrose to induce a (pre)diabetic phenotype have a similar brain mitochondrial bioenergetic profile, oxidative stress status, and Aβ burden in comparison with 3xTg-AD mice (Carvalho et al. 2012). Such findings prompted authors to suggest that the metabolic alterations associated with diabetes contribute to the development of AD-like pathologic features (Carvalho et al. 2012). Importantly, 3xTg-AD and sucrose-treated mice showed similar behavioral and cognitive anomalies characterized by increased fear and anxiety and decreased learning and memory abilities (Carvalho

et al. 2013). In the same line, a recent study from Petrov and collaborators observed that the metabolic alterations induced by high fat diet (HFD) in order to mimic T2D have direct effects on brain mitochondrial function contributing to AD pathology, with mitochondria as a key culprit leading to cognitive decline in both the HFD-treated and AD-like rodents at a relatively young age (Petrov et al. 2015). Using a rat model of sporadic AD induced by the intracerebroventricular (icv) administration of a sub-diabetogenic dose of STZ, Correia and collaborators found that the insulin-resistant brain state that characterizes the pathological course of the disease is accompanied by the occurrence of mitochondrial abnormalities (Correia et al. 2013). In that study the authors found that icv administration of STZ promotes a significant decline in both brain cortical and hippocampal bioenergetics function as reflected by impaired mitochondrial respiration and OXPHOS, increased susceptibility to Ca^{2+}-induced MPTP opening and oxidative stress (Correia et al. 2013).

Despite the progress that has been made to dissect the causal relation between AD and diabetes, future research is needed to better understand the "mito-mechanisms" underlying the connection between both pathologies.

6 Mitochondrial Directed Drugs in Alzheimer's Disease and Diabetes: The Quest for a Cure

Despite the overwhelming evidence that mitochondrial dysfunction is a contributing factor for the neurodegenerative events that occur in AD and diabetes, mitochondrial biology represents a potentially useful, but relatively unexploited area of therapeutic innovation. Till now, there are no studies exploring the effectiveness of mitochondrial-targeted strategies in both AD and diabetes. Therefore, this section is devoted to highlight the most relevant scientific breakthroughs in the field and propel future research efforts essential for the development of "double-impact" therapeutics for both disorders.

Taking into account that oxidative stress is an early event in AD pathology, the first line of mitochondrial therapeutic strategies tested in the past encompasses a series of natural antioxidants (e.g., vitamins C and E, CoQ_{10}) believed to improve mitochondrial bioenergetics efficiency and neutralize mitochondrial-derived ROS. However, natural antioxidant therapy failed to show efficacy in AD clinical trials despite the positive outcomes in preclinical models. But, why did natural antioxidant therapy fail? First and foremost, the capacity of these natural antioxidants in reaching mitochondria and neutralize free radicals overproduction is questionable. Additionally, these compounds have a poor ability to cross the blood–brain barrier (BBB), which has as a direct consequence the administration of these natural substances at high doses. Lastly, most clinical trials are conducted in the late stages of AD when the pathological course of the disease attains a point of no return (Persson et al. 2014).

In a step further, multiple mitochondria-targeted molecules emerged to circunvent the limited efficacy of natural antioxidant therapy by enhancing the delivery and accumulation of antioxidants into mitochondria (Reddy and Reddy

2011). The idea of selective targeting of a specific drug to concentrate in mitochondria results in a decrease in the dose required and may limit toxic side effects and minimize metabolism of the compound (Smith et al. 2011). However, two major limitations should be taken into account: (1) the lack of organ specificity, leading to superior accumulation in mitochondria-rich tissues; and (2) the typically used chemicals tend to accumulate in the matrix and the matrix-facing surface of the inner mitochondrial membrane over other important mitochondrial compartments (Leitao-Rocha et al. 2015).

Considering all those facts, current strategies for delivering drugs to the mitochondria can be integrated in two categories: (1) active (through specific interactions at mitochondrial sites, such as antigen–antibody and ligand–receptor interactions) and (2) passive targeting (where physicochemical properties such as electric charge, hydrophilicity, size, and mass of the carrier lead to preferential accumulation on mitochondrial compartment) (Serviddio et al. 2010; Rocha et al. 2014).

One of the most common approaches used in mitochondrial medicine is the coupling of the drug of interest to lipophilic cations such as triphenylphosphonium (TPP^+) (Murphy 2008). A perfect example that illustrates this approach is MitoQ that resulted from the coupling of TPP^+ to CoQ_{10}, a vitamin-like lipid-soluble component of the ETC, that could act as an important antioxidant in mitochondria in order to scavenge free radicals. As expected, MitoQ has a proven efficacy in reducing oxidative stress without causing adverse effects (Rodriguez-Cuenca et al. 2010; Smith et al. 2011). Recent breakthroughs revealed that MitoQ is able to reduce Aβ-related cognitive decline and neuronal demise in part by preventing ROS overproduction and loss of mitochondrial membrane potential ($\Delta\Psi m$) (McManus et al. 2011). Moreover, MitoQ has been shown to protect pancreatic β-cells against oxidative stress, leading to their survival and improvement in insulin secretion in cell models of glucotoxicity and glucolipotoxicity associated with T2D (Lim et al. 2011). Hitherto, the potential protective effects of MitoQ in tackling diabetes-related neurodegenerative events were not explored. More recently, the use of MitoTempo, a new and promising mitochondria-targeted antioxidant, resulting from the coupling of antioxidant Tempol with TPP^+, emerged as a possible therapeutic strategy against mitochondrial-related pathologies. Indeed, the use of MitoTempo was able to reverse hyperglycemia-induced increased susceptibility to Aβ peptide in brain microvascular endothelial cells (Carvalho et al. 2014). Furthermore, MitoTempo was able to improve mitochondrial function and coronary collateral growth after ischemia/reperfusion in Zucher obese fatty rats (Pung et al. 2012). Like MitoQ, it is important to mention that MitoTempo has very low toxicity, with no known side effects, making it a perfect candidate for in vivo use (Dikalov 2011). However, more studies regarding its use should be performed to evaluate its efficacy in vivo.

As an alternative to TPP^+ strategy other mitochondria therapeutic strategies have gained new insights in the field of neurodegenerative events and diabetes-associated complications, the Szeto-Schiller (SS) compounds. SS peptides, due to

their chemical structure based on alternating aromatic-cationic amino acids, seem to have a random preference for mitochondrial accumulation rather than cytosolic or other cellular structures (>1,000-fold) (Zhao et al. 2004; Doughan and Dikalov 2007; Bakeeva et al. 2008). Indeed, this increased mitochondrial uptake seems ΔΨm-independent, having the capacity to accumulate even in depolarized mitochondria (Zhao et al. 2004; Doughan and Dikalov 2007). In this context, studies using SS-31, the most effective SS peptide, brought new evidence about this preferential accumulation, showing the selectivity of this peptide to bind cardiolipin through both electrostatic and hydrophobic interactions (Zhang et al. 2002; Pfeiffer et al. 2003; Acehan et al. 2007). The efficacy of SS-31 against AD-related mitochondrial bioenergetics deficits and abnormal mitochondrial dynamics has been demonstrated in several in vivo models of the disease. It was demonstrated that SS-31 partially prevented Aβ-induced ATP depletion, reduced COX activity and increased hydrogen peroxide (H_2O_2) production and mitochondrial fragmentation (Di Carlo et al. 2012). Additionally, the treatment of primary neurons overexpressing Aβ peptide with SS-31 was also able to restore mitochondrial transport and mitochondrial fusion–fission balance (Calkins et al. 2012; Cabezas-Opazo et al. 2015) and decrease the percentage of defective mitochondria, improving neurite outgrowth and synaptic integrity (Calkins et al. 2012). Concerning T2D, similar results were obtained, with SS-31 leading to a decrease in mitochondrial ROS production, release of cytochrome c from the mitochondrion to the cytosol and caspase-3 expression, and an increase in ΔΨm stabilization in hyperglycemia-induced damage in human retinal endothelial cells (Li et al. 2011). Overall, it is tempting to affirm that SS-31 is emerging as a potential therapeutic in mitochondrial-related pathologies such as AD and diabetes due to its promising results in several in vitro and in vivo studies.

Outside the field of mitochondrial-targeted compounds few drugs were identified as exerting their functions directly on mitochondria. Although it is not clear if the mechanism underlying its beneficial effects is due to its action on mitochondria, lipoic acid (LA) therapy has shown promising results concerning mitochondrial-related diseases. Naturally occurring in cells, LA is the cofactor of two mitochondrial enzymes, pyruvate and α-ketoglutarate dehydrogenases (Di Domenico et al. 2015), and has a major role in the regulation of mitochondrial metabolism (Rochette et al. 2013). The use of LA supplementation has shown to improve cognitive decline in in vivo and in vitro models of AD (Quinn et al. 2007). It was also demonstrated that LA can lead to a decrease in mitochondrial-related oxidative stress in fibroblasts isolated from AD patients (Moreira et al. 2007a). In a clinical trial, Alzheimer's patients who took a combination of 600 mg of alpha-lipoic acid with omega-3 fatty acids for one year showed less cognitive and functional decline (Shinto et al. 2014). Regarding diabetes, LA was effective in decreasing mitochondria-dependent cardiac apoptosis (Li et al. 2009) and promoting mitochondrial biogenesis in retinal endothelial cells (Santos and Kowluru 2011). Promising results are also emerging from some clinical studies with improvement of mitochondrial function, reduction of inflammation and insulin

resistance, resulting in a better metabolic control in diabetes and management/prevention of diabetic neuropathy and cardiovascular disease (Padmalayam 2012).

Likewise, strategies designed to improve mitochondrial biogenesis is also achieving great importance in mitochondrial medicine. Besides physical exercise, good examples are Bezafibrate, resveratrol, and (–)-epicatechin whose mechanisms of action, although not 100% proven, seem to focus on the stimulation of PGC1α, leading to the increased transcription of genes involved in energy metabolism (Cabrero et al. 1999; Koene and Smeitink 2011; Enns 2014). Bezafibrate and resveratrol have similar effects with Bezafibrate directly inducing UCP1 and UCP3 expression in diabetic conditions (Cabrero et al. 1999) while resveratrol leads to sirtuin-1 activation with concomitant increase in UCPs expression, modulating cellular processes involved in fatty acid oxidation, ETC function, and urea cycle metabolism (Verdin et al. 2010). Interestingly, (–)-epicatechin seems to increase the levels of ETC proteins and TFAM, confirming the increase in mitochondrial biogenesis. Furthermore, increased mitochondrial volume and cristae abundance occurs under (–)-epicatechin supplementation (Nogueira et al. 2011).

Noteworthy, the use of mitochondrial fission inhibitors such as mitochondrial division inhibitor 1 (mdivi-1) has also being explored as a therapeutic strategy (Jheng et al. 2012; Gan et al. 2014). Indeed, the use of mdivi-1 in AD cybrid cells showed efficacy in maintaining normal mitochondrial structure and function (Gan et al. 2014). However, there are no studies unveiling the efficacy of mdivi-1 in diabetes-related neuronal deterioration.

Lastly, preliminary findings are arising from the study of neural stem cells (NSC) and genetic manipulation of mitochondrial-related genes. For instance, a recent study with NSC transplantation revealed an increase in mitochondrial content and expression of mitochondrial fusion protein OPA1 as well as improved cognitive performance in a transgenic mouse model of AD (Zhang et al. 2015). The use of stem cells transplantation in diabetes is limited to the use of human umbilical cord matrix-derived mesenchymal cells to be differentiated in insulin-producing cells in T1D (Moshrefi et al. 2015) with no studies in the field of mitochondrial-related therapies. Also, genetic manipulation of voltage dependent anion channel (VDAC1), a component of MPTP, decreases the occurrence of the key pathological hallmarks of AD and mitochondrial fission by hampering DRP-1 activity, ultimately preventing neuronal death (Manczak et al. 2013; Cabezas-Opazo et al. 2015).

7 Conclusion

A healthy mitochondrial pool is vital to sustain normal synaptic and neuronal functioning. Throughout this chapter, the importance of mitochondria to normal neuronal function and its engagement with neuronal deterioration in AD and diabetes – two giants overshadowing global health – was highlighted. Furthermore, this chapter also gathers some of the most recent mitochondrial therapeutics that are being developed as an attempt to overcome the ongoing challenge of finding a "cure" for neurodegeneration (Table 1). Nevertheless, it is worth mentioning that so

Table 1 Mitochondrial directed therapeutic approaches in Alzheimer's disease (AD) and diabetes

	Protective effects	AD	Diabetes	References
MitoQ	↓Oxidative stress ↑ΔΨm ↓Caspase activation	✓	✓	Rodriguez-Cuenca et al. (2010); McManus et al. (2011); Lim et al. (2011); Rodriguez-Cuenca et al. (2010)
MitoTempo	↑Mitochondrial function ↓ROS production	✓	✓	Carvalho et al. (2014); Pung et al. (2012)
SS-31	↑Mitochondrial function (COX activity, ATP levels and ΔΨm) ↓ROS production and apoptotic markers ↓Mitochondrial fission ↑Mitochondrial transport and clearance	✓	✓	Di Carlo et al. (2012); Min et al. (2011); Calkins et al. (2011); Cabezas-Opazo et al. (2015); Calkins et al. (2012); Li et al. (2011)
LA	↓Oxidative stress ↓Damaged mitochondria ↑Mitochondrial bioenergetics and biogenesis	✓	✓	Moreira et al. (2007); Quinn et al. (2007); Di Domenico et al. (2015); Shinto et al. (2014); Li et al. (2009); Santos and Kowluru (2011); Padmalayam (2012)
Bezafibrate	↑PGC-1α pathway ↑UCP1 and 3	✓	✓	Cabrero et al. (1999); Enns (2014); Koene and Smeitink (2011)
Resveratrol	↑ UCPs expression and sirtuin-1 activation ↑	✓	✓	Verdin et al. (2010); Enns (2014); Koene and Smeitink (2011)
(−)-Epicatechin	↑ETC proteins expression ↑TFAM ↑Mitochondrial volume and cristae abundance	-------	-------	Nogueira et al. (2011)
mdivi-1	↑Mitochondrial structure and function	✓	-------	Gan et al. (2014); Jheng et al. (2012)
NSC transplantation	↑Number of mitochondria and OPA1 levels	✓	-------	Zhang et al. (2015); Moshrefi et al. (2015)
VDAC1 genetic manipulation	↓DRP-1 activity	✓	-------	Cabezas-Opazo et al. (2015); Manczak et al. (2013)

During the last few years several therapeutic approaches directed to mitochondria were designed and their effects evaluated in several mitochondria-related pathologies. This table summarizes the results obtained from several studies with mitochondrial-targeted drugs in AD and diabetes research

far, the quest for a cure is still a utopia with more mechanistic studies being necessary to clarify where and which aspects of mitochondrial quality control axis should be manipulated in order to tackle the neurodegenerative events associated with AD and diabetes.

References

Abdul-Rahman O, Sasvari-Szekely M, Ver A, Rosta K, Szasz BK, Kereszturi E, Keszler G (2012) Altered gene expression profiles in the hippocampus and prefrontal cortex of type 2 diabetic rats. BMC Genomics 13:81. doi:10.1186/1471-2164-13-81

Acehan D, Xu Y, Stokes DL, Schlame M (2007) Comparison of lymphoblast mitochondria from normal subjects and patients with Barth syndrome using electron microscopic tomography. Lab Invest 87:40–48. doi:10.1038/labinvest.3700480

Atamna H (2006) Heme binding to amyloid-beta peptide: mechanistic role in Alzheimer's disease. J Alzheimers Dis 10:255–266

Atamna H, Frey WH 2nd (2004) A role for heme in Alzheimer's disease: heme binds amyloid beta and has altered metabolism. Proc Natl Acad Sci U S A 101:11153–11158. doi:10.1073/pnas.0404349101

Bakeeva LE, Barskov IV, Egorov MV et al (2008) Mitochondria-targeted plastoquinone derivatives as tools to interrupt execution of the aging program. 2. Treatment of some ROS- and age-related diseases (heart arrhythmia, heart infarctions, kidney ischemia, and stroke). Biochemistry (Mosc) 73:1288–1299

Ballesteros JR, Mishra OP, McGowan JE (2003) Alterations in cerebral mitochondria during acute hypoglycemia. Biol Neonate 84:159–163

Baloyannis SJ (2006) Mitochondrial alterations in Alzheimer's disease. J Alzheimers Dis 9:119–126

Bechmann I, Diano S, Warden CH, Bartfai T, Nitsch R, Horvath TL (2002) Brain mitochondrial uncoupling protein 2 (UCP2): a protective stress signal in neuronal injury. Biochem Pharmacol 64:363–367

Biessels GJ, Kappelle AC, Bravenboer B, Erkelens DW, Gispen WH (1994) Cerebral function in diabetes mellitus. Diabetologia 37:643–650

Braak H, Braak E (1995) Staging of Alzheimer's disease-related neurofibrillary changes. Neurobiol Aging 16:271–278. Discussion 278–284

Breuer ME, Willems PH, Russel FG, Koopman WJ, Smeitink JA (2012) Modeling mitochondrial dysfunctions in the brain: from mice to men. J Inherit Metab Dis 35:193–210. doi:10.1007/s10545-011-9375-8

Bubber P, Haroutunian V, Fisch G, Blass JP, Gibson GE (2005) Mitochondrial abnormalities in Alzheimer brain: mechanistic implications. Ann Neurol 57:695–703. doi:10.1002/ana.20474

Burte F, Carelli V, Chinnery PF, Yu-Wai-Man P (2015) Disturbed mitochondrial dynamics and neurodegenerative disorders. Nat Rev Neurol 11:11–24. doi:10.1038/nrneurol.2014.228

Cabezas-Opazo FA, Vergara-Pulgar K, Perez MJ, Jara C, Osorio-Fuentealba C, Quintanilla RA (2015) Mitochondrial dysfunction contributes to the pathogenesis of Alzheimer's disease. Oxid Med Cell Longev 2015:509654. doi:10.1155/2015/509654

Cabrero A, Llaverias G, Roglans N et al (1999) Uncoupling protein-3 mRNA levels are increased in white adipose tissue and skeletal muscle of bezafibrate-treated rats. Biochem Biophys Res Commun 260:547–556. doi:10.1006/bbrc.1999.0926

Calkins MJ, Manczak M, Mao P, Shirendeb U, Reddy PH (2011) Impaired mitochondrial biogenesis, defective axonal transport of mitochondria, abnormal mitochondrial dynamics and synaptic degeneration in a mouse model of Alzheimer's disease. Hum Mol Genet 20:4515–4529. doi:10.1093/hmg/ddr381

Calkins MJ, Manczak M, Reddy PH (2012) Mitochondria-targeted antioxidant SS31 prevents amyloid beta-induced mitochondrial abnormalities and synaptic degeneration in Alzheimer's disease. Pharmaceuticals (Basel) 5:1103–1119. doi:10.3390/ph5101103

Cardoso S, Santos MS, Seica R, Moreira PI (2010) Cortical and hippocampal mitochondria bioenergetics and oxidative status during hyperglycemia and/or insulin-induced hypoglycemia. Biochim Biophys Acta 1802:942–951. doi:10.1016/j.bbadis.2010.07.001

Cardoso S, Carvalho C, Santos R et al (2011) Impact of STZ-induced hyperglycemia and insulin-induced hypoglycemia in plasma amino acids and cortical synaptosomal neurotransmitters. Synapse 65:457–466. doi:10.1002/syn.20863

Cardoso S, Santos MS, Moreno A, Moreira PI (2013a) UCP2 and ANT differently modulate proton-leak in brain mitochondria of long-term hyperglycemic and recurrent hypoglycemic rats. J Bioenerg Biomembr 45:397–407. doi:10.1007/s10863-013-9503-2

Cardoso S, Santos RX, Correia SC et al (2013b) Insulin-induced recurrent hypoglycemia exacerbates diabetic brain mitochondrial dysfunction and oxidative imbalance. Neurobiol Dis 49:1–12. doi:10.1016/j.nbd.2012.08.008

Cardoso S, Correia S, Carvalho C et al (2015) Perspectives on mitochondrial uncoupling proteins-mediated neuroprotection. J Bioenerg Biomembr 47:119–131. doi:10.1007/s10863-014-9580-x

Carvalho C, Cardoso S, Correia SC et al (2012) Metabolic alterations induced by sucrose intake and Alzheimer's disease promote similar brain mitochondrial abnormalities. Diabetes 61:1234–1242. doi:10.2337/db11-1186

Carvalho C, Machado N, Mota PC et al (2013) Type 2 diabetic and Alzheimer's disease mice present similar behavioral, cognitive, and vascular anomalies. J Alzheimers Dis 35:623–635. doi:10.3233/JAD-130005

Carvalho C, Katz PS, Dutta S, Katakam PV, Moreira PI, Busija DW (2014) Increased susceptibility to amyloid-beta toxicity in rat brain microvascular endothelial cells under hyperglycemic conditions. J Alzheimers Dis 38:75–83. doi:10.3233/JAD-130464

Carvalho C, Santos MS, Oliveira CR, Moreira PI (2015) Alzheimer's disease and type 2 diabetes-related alterations in brain mitochondria, autophagy and synaptic markers. Biochim Biophys Acta 1852:1665–1675. doi:10.1016/j.bbadis.2015.05.001

Caspersen C, Wang N, Yao J et al (2005) Mitochondrial Abeta: a potential focal point for neuronal metabolic dysfunction in Alzheimer's disease. FASEB J 19:2040–2041. doi:10.1096/fj.05-3735fje

Chen H, Chan DC (2009) Mitochondrial dynamics – fusion, fission, movement, and mitophagy – in neurodegenerative diseases. Hum Mol Genet 18:R169–R176. doi:10.1093/hmg/ddp326

Chen X, Wei S, Yang F (2012) Mitochondria in the pathogenesis of diabetes: a proteomic view. Protein Cell 3:648–660. doi:10.1007/s13238-012-2043-4

Cho DH, Nakamura T, Fang J, Cieplak P, Godzik A, Gu Z, Lipton SA (2009) S-nitrosylation of Drp1 mediates beta-amyloid-related mitochondrial fission and neuronal injury. Science 324:102–105. doi:10.1126/science.1171091

Chou JL, Shenoy DV, Thomas N, Choudhary PK, Laferla FM, Goodman SR, Breen GA (2011) Early dysregulation of the mitochondrial proteome in a mouse model of Alzheimer's disease. J Proteomics 74:466–479. doi:10.1016/j.jprot.2010.12.012

Correia SC, Santos RX, Cardoso S et al (2012a) Alzheimer disease as a vascular disorder: where do mitochondria fit? Exp Gerontol 47:878–886. doi:10.1016/j.exger.2012.07.006

Correia SC, Santos RX, Carvalho C et al (2012b) Insulin signaling, glucose metabolism and mitochondria: major players in Alzheimer's disease and diabetes interrelation. Brain Res 1441:64–78. doi:10.1016/j.brainres.2011.12.063

Correia SC, Santos RX, Santos MS et al (2013) Mitochondrial abnormalities in a streptozotocin-induced rat model of sporadic Alzheimer's disease. Curr Alzheimer Res 10:406–419

Correia SC, Resende R, Moreira PI, Pereira CM (2015) Alzheimer's disease-related misfolded proteins and dysfunctional organelles on autophagy menu. DNA Cell Biol 34:261–273. doi:10.1089/dna.2014.2757

Correia SC, Perry G, Moreira PI (2016) Mitochondrial traffic jams in Alzheimer's disease – pinpointing the roadblocks. Biochim Biophys Acta 1862:1909–1917. doi:10.1016/j.bbadis.2016.07.010

Crane PK, Walker R, Larson EB (2013) Glucose levels and risk of dementia. N Engl J Med 369:1863–1864. doi:10.1056/NEJMc1311765

Crouch PJ, Blake R, Duce JA et al (2005) Copper-dependent inhibition of human cytochrome c oxidase by a dimeric conformer of amyloid-beta1-42. J Neurosci 25:672–679. doi:10.1523/JNEUROSCI.4276-04.2005

Dave KR, Tamariz J, Desai KM et al (2011) Recurrent hypoglycemia exacerbates cerebral ischemic damage in streptozotocin-induced diabetic rats. Stroke 42:1404–1411. doi:10.1161/STROKEAHA.110.594937

Devi L, Prabhu BM, Galati DF, Avadhani NG, Anandatheerthavarada HK (2006) Accumulation of amyloid precursor protein in the mitochondrial import channels of human Alzheimer's disease brain is associated with mitochondrial dysfunction. J Neurosci 26:9057–9068. doi:10.1523/JNEUROSCI.1469-06.2006

Di Carlo M, Giacomazza D, Picone P, Nuzzo D, San Biagio PL (2012) Are oxidative stress and mitochondrial dysfunction the key players in the neurodegenerative diseases? Free Radic Res 46:1327–1338. doi:10.3109/10715762.2012.714466

Di Domenico F, Barone E, Perluigi M, Butterfield DA (2015) Strategy to reduce free radical species in Alzheimer's disease: an update of selected antioxidants. Expert Rev Neurother 15:19–40. doi:10.1586/14737175.2015.955853

Dikalov S (2011) Cross talk between mitochondria and NADPH oxidases. Free Radic Biol Med 51:1289–1301 doi: S0891-5849(11)00427-8 [pii] 10.1016/j.freeradbiomed.2011.06.033

Doughan AK, Dikalov SI (2007) Mitochondrial redox cycling of mitoquinone leads to superoxide production and cellular apoptosis. Antioxid Redox Signal 9:1825–1836. doi:10.1089/ars.2007.1693

Edwards JL, Quattrini A, Lentz SI et al (2010) Diabetes regulates mitochondrial biogenesis and fission in mouse neurons. Diabetologia 53:160–169. doi:10.1007/s00125-009-1553-y

Enns GM (2014) Treatment of mitochondrial disorders: antioxidants and beyond. J Child Neurol 29:1235–1240. doi:10.1177/0883073814538509

Finkel T, Holbrook NJ (2000) Oxidants, oxidative stress and the biology of ageing. Nature 408:239–247. doi:10.1038/35041687

Friedland RP, Jagust WJ, Huesman RH et al (1989) Regional cerebral glucose transport and utilization in Alzheimer's disease. Neurology 39:1427–1434

Gan X, Huang S, Wu L et al (2014) Inhibition of ERK-DLP1 signaling and mitochondrial division alleviates mitochondrial dysfunction in Alzheimer's disease cybrid cell. Biochim Biophys Acta 1842:220–231. doi:10.1016/j.bbadis.2013.11.009

Hagberg H, Mallard C, Rousset CI, Thornton C (2014) Mitochondria: hub of injury responses in the developing brain. Lancet Neurol 13:217–232. doi:10.1016/S1474-4422(13)70261-8

Hansson Petersen CA, Alikhani N, Behbahani H et al (2008) The amyloid beta-peptide is imported into mitochondria via the TOM import machinery and localized to mitochondrial cristae. Proc Natl Acad Sci U S A 105:13145–13150. doi:10.1073/pnas.0806192105

Hirai K, Aliev G, Nunomura A et al (2001) Mitochondrial abnormalities in Alzheimer's disease. J Neurosci 21:3017–3023

Hirokawa N, Niwa S, Tanaka Y (2010) Molecular motors in neurons: transport mechanisms and roles in brain function, development, and disease. Neuron 68:610–638. doi:10.1016/j.neuron.2010.09.039

Hoffman WH, Shacka JJ, Andjelkovic AV (2012) Autophagy in the brains of young patients with poorly controlled T1DM and fatal diabetic ketoacidosis. Exp Mol Pathol 93:273–280. doi:10.1016/j.yexmp.2011.10.007

Huang S, Wang Y, Gan X et al (2015) Drp1-mediated mitochondrial abnormalities link to synaptic injury in diabetes model. Diabetes 64:1728–1742. doi:10.2337/db14-0758

Jagust WJ, Seab JP, Huesman RH et al (1991) Diminished glucose transport in Alzheimer's disease: dynamic PET studies. J Cereb Blood Flow Metab 11:323–330. doi:10.1038/jcbfm.1991.65

Janson J, Laedtke T, Parisi JE, O'Brien P, Petersen RC, Butler PC (2004) Increased risk of type 2 diabetes in Alzheimer disease. Diabetes 53:474–481

Jheng HF, Tsai PJ, Guo SM et al (2012) Mitochondrial fission contributes to mitochondrial dysfunction and insulin resistance in skeletal muscle. Mol Cell Biol 32:309–319. doi:10.1128/MCB.05603-11

Jolivalt CG, Lee CA, Beiswenger KK, Smith JL, Orlov M, Torrance MA, Masliah E (2008) Defective insulin signaling pathway and increased glycogen synthase kinase-3 activity in the brain of diabetic mice: parallels with Alzheimer's disease and correction by insulin. J Neurosci Res 86:3265–3274. doi:10.1002/jnr.21787

Kandimalla R, Thirumala V, Reddy PH (2016) Is Alzheimer's disease a type 3 diabetes? A critical appraisal. Biochim Biophys Acta. doi: 10.1016/j.bbadis.2016.08.018

Kann O, Kovacs R (2007) Mitochondria and neuronal activity. Am J Physiol Cell Physiol 292:C641–C657. doi:10.1152/ajpcell.00222.2006

Karbowski M, Neutzner A (2012) Neurodegeneration as a consequence of failed mitochondrial maintenance. Acta Neuropathol 123:157–171. doi:10.1007/s00401-011-0921-0

Katyare SS, Patel SP (2006) Insulin status differentially affects energy transduction in cerebral mitochondria from male and female rats. Brain Res Bull 69:458–464. doi:10.1016/j.brainresbull.2006.02.012

Kim B, Backus C, Oh S, Hayes JM, Feldman EL (2009) Increased tau phosphorylation and cleavage in mouse models of type 1 and type 2 diabetes. Endocrinology 150:5294–5301. doi:10.1210/en.2009-0695

Koene S, Smeitink J (2011) Metabolic manipulators: a well founded strategy to combat mitochondrial dysfunction. J Inherit Metab Dis 34:315–325. doi:10.1007/s10545-010-9162-y

Lam DW, LeRoith D (2012) The worldwide diabetes epidemic. Curr Opin Endocrinol Diabetes Obes 19:93–96. doi:10.1097/MED.0b013e328350583a

Lane N (2006) Mitochondrial disease: powerhouse of disease. Nature 440:600–602. doi:10.1038/440600a

Leinninger GM, Backus C, Sastry AM, Yi YB, Wang CW, Feldman EL (2006) Mitochondria in DRG neurons undergo hyperglycemic mediated injury through Bim, Bax and the fission protein Drp1. Neurobiol Dis 23:11–22. doi:10.1016/j.nbd.2006.01.017

Leitao-Rocha A, Guedes-Dias P, Pinho BR, Oliveira JM (2015) Trends in mitochondrial therapeutics for neurological disease. Curr Med Chem 22(20):2458–2467

Li CJ, Zhang QM, Li MZ, Zhang JY, Yu P, Yu DM (2009) Attenuation of myocardial apoptosis by alpha-lipoic acid through suppression of mitochondrial oxidative stress to reduce diabetic cardiomyopathy. Chin Med J (Engl) 122:2580–2586

Li J, Chen X, Xiao W et al (2011) Mitochondria-targeted antioxidant peptide SS31 attenuates high glucose-induced injury on human retinal endothelial cells. Biochem Biophys Res Commun 404:349–356. doi:10.1016/j.bbrc.2010.11.122

Liang WS, Reiman EM, Valla J et al (2008) Alzheimer's disease is associated with reduced expression of energy metabolism genes in posterior cingulate neurons. Proc Natl Acad Sci U S A 105:4441–4446. doi:10.1073/pnas.0709259105

Lim S, Rashid MA, Jang M et al (2011) Mitochondria-targeted antioxidants protect pancreatic beta-cells against oxidative stress and improve insulin secretion in glucotoxicity and glucolipotoxicity. Cell Physiol Biochem 28:873–886. doi:10.1159/000335802

Lin MY, Sheng ZH (2015) Regulation of mitochondrial transport in neurons. Exp Cell Res 334:35–44. doi:10.1016/j.yexcr.2015.01.004

Luft R, Ikkos D, Palmieri G, Ernster L, Afzelius B (1962) A case of severe hypermetabolism of nonthyroid origin with a defect in the maintenance of mitochondrial respiratory control: a correlated clinical, biochemical, and morphological study. J Clin Invest 41:1776–1804. doi:10.1172/JCI104637

Manczak M, Anekonda TS, Henson E, Park BS, Quinn J, Reddy PH (2006) Mitochondria are a direct site of A beta accumulation in Alzheimer's disease neurons: implications for free radical generation and oxidative damage in disease progression. Hum Mol Genet 15:1437–1449. doi:10.1093/hmg/ddl066

Manczak M, Sheiko T, Craigen WJ, Reddy PH (2013) Reduced VDAC1 protects against Alzheimer's disease, mitochondria, and synaptic deficiencies. J Alzheimers Dis 37:679–690. doi:10.3233/JAD-130761

Mastrocola R, Restivo F, Vercellinatto I, Danni O, Brignardello E, Aragno M, Boccuzzi G (2005) Oxidative and nitrosative stress in brain mitochondria of diabetic rats. J Endocrinol 187:37–44. doi:10.1677/joe.1.06269

McGowan JE, Chen L, Gao D, Trush M, Wei C (2006) Increased mitochondrial reactive oxygen species production in newborn brain during hypoglycemia. Neurosci Lett 399:111–114. doi:10.1016/j.neulet.2006.01.034

McManus MJ, Murphy MP, Franklin JL (2011) The mitochondria-targeted antioxidant MitoQ prevents loss of spatial memory retention and early neuropathology in a transgenic mouse model of Alzheimer's disease. J Neurosci 31:15703–15715. doi:10.1523/JNEUROSCI.0552-11.2011

Min K, Smuder AJ, Kwon OS, Kavazis AN, Szeto HH, Powers SK (2011) Mitochondrial-targeted antioxidants protect skeletal muscle against immobilization-induced muscle atrophy. J Appl Physiol 111:1459–1466. doi:10.1152/japplphysiol.00591.2011

Misko A, Jiang S, Wegorzewska I, Milbrandt J, Baloh RH (2010) Mitofusin 2 is necessary for transport of axonal mitochondria and interacts with the Miro/Milton complex. J Neurosci 30:4232–4240. doi:10.1523/JNEUROSCI.6248-09.2010

Moheet A, Mangia S, Seaquist ER (2015) Impact of diabetes on cognitive function and brain structure. Ann N Y Acad Sci 1353:60–71. doi:10.1111/nyas.12807

Mootha VK, Bunkenborg J, Olsen JV et al (2003) Integrated analysis of protein composition, tissue diversity, and gene regulation in mouse mitochondria. Cell 115:629–640

Moreira PI (2012) Alzheimer's disease and diabetes: an integrative view of the role of mitochondria, oxidative stress, and insulin. J Alzheimers Dis 30(Suppl 2):S199–S215. doi:10.3233/JAD-2011-111127

Moreira PI, Santos MS, Moreno AM, Seica R, Oliveira CR (2003) Increased vulnerability of brain mitochondria in diabetic (Goto-Kakizaki) rats with aging and amyloid-beta exposure. Diabetes 52:1449–1456

Moreira PI, Santos MS, Sena C, Seica R, Oliveira CR (2005) Insulin protects against amyloid beta-peptide toxicity in brain mitochondria of diabetic rats. Neurobiol Dis 18:628–637. doi:10.1016/j.nbd.2004.10.017

Moreira PI, Rolo AP, Sena C, Seica R, Oliveira CR, Santos MS (2006) Insulin attenuates diabetes-related mitochondrial alterations: a comparative study. Med Chem 2:299–308

Moreira PI, Harris PL, Zhu X, Santos MS, Oliveira CR, Smith MA, Perry G (2007a) Lipoic acid and N-acetyl cysteine decrease mitochondrial-related oxidative stress in Alzheimer disease patient fibroblasts. J Alzheimers Dis 12:195–206

Moreira PI, Siedlak SL, Wang X et al (2007b) Autophagocytosis of mitochondria is prominent in Alzheimer disease. J Neuropathol Exp Neurol 66:525–532. doi:10.1097/01.jnen.0000240476.73532.b0

Moreira PI, Siedlak SL, Wang X et al (2007c) Increased autophagic degradation of mitochondria in Alzheimer disease. Autophagy 3:614–615

Moreira PI, Zhu X, Wang X et al (2010) Mitochondria: a therapeutic target in neurodegeneration. Biochim Biophys Acta 1802:212–220. doi:10.1016/j.bbadis.2009.10.007

Moshrefi M, Yari N, Nabipour F, Bazrafshani MR, Nematollahi-Mahani SN (2015) Transplantation of differentiated umbilical cord mesenchymal cells under kidney capsule for control of type I diabetes in rat. Tissue Cell 47:395–405. doi:10.1016/j.tice.2015.04.008

Muriach M, Flores-Bellver M, Romero FJ, Barcia JM (2014) Diabetes and the brain: oxidative stress, inflammation, and autophagy. Oxid Med Cell Longev 2014:102158. doi:10.1155/2014/102158

Murphy MP (2008) Targeting lipophilic cations to mitochondria. Biochim Biophys Acta 1777:1028–1031. doi:10.1016/j.bbabio.2008.03.029

Mutisya EM, Bowling AC, Beal MF (1994) Cortical cytochrome oxidase activity is reduced in Alzheimer's disease. J Neurochem 63:2179–2184

Ni HM, Williams JA, Ding WX (2015) Mitochondrial dynamics and mitochondrial quality control. Redox Biol 4:6–13. doi:10.1016/j.redox.2014.11.006

Nishikawa T, Edelstein D, Du XL et al (2000) Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. Nature 404:787–790. doi:10.1038/35008121

Nogueira L, Ramirez-Sanchez I, Perkins GA et al (2011) (−)-Epicatechin enhances fatigue resistance and oxidative capacity in mouse muscle. J Physiol 589:4615–4631. doi:10.1113/jphysiol.2011.209924

Nunomura A, Perry G, Aliev G et al (2001) Oxidative damage is the earliest event in Alzheimer disease. J Neuropathol Exp Neurol 60:759–767

Obrosova IG (2005) Increased sorbitol pathway activity generates oxidative stress in tissue sites for diabetic complications. Antioxid Redox Signal 7:1543–1552. doi:10.1089/ars.2005.7.1543

Obrosova IG, Van Huysen C, Fathallah L, Cao XC, Greene DA, Stevens MJ (2002) An aldose reductase inhibitor reverses early diabetes-induced changes in peripheral nerve function, metabolism, and antioxidative defense. FASEB J 16:123–125. doi:10.1096/fj.01-0603fje

Otera H, Mihara K (2011) Molecular mechanisms and physiologic functions of mitochondrial dynamics. J Biochem 149:241–251. doi:10.1093/jb/mvr002

Padmalayam I (2012) Targeting mitochondrial oxidative stress through lipoic acid synthase: a novel strategy to manage diabetic cardiovascular disease. Cardiovasc Hematol Agents Med Chem 10:223–233

Palmeira CM, Rolo AP, Berthiaume J, Bjork JA, Wallace KB (2007) Hyperglycemia decreases mitochondrial function: the regulatory role of mitochondrial biogenesis. Toxicol Appl Pharmacol 225:214–220. doi:10.1016/j.taap.2007.07.015

Pelligrino DA, Becker GL, Miletich DJ, Albrecht RF (1989) Cerebral mitochondrial respiration in diabetic and chronically hypoglycemic rats. Brain Res 479:241–246

Persson T, Popescu BO, Cedazo-Minguez A (2014) Oxidative stress in Alzheimer's disease: why did antioxidant therapy fail? Oxid Med Cell Longev 2014:427318. doi:10.1155/2014/427318

Petrov D, Pedros I, Artiach G et al (2015) High-fat diet-induced deregulation of hippocampal insulin signaling and mitochondrial homeostasis deficiences contribute to Alzheimer disease pathology in rodents. Biochim Biophys Acta 1852:1687–1699. doi:10.1016/j.bbadis.2015.05.004

Pfeiffer K, Gohil V, Stuart RA, Hunte C, Brandt U, Greenberg ML, Schagger H (2003) Cardiolipin stabilizes respiratory chain supercomplexes. J Biol Chem 278:52873–52880. doi:10.1074/jbc.M308366200

Pivovarova NB, Andrews SB (2010) Calcium-dependent mitochondrial function and dysfunction in neurons. FEBS J 277:3622–3636. doi:10.1111/j.1742-4658.2010.07754.x

Pung YF, Rocic P, Murphy MP et al (2012) Resolution of mitochondrial oxidative stress rescues coronary collateral growth in Zucker obese fatty rats. Arterioscler Thromb Vasc Biol 32:325–334. doi:10.1161/ATVBAHA.111.241802

Qin W, Haroutunian V, Katsel P, Cardozo CP, Ho L, Buxbaum JD, Pasinetti GM (2009) PGC-1alpha expression decreases in the Alzheimer disease brain as a function of dementia. Arch Neurol 66:352–361. doi:10.1001/archneurol.2008.588

Querfurth HW, LaFerla FM (2010) Alzheimer's disease. N Engl J Med 362:329–344. doi:10.1056/NEJMra0909142

Quinn JF, Bussiere JR, Hammond RS, Montine TJ, Henson E, Jones RE, Stackman RW Jr (2007) Chronic dietary alpha-lipoic acid reduces deficits in hippocampal memory of aged Tg2576 mice. Neurobiol Aging 28:213–225. doi:10.1016/j.neurobiolaging.2005.12.014

Reddy PH, Reddy TP (2011) Mitochondria as a therapeutic target for aging and neurodegenerative diseases. Curr Alzheimer Res 8:393–409

Resende R, Moreira PI, Proenca T, Deshpande A, Busciglio J, Pereira C, Oliveira CR (2008) Brain oxidative stress in a triple-transgenic mouse model of Alzheimer disease. Free Radic Biol Med 44:2051–2057. doi:10.1016/j.freeradbiomed.2008.03.012

Rhein V, Song X, Wiesner A et al (2009) Amyloid-beta and tau synergistically impair the oxidative phosphorylation system in triple transgenic Alzheimer's disease mice. Proc Natl Acad Sci U S A 106:20057–20062. doi:10.1073/pnas.0905529106

Rocha M, Apostolova N, Herance JR, Rovira-Llopis S, Hernandez-Mijares A, Victor VM (2014) Perspectives and potential applications of mitochondria-targeted antioxidants in cardiometabolic diseases and type 2 diabetes. Med Res Rev 34:160–189. doi:10.1002/med.21285

Rochette L, Ghibu S, Richard C, Zeller M, Cottin Y, Vergely C (2013) Direct and indirect antioxidant properties of alpha-lipoic acid and therapeutic potential. Mol Nutr Food Res 57:114–125. doi:10.1002/mnfr.201200608

Rodriguez-Cuenca S, Cocheme HM, Logan A et al (2010) Consequences of long-term oral administration of the mitochondria-targeted antioxidant MitoQ to wild-type mice. Free Radic Biol Med 48:161–172. doi:10.1016/j.freeradbiomed.2009.10.039

Rudofsky G Jr, Schroedter A, Schlotterer A et al (2006) Functional polymorphisms of UCP2 and UCP3 are associated with a reduced prevalence of diabetic neuropathy in patients with type 1 diabetes. Diabetes Care 29:89–94

Russell JW, Golovoy D, Vincent AM, Mahendru P, Olzmann JA, Mentzer A, Feldman EL (2002) High glucose-induced oxidative stress and mitochondrial dysfunction in neurons. FASEB J 16:1738–1748. doi:10.1096/fj.01-1027com

Santos JM, Kowluru RA (2011) Role of mitochondria biogenesis in the metabolic memory associated with the continued progression of diabetic retinopathy and its regulation by lipoic acid. Invest Ophthalmol Vis Sci 52:8791–8798 doi: iovs.11-8203 [pii] 10.1167/iovs.11-8203

Santos MS, Santos DL, Palmeira CM, Seica R, Moreno AJ, Oliveira CR (2001) Brain and liver mitochondria isolated from diabetic Goto-Kakizaki rats show different susceptibility to induced oxidative stress. Diabetes Metab Res Rev 17:223–230

Santos RX, Correia SC, Zhu X et al (2012) Nuclear and mitochondrial DNA oxidation in Alzheimer's disease. Free Radic Res 46:565–576. doi:10.3109/10715762.2011.648188

Santos RX, Correia SC, Alves MG et al (2014a) Insulin therapy modulates mitochondrial dynamics and biogenesis, autophagy and tau protein phosphorylation in the brain of type 1 diabetic rats. Biochim Biophys Acta 1842:1154–1166. doi:10.1016/j.bbadis.2014.04.011

Santos RX, Correia SC, Alves MG et al (2014b) Mitochondrial quality control systems sustain brain mitochondrial bioenergetics in early stages of type 2 diabetes. Mol Cell Biochem 394:13–22. doi:10.1007/s11010-014-2076-5

Scarpulla RC (2002) Nuclear activators and coactivators in mammalian mitochondrial biogenesis. Biochim Biophys Acta 1576:1–14

Schultes B, Kern W, Oltmanns K, Peters A, Gais S, Fehm HL, Born J (2005) Differential adaptation of neurocognitive brain functions to recurrent hypoglycemia in healthy men. Psychoneuroendocrinology 30:149–161. doi:10.1016/j.psyneuen.2004.06.007

Serviddio G, Bellanti F, Sastre J, Vendemiale G, Altomare E (2010) Targeting mitochondria: a new promising approach for the treatment of liver diseases. Curr Med Chem 17:2325–2337

Shaerzadeh F, Motamedi F, Minai-Tehrani D, Khodagholi F (2014) Monitoring of neuronal loss in the hippocampus of Abeta-injected rat: autophagy, mitophagy, and mitochondrial biogenesis stand against apoptosis. Neuromolecular Med 16:175–190. doi:10.1007/s12017-013-8272-8

Shah K, Desilva S, Abbruscato T (2012) The role of glucose transporters in brain disease: diabetes and Alzheimer's disease. Int J Mol Sci 13:12629–12655. doi:10.3390/ijms131012629

Sheng ZH (2014) Mitochondrial trafficking and anchoring in neurons: new insight and implications. J Cell Biol 204:1087–1098. doi:10.1083/jcb.201312123

Sheng ZH, Cai Q (2012) Mitochondrial transport in neurons: impact on synaptic homeostasis and neurodegeneration. Nat Rev Neurosci 13:77–93. doi:10.1038/nrn3156

Sheng B, Wang X, Su B, Lee HG, Casadesus G, Perry G, Zhu X (2012) Impaired mitochondrial biogenesis contributes to mitochondrial dysfunction in Alzheimer's disease. J Neurochem 120:419–429. doi:10.1111/j.1471-4159.2011.07581.x

Shinto L, Quinn J, Montine T et al (2014) A randomized placebo-controlled pilot trial of omega-3 fatty acids and alpha lipoic acid in Alzheimer's disease. J Alzheimers Dis 38:111–120. doi:10.3233/JAD-130722

Silva JP, Kohler M, Graff C, Oldfors A, Magnuson MA, Berggren PO, Larsson NG (2000) Impaired insulin secretion and beta-cell loss in tissue-specific knockout mice with mitochondrial diabetes. Nat Genet 26:336–340. doi:10.1038/81649

Sims-Robinson C, Kim B, Rosko A, Feldman EL (2010) How does diabetes accelerate Alzheimer disease pathology? Nat Rev Neurol 6:551–559. doi:10.1038/nrneurol.2010.130

Sivitz WI, Yorek MA (2010) Mitochondrial dysfunction in diabetes: from molecular mechanisms to functional significance and therapeutic opportunities. Antioxid Redox Signal 12:537–577. doi:10.1089/ars.2009.2531

Smith RA, Hartley RC, Murphy MP (2011) Mitochondria-targeted small molecule therapeutics and probes. Antioxid Redox Signal 15:3021–3038. doi:10.1089/ars.2011.3969

Sparre T, Larsen MR, Heding PE, Karlsen AE, Jensen ON, Pociot F (2005) Unraveling the pathogenesis of type 1 diabetes with proteomics: present and future directions. Mol Cell Proteomics 4:441–457. doi:10.1074/mcp.R500002-MCP200

Strachan MW, Deary IJ, Ewing FM, Frier BM (2000) Recovery of cognitive function and mood after severe hypoglycemia in adults with insulin-treated diabetes. Diabetes Care 23:305–312

Sultana R, Butterfield DA (2013) Oxidative modification of brain proteins in Alzheimer's disease: perspective on future studies based on results of redox proteomics studies. J Alzheimers Dis 33 (Suppl 1):S243–S251. doi:10.3233/JAD-2012-129018

Swerdlow RH, Khan SM (2004) A "mitochondrial cascade hypothesis" for sporadic Alzheimer's disease. Med Hypotheses 63:8–20. doi:10.1016/j.mehy.2003.12.045

Swerdlow RH, Burns JM, Khan SM (2014) The Alzheimer's disease mitochondrial cascade hypothesis: progress and perspectives. Biochim Biophys Acta 1842:1219–1231. doi:10.1016/j.bbadis.2013.09.010

Tretter L, Adam-Vizi V (2000) Inhibition of Krebs cycle enzymes by hydrogen peroxide: a key role of [alpha]-ketoglutarate dehydrogenase in limiting NADH production under oxidative stress. J Neurosci 20:8972–8979

Trimmer PA, Borland MK (2005) Differentiated Alzheimer's disease transmitochondrial cybrid cell lines exhibit reduced organelle movement. Antioxid Redox Signal 7:1101–1109. doi:10.1089/ars.2005.7.1101

Tuomi T (2005) Type 1 and type 2 diabetes: what do they have in common? Diabetes 54(Suppl 2): S40–S45

Um HS, Kang EB, Koo JH et al (2011) Treadmill exercise represses neuronal cell death in an aged transgenic mouse model of Alzheimer's disease. Neurosci Res 69:161–173. doi:10.1016/j.neures.2010.10.004

Verdin E, Hirschey MD, Finley LW, Haigis MC (2010) Sirtuin regulation of mitochondria: energy production, apoptosis, and signaling. Trends Biochem Sci 35:669–675 . doi:10.1016/j.tibs.2010.07.003S0968-0004(10)00135-0 [pii]

Vos M, Lauwers E, Verstreken P (2010) Synaptic mitochondria in synaptic transmission and organization of vesicle pools in health and disease. Front Synaptic Neurosci 2:139. doi:10.3389/fnsyn.2010.00139

Wang X, Su B, Lee HG, Li X, Perry G, Smith MA, Zhu X (2009) Impaired balance of mitochondrial fission and fusion in Alzheimer's disease. J Neurosci 29:9090–9103. doi:10.1523/JNEUROSCI.1357-09.2009

Westermann B (2010) Mitochondrial fusion and fission in cell life and death. Nat Rev Mol Cell Biol 11:872–884. doi:10.1038/nrm3013

Wild P, Dikic I (2010) Mitochondria get a Parkin' ticket. Nat Cell Biol 12:104–106. doi:10.1038/ncb0210-104

Yan MH, Wang X, Zhu X (2013) Mitochondrial defects and oxidative stress in Alzheimer disease and Parkinson disease. Free Radic Biol Med 62:90–101. doi:10.1016/j.freeradbiomed.2012.11.014

Yao J, Irwin RW, Zhao L, Nilsen J, Hamilton RT, Brinton RD (2009) Mitochondrial bioenergetic deficit precedes Alzheimer's pathology in female mouse model of Alzheimer's disease. Proc Natl Acad Sci U S A 106:14670–14675. doi:10.1073/pnas.0903563106

Ye X, Sun X, Starovoytov V, Cai Q (2015) Parkin-mediated mitophagy in mutant hAPP neurons and Alzheimer's disease patient brains. Hum Mol Genet 24:2938–2951. doi:10.1093/hmg/ddv056

Yu T, Sheu SS, Robotham JL, Yoon Y (2008) Mitochondrial fission mediates high glucose-induced cell death through elevated production of reactive oxygen species. Cardiovasc Res 79:341–351. doi:10.1093/cvr/cvn104

Zhang M, Mileykovskaya E, Dowhan W (2002) Gluing the respiratory chain together. Cardiolipin is required for supercomplex formation in the inner mitochondrial membrane. J Biol Chem 277:43553–43556. doi:10.1074/jbc.C200551200

Zhang W, Gu GJ, Shen X, Zhang Q, Wang GM, Wang PJ (2015) Neural stem cell transplantation enhances mitochondrial biogenesis in a transgenic mouse model of Alzheimer's disease-like pathology. Neurobiol Aging 36:1282–1292 doi: S0197-4580(14)00706-4 [pii] 10.1016/j.neurobiolaging.2014.10.040

Zhao WQ, Townsend M (2009) Insulin resistance and amyloidogenesis as common molecular foundation for type 2 diabetes and Alzheimer's disease. Biochim Biophys Acta 1792:482–496. doi:10.1016/j.bbadis.2008.10.014

Zhao K, Zhao GM, Wu D, Soong Y, Birk AV, Schiller PW, Szeto HH (2004) Cell-permeable peptide antioxidants targeted to inner mitochondrial membrane inhibit mitochondrial swelling, oxidative cell death, and reperfusion injury. J Biol Chem 279:34682–34690. doi:10.1074/jbc.M402999200

Zimmet P, Alberti KG, Shaw J (2001) Global and societal implications of the diabetes epidemic. Nature 414:782–787. doi:10.1038/414782a

Leber Hereditary Optic Neuropathy: A Mitochondrial Disease Unique in Many Ways

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Handbook of Experimental Pharmacology 240, DOI 10.1007/164_2016_1

Abstract

Leber hereditary optic neuropathy (LHON) was the first mitochondrial disease to be identified as being caused by mutations in the mitochondrial DNA (mtDNA). This disease has been studied extensively in the past two decades, particularly in Brazilian, Chinese and European populations; and many primary mutations have been reported. However, the disease is enigmatic with many unique features, and there still are several important questions to be resolved. The incomplete penetrance, the male-biased disease expression and the prevalence in young adults all defy a proper explanation. It has been reported that the development of LHON is affected by the interaction between mtDNA mutations, mtDNA haplogroup background, nuclear genes, environmental factors and epigenetics. Furthermore, with the help of new animal models for LHON that have been created in recent years, we are continuing to learn more about the mechanism of this disease. The stage has now been reached at which there is a good understanding of both the genetic basis of the disease and its epidemiology, but just how the blindness that follows from the death of cells in the optic nerve can be prevented remains to be a pharmacological challenge. In this chapter, we summarize the progress that has been made in various recent studies on LHON, focusing on the molecular pathogenic mechanisms, clinical features, biochemical effects, the pharmacology and its treatment.

Keywords

Animal model • LHON • mtDNA • Nuclear genes • Therapy

Leber hereditary optic neuropathy (LHON, MIM 535000) is a common cause of acute or subacute central visual loss in young adults, with a high male-to-female ratio for the clinical expression of symptoms (Carelli et al. 2004; Man et al. 2002b; Yen et al. 2006; Yu-Wai-Man et al. 2009). LHON was first described by German ophthalmologist Theodore Leber in 1871 (Erickson 1972; Leber 1871) and was later recognized to be a mitochondrial disease (Wallace et al. 1988). In the past two decades, our knowledge about this disease was mainly derived from studies involving patients with European roots; although it is generally considered the disease occurs worldwide. We now know that the majority of LHON cases are caused by the presence of one of three primary mitochondrial DNA (mtDNA) mutations (m.11778G>A, m.14484T>C and m.3460G>A) (Howell et al. 1991; Huoponen et al. 1991; Mackey and Howell 1992; Wallace et al. 1988). From the 1990s, researchers all around the world have carried out large number of studies to characterize the genetic and clinical features of LHON (Carelli et al. 2004; Ji et al. 2008; Man et al. 2002b; Yen et al. 2006; Yu-Wai-Man et al. 2009; Yu et al. 2010b; Zhang et al. 2009). These studies have greatly increased our understanding of the pathology and risk factors of LHON. However, the two major features of the disease, incomplete penetrance (not all mutation carriers demonstrate clinical expression of disease) and gender bias, still are the most urgent

challenges for understanding the pathogenesis of this disease (Carelli et al. 2004; Man et al. 2002b; Yen et al. 2006; Yu-Wai-Man et al. 2009). Furthermore, medical treatment for this disease has still not been satisfactorily resolved (La Morgia et al. 2014).

1 LHON Clinical Features

The most typical characteristics of patients with LHON are the following: (1) a family history that shows a matrilineal inheritance pattern, although there are a considerable number of sporadic LHON cases; (2) the visual loss is painless, affecting just one eye initially and then involving the other eye and going on to cause a significant loss of vision or even total blindness, within a short time; and (3) more male carriers of the pathogenic mutations develop symptoms of LHON than maternally related female carriers of the same primary LHON mutations. About 50% of the male carriers and 10% of the female carriers will be affected, while males from 15 to 35 years old are the main affected population (Seedorff 1985). The progress of LHON can be divided into an acute phase and a chronic phase based on the clinical features. In the majority of patients, the deterioration of vision is acute and affects the two eyes simultaneously (25%) or sequentially (75%) within about 8 weeks (Harding et al. 1995). This stage of the disease is associated with retinal vascular engorgement, deformation of the optic nerve and oedema of the retina (Yen et al. 2006), while there also are some patients with no pathological changes to be observed (Riordan-Eva and Harding 1995). In addition, the disease progression may present slowly in some patients, lasting for 6 months or more. In this kind of patient, the visual field changes are not significant, thus increasing the difficulty of diagnosis (Nikoskelainen et al. 1996), particularly in the cases which lack the typical family history of LHON. In addition to typical LHON symptoms, the patient may also suffer from other problems, such as muscle atrophy, multiple sclerosis, heart disease and cerebrovascular abnormalities (Jaros et al. 2007; La Morgia et al. 2008; Sorajja et al. 2003).

Because the clinical manifestations of LHON vary widely, patients with complicated symptoms are often not diagnosed with LHON accurately. An important diagnostic marker for LHON is the matrilineal inheritance pattern, but for those patients with the clinical features of LHON but who lack a family history, screening the mtDNA for the three primary mutations (m.3460G>A, m.11778G>A and m.14484T>C) is necessary for an accurate diagnosis (Jia et al. 2006). It is worth noting that there are many suspected LHON patients who have symptoms but no family history and with no known primary mutations, and this suggests other genetic or environmental factors may influence the onset of LHON, or produce similar, and as yet unidentified, diseases (Carelli et al. 2004; Kirkman et al. 2009; Yen et al. 2006).

2 Epidemiology

The reported prevalence of LHON varied among European countries: about 1/25,000 in the north-east of England (Chinnery et al. 2000; Man et al. 2003), 1/39,000 in the Netherlands (Spruijt et al. 2006) and 1/50,000 in Finland (Puomila et al. 2007). But in China and other regions of Asia, there has not been a comprehensive epidemiological survey. In 2008, Elliott et al. screened for LHON primary mutations in the neonatal umbilical cord blood samples of 3,000 normal newborns and identified a relatively high frequency of 0.107% for m.3460G>A (3/2807), 0.108% for m.11778G>A (3/2770) and 0.105% for m.14484T>C (3/2855) (Elliott et al. 2008). This study indicated that the primary LHON mutations do exist in normal populations. We screened for the three LHON primary mutations by using a sensitive allele-specific PCR technique in the general Han Chinese population (1,555 healthy samples), but found no LHON primary mutation carriers, suggesting that the frequency of the LHON primary mutation in Han Chinese general populations is very low (Bi et al. 2010). Large-scale epidemiological surveys for LHON still need to be carried out in Asian populations and populations from other continents to get a complete profile of LHON prevalence.

3 mtDNA Primary Mutations

3.1 Three Well-Known Primary Mutations

In 1988, Wallace and his co-workers were the first to propose an mtDNA mutation as the cause of LHON (Wallace et al. 1988). Since then many mtDNA mutations have been reported as being LHON primary mutations (cf. http://www.mitomap.org) (Fig. 1). Among these mutations, three mutations (m.3460G>A in the *MT-ND1* gene, m.11778G>A in the *MT-ND4* gene and m.14484T>C in the *MT-ND6* gene) are reported to account for more than 95% of the LHON patients (Mackey et al. 1996).

These three primary mutations show considerable variation in their levels of evolutionary conservation. Mutation m.11778A>G is highly conserved, while mutations m.3460G>A and m.14484T>C are less conserved, which suggests that not all pathogenic mutations occur in highly conserved regions. The frequency of these three mutations varies among different geographical regions. In Europe, mutation m.11778G>A is found in 56.6% of patients, and mutations m.3460G>A and m.14484T>C are found in 22.6 and 20.8% of patients, respectively (Hudson et al. 2007b). In Chinese populations, the frequency of m.11778G>A is much higher than that of European populations and populations of European origin and can be up to 90.2%. The frequencies of m.3460G>A and m.14484T>C are much lower than those of European populations and populations of European origin, with a frequency of 1.1 and 8.7%, respectively (Ji et al. 2008; Jia et al. 2006; Yu et al. 2010a,b) (Fig. 2). A founder effect (the fixation of an allele after the migration and population expansion of a small population in a new area)

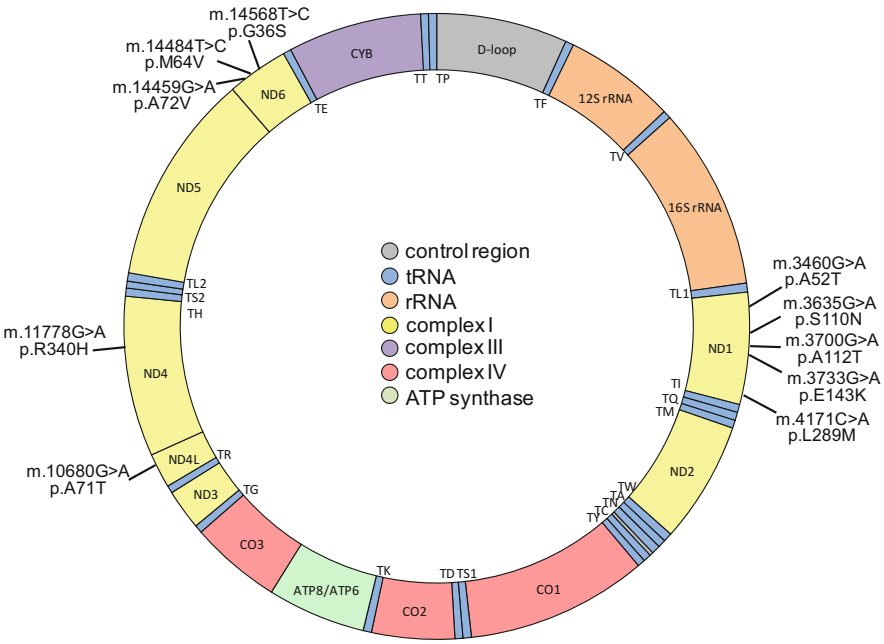

Fig. 1 Schematic profile of LHON mutations. The three primary mutations and the other rare mtDNA mutations that have been reported in at least three independent LHON families, as summarized in MITOMAP, http://www.mitomap.org/bin/view.pl/MITOMAP/MutationsLHON, are shown in their relative positions on the mtDNA molecule (Achilli et al. 2012; Brown et al. 2001b; Bu and Rotter 1991; Fauser et al. 2002a; Fauser et al. 2002b; Gropman et al. 2004; Horvath et al. 2002; Kim et al. 2002; Shoffner et al. 1995; Valentino et al. 2004; Wissinger et al. 1997; Zhang et al. 2012)

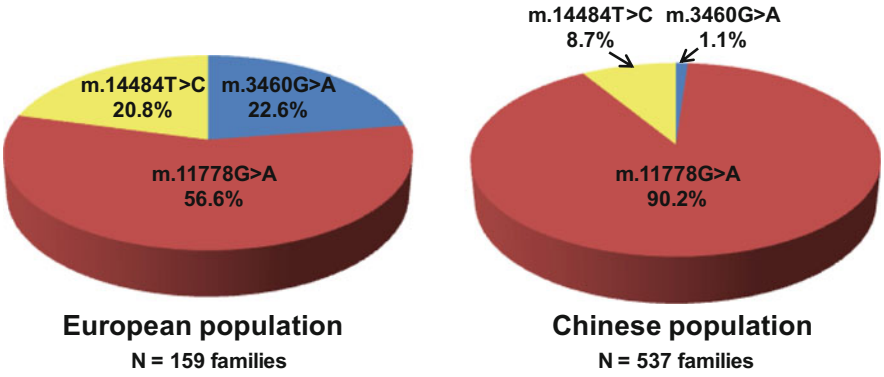

Fig. 2 Mutation spectra of known primary LHON mutations in Chinese and European populations. Frequency of the three LHON primary mutations in European (*left*) (Hudson et al. 2007b) and Han Chinese (*right*) populations (Ji et al. 2008; Jia et al. 2006; Yu et al. 2010a, b)

was observed for m.14484T>C in Canada, which accounts for 87% of the cases and appears to have had an origin in France (Macmillan et al. 2000; Macmillan et al. 1998). However, the best founder effect has been reported from Brazil, where many hundreds of persons can trace their maternal ancestry and the carriage of mutation m.11778G>A, back to an immigrant family that came from Italy (Sadun et al. 2002). The different frequencies of the three primary mutations in different populations may be caused by the past demographic history.

3.2 Rare Primary Mutations

In addition to the three primary mutations, some rare mutations, i.e. m.3635G>A, m.3700G>A, m.14459G>A and so on, as summarized in MITOMAP (http://www.mitomap.org/bin/view.pl/MITOMAP/MutationsLHON) from previous studies (Achilli et al. 2012; Brown et al. 2001b; Fauser et al. 2002b; Gropman et al. 2004; Horvath et al. 2002; Shoffner et al. 1995) have also been reported (Fig. 1). Many of these rare primary mutations were reported in single patients with the typical features of LHON. We have shown how we identified mutations m.3635G>A and m.10680G>A as being pathogenic as follows.

Mutation m.3635G>A was first identified in a Russian LHON family (Brown et al. 2001b) and was confirmed to cause dysfunction of the mitochondrial respiratory chain. Recently, we identified mutation m.3635G>A in a family that had no known primary mutation but had a clear matrilineal inheritance and typical clinical pattern of LHON (Zhang et al. 2009). At about the same time, Yang et al. (2009a) also identified m.3635G>A in two Chinese LHON families. Subsequently, Jia et al. (2010) screened for this mutation in a large sample set of suspected LHON patients who showed the clinical features of LHON but without any of the three primary mutations and identified eight families with the m.3635G>A mutation. These authors further characterized the clinical symptoms of the representative probands with the mutation and provided evidence for a causal association of m.3635G>A with LHON (Jia et al. 2010). We analyzed the complete mtDNA genome sequences of these families and found that m.3635G>A had multiple origins in Chinese (Bi et al. 2012). Furthermore, m.3635G>A is highly conserved in vertebrates, suggesting the importance of this site in the MT-ND1 protein (Zhang et al. 2009). Recently, mutation m.3635G>A was again reported in nine Chinese families and one family from Poland (Kodron et al. 2014; Zhang et al. 2014). Therefore, we can now propose that the mutation m.3635G>A does cause LHON and has a frequency similar to the m.3460G>A mutation (0.57 vs. 0.50%) in Chinese LHON patients (Bi et al. 2012; Jia et al. 2010; Zhang et al. 2009). This mutation should be considered routinely when testing Chinese patients with the symptoms and signs of LHON disease.

Mutation m.10680G>A in the *MT-ND4L* gene can be given as another good example for the identification of additional LHON mutations. This mutation was

first reported in a Chinese family in which all the members had a partial reduction in their vision or had complete blindness, while the mutation was not found in unaffected family members, nor in 100 controls (Yang et al. 2009b). Through complete mitochondrial genome sequencing, they found that the matrilineal family members had the primary mutation m.14484T>C and one novel mutation m.10680G>A. Accordingly, Yang et al. (2009b) suggested that mutation m.10680G>A might be the reason for the complete penetrance of LHON symptoms in this family, although this symptom severity may also be caused by m.14484T>C. Later on, we identified mutation m.10680G>A in one suspected LHON family without any of the three well-known primary mutations and performed an analysis from an evolutionary perspective which suggested a potentially pathogenic role of m.10680G>A in LHON (Zou et al. 2010). Recently, we optimized an allele-specific PCR technique to screen for m.10680G>A in 774 patients with suspected LHON and identified two patients with the mutation (Zhang et al. 2012). The predicted protein structure revealed that mutation m.10680G>A may lead to LHON by changing the structure of the transmembrane region in the MT-ND4L protein (Zhang et al. 2012). However, there remains to be a doubt concerning whether this mutation is significant outside China, as there are eight mitochondrial sequences in the GenBank database from other parts of the world which show this mutation but are not specifically linked to LHON disease (data searched on June 25, 2015).

3.3 Mutation Heteroplasmy and Occurrence of Double Primary LHON Mutations

Depending on its particular type, a cell may contain hundreds or thousands of mitochondria. The presence of a mutation only in some of cells of an individual or in some of the mitochondria within a cell indicates heteroplasmy for that mutation. The existence of wild-type and mutant mtDNA within cells (intracell heteroplasmy) and among cells (intercell heteroplasmy) leads to different levels of mutation load (0–100%) within a cell or tissue (Yao et al. 2015). The heteroplasmy level of a pathogenic mutation may affect the onset of a mitochondrial disease, and there is a threshold effect (DiMauro and Schon 2003). Most families with LHON primary mutation are homoplasmic, but about 10–15% of families do show heteroplasmy (Carelli et al. 2004; Man et al. 2002b; Yen et al. 2006; Yu-Wai-Man et al. 2009). However, a high prevalence of LHON family with heteroplasmic m.11778G>A (11 in 30 pedigrees, 37%) was observed in Thai population (Phasukkijwatana et al. 2006). The level of heteroplasmy affects the presence of LHON symptoms, and it is reported that when the level of heteroplasmy is less than 60%, there is a lower probability of disease (Chinnery et al. 2001a). But there are also some reports showing cases with disease symptoms even if the heteroplasmy is at a low level, suggesting a modulation effect from the nuclear genetic background (Black et al. 1996; Jacobi et al. 2001).

In our previous studies, we have found that only a small part of Chinese LHON patients (about 0.2% of patients with m.11778G>A and 5.8% of patients with m.14484T>C) carried heteroplasmic primary mutations, while most of the patients were homoplasmic (Yu et al. 2010b; Zhang et al. 2011b). The pattern of extremely low frequency of heteroplasmic m.11778G>A in Chinese patients appears to be different from that of European patients or patients of European origin. In six LHON families with the mutation m.3460G>A that we analyzed, we only identified one family with a heteroplasmic mutation, and the proband of this family had a heteroplasmic mutation load of about 40%. Interestingly, the penetrance of LHON symptoms in this family was 12.5%, which was significantly lower than the average penetrance of families (25.6%) with a homoplasmic mutation (Yu et al. 2010a). The heteroplasmy status of m.3460G>A may be one of the reasons for the low penetrance of LHON in this family (Yu et al. 2010a), which is consistent with previous findings that only 20% of heteroplasmic carriers, but 47% of homoplasmic carriers, manifested the disease in a Thai population (Phasukkijwatana et al. 2006).

In addition, there are sporadic reports of the presence of two LHON mutations in one family. Brown et al. (2001a) reported a 9-year-old Caucasian girl carrying both the m.11778G>A and m.14484T>C mutations, with m.11778G>A in a heteroplasmic status. Despite carrying two pathogenic mutations, the patient's mother was apparently normal (Brown et al. 2001a). Tonska et al. (2008) reported a Polish LHON family with both the m.11778G>A and m.3460G>A mutations, with m.3460G>A in a heteroplasmic status. Except for typical LHON symptoms, no other nervous system disorders were found in this family, and all the female members were unaffected (Tonska et al. 2008). These studies seem to indicate that heteroplasmic mutations may be linked to fewer manifestations of LHON.

4 Effect of Secondary Mutations and mtDNA Genetic Background

According to the clinical observation, different families present a variable penetrance of LHON symptoms, ranging from 10 to 100%, albeit harbouring the same primary mutation, as shown by the two families with m.11778G>A in Han Chinese (Wang et al. 2008). Secondary mtDNA mutations and mtDNA haplogroup genetic background appear to have some effect on the penetrance of LHON. These two factors may not lead to LHON directly, but can act in synergy with primary mutations to affect the onset of LHON.

4.1 The Secondary Mutations May Act in Synergy with the Primary Mutations

Most of the secondary mutations are common variants. For instance, variant m.12811T>C, which is the defining variants of haplogroup M7b1′2 and has a

high frequency in the general population (Kong et al. 2006), was considered to be one of the secondary mutations of LHON (Huoponen et al. 1993). We have obtained consistent results to show that haplogroup M7b1′2 is an important risk factor for the development of LHON in Han Chinese patients with m.11778G>A mutation (Ji et al. 2008; Zhang et al. 2011b). Intriguingly, m.12811T>C also defined a clade of H3h in European populations or populations of European origin, which also shows an increased prevalence of LHON (Bandelt H-J et al. unpublished data). All these results suggested m.12811T>C as being a secondary mutation for LHON, both in Chinese and European populations. Also the mutation m.593T>C is a common variant found in the *MT-tRNA*^{Phe} gene. Our recent study revealed that this variant may lead to altered structure of *MT-tRNA*^{Phe} and thus acts in synergy with m.11778G>A to increase the penetrance of LHON (Zhang et al. 2011a). Note that many of the secondary mutations of LHON are haplogroup-defining variants and have a much higher frequency than the primary LHON mutations. Just why some of these secondary mutations are associated with LHON only in some populations, but not others, is unresolved.

4.2 mtDNA Haplogroup Background Effect

Because of high mutation rate and lack of recombination, mtDNA accumulates mutations in a time order within a relatively short span of time and forms different haplotypes (Pakendorf and Stoneking 2005). In the process of human evolution and migration, descendants of an original mtDNA haplotype would sequentially accumulate additional mutations to generate a group of related haplotypes that was termed as a haplogroup (Achilli et al. 2008; Kong et al. 2006; Torroni et al. 2000). The current global mtDNA phylogenetic tree contains over 4,000 different haplogroups, which form a region-specific distribution pattern (Achilli et al. 2008; Kong et al. 2010; Kong et al. 2006; van Oven and Kayser 2009). mtDNA haplogroups have become important genetic markers for different populations. In the past decades, mtDNA haplogroups have also been identified to be associated with many different metabolic and neurodegenerative diseases. As early as in 1992, Brown et al. (1992) found that mtDNA haplotype (5244-13708-15257-15812) was associated with significantly increased risk for the onset of LHON. Later, Torroni et al. (1997) analyzed mtDNA haplogroup in Italian LHON patients and identified haplogroup J as a risk factor for patients with m.11778G>A and m.14484T>C. Shafa Shariat Panahi et al. (2006) found that haplogroups J and W increased disease risk in Iranian LHON patients carrying m.11778G>A and m.3460G>A, respectively. However, the sample size of these studies was too small to obtain a firm conclusion. In recent years, further studies have conducted to investigate the association between mtDNA haplogroup and LHON. One of the most comprehensive studies was carried out by Hudson et al. (2007b), who analyzed mtDNA haplogroups in 3,613 individuals from 159 - European LHON families and found that haplogroups J2, J1 and K increased the LHON risk in patients with m.11778G>A, m.14484T>C and m.3460G>A,

respectively, while haplogroup H was a protective factor for patients with m.11778G>A. Phylogenetic analysis indicated that the nonsynonymous variants located in the root of haplogroups J and K may account for the association between these haplogroups and LHON (Hudson et al. 2007b).

Because the genetic structure is different between Asian and European populations, we have performed a comprehensive study, with the largest number of LHON patients in the Han Chinese population including 1,859 individuals from 182 Chinese families (including one from Cambodia) with the m.11778G>A mutation, to investigate whether mtDNA haplogroups affect the penetrance of LHON in East Asian families. Haplogroup M7b1′2 was identified to be significantly associated with visual loss, whereas haplogroup M8a showed a protective effect (Ji et al. 2008). Further analyses of the complete mtDNA sequences from LHON families with m.11778G>A suggested that variants m.12811T>C (p.Y159H) in the *MT-ND5* gene and m.8584G>A – m.8684C>T (p.A20T – p.T53I) in the *MT-ATP6* gene, which are haplogroup-defining variants for haplogroups M7b1′2 and M8a, respectively, might account for the association of these two haplogroups with LHON (Ji et al. 2008). Subsequently, we analyzed the matrilineal structure of further 304 Chinese patients with m.11778G>A and 843 patients with suspected LHON, to determine the effect of the mtDNA genetic background on the onset of disease (Zhang et al. 2011b). The enlarged sample size of LHON patients confirmed the results that were obtained in our previous study based on 182 families (Ji et al. 2008). Specifically, the LHON samples differed from the general Chinese or samples with suspected LHON by harbouring fewer lineages belonging to haplogroup F and more lineages belonging to M7b and D4. In contrast, the matrilineal structure of the suspected LHON population resembled that of the general Chinese population, suggesting no role of mtDNA haplogroup in these suspected LHON patients (Zhang et al. 2011b). In Southeast Asian population, haplogroup B5a1 was identified to be significantly associated with m.11778G>A and appeared to modify the risk of visual loss (Kaewsutthi et al. 2011).

Although association studies have indicated that the onset of LHON is influenced by mtDNA haplogroup, functional characterization of this effect is a daunting task. There have been several attempts that have increased our knowledge regarding the background effect. For instance, Suissa et al. (2009) found that ancient mtDNA variants of haplogroup J reduced the replication and stability of mtDNA. Using cybrid cells with different mtDNA haplogroup backgrounds, Ghelli et al. (2009) found that haplogroup J increases the sensitivity of cells to 2,5-hexanedione toxicity, indicating that the mtDNA haplogroup background affects the development of LHON in combination with environmental factors. However, tests of respiratory function in cybrid cell lines carrying European mtDNA haplogroups (including J) that were demonstrated to be associated with LHON showed no detectable differences in multiple parameters, suggesting that the effect of the mtDNA haplogroup may be more complex than what we had thought (Carelli et al. 2002).

Note that a search of the 30,000 mtDNA sequences (data searched on June 25, 2015) held by the GenBank database shows that many of the European sequences

containing a primary LHON mutation do come from the subgroups of haplogroup U, a feature which as yet has not raised the attention of researchers.

4.3 Occurrence of mtDNA Pathogenic Mutations with the Primary Mutations

Besides common variants, there were some pathogenic mutations for other mitochondrial diseases coexisting with the LHON primary mutations. Mimaki et al. (2003) reported one 51-year-old Japanese patient carrying both m.11778G>A and m.12192G>A (a pathogenic mutation for cardiomyopathy). The initial symptom of this patient was rapid reduction of weight, with acute visual loss developing 6 months later. We reported a LHON family with two pathogenic mutations m.11778G>A and m.1555A>G, which may account for the high penetrance (78.6%) of LHON in this family (Zhang et al. 2008). Mutation m.1555A>G is the major cause of aminoglycoside-induced and non-syndromic hearing loss (Guan 2011; Kokotas et al. 2007; Petit et al. 2001). However, none of the matrilineal relatives showed problems with their hearing, and we therefore inferred that the mutation m.1555A>G in this family may act in synergy with m.11778G>A to significantly increase the penetrance of LHON (Zhang et al. 2008). Interestingly, the co-occurrence of m.1555A>G and m.11778G>A was also observed in two Indian families recently, with one family showing a high penetrance of LHON (62.5%) (Khan et al. 2013).

4.4 mtDNA Mutational Hotspots in Suspected LHON Patients

Though most of the LHON patients have been identified to carry known mtDNA primary mutations, there still are many patients with typical clinical features of LHON but without known mtDNA primary mutations. These patients have not been fully investigated, and genetic analysis has revealed that there might be mtDNA mutation hotspots in these patients. Studies in European populations revealed that the *MT-ND1* and *MT-ND6* genes were mutational hotspots for nonsynonymous mutations (Chinnery et al. 2001b; Valentino et al. 2004), whereas in our study, albeit with a limited number of Han Chinese families, we showed that the *MT-ND1* and *MT-ND5* genes are mutational hotspots in families with suspected LHON (Zou et al. 2010). However, it remains to be answered why the mutations occur mainly in these gene regions, how these mutations affect the onset of LHON and if there are any other nuclear modifiers to these mtDNA mutations (Yen et al. 2006).

5 Effect of Nuclear Genes on LHON

5.1 Chromosome X

The gender bias of LHON suggests the chromosome X genes may be involved in this disease (Bu and Rotter 1991, 1992). According to the proposed two-locus mitochondrial and X-chromosome-linked nuclear gene model (Bu and Rotter 1991, 1992), LHON symptoms occur only when the mtDNA pathogenic mutations and chromosome X-sensitive gene(s) are present at the same time and the onset in female carriers was dependent on the homozygous status of susceptible gene(s) in chromosome X. This model is a reasonable explanation of the gender bias of LHON, and different studies have identified several regions in chromosome X showing linkage with LHON. The pioneer linkage study in six LHON families performed by Vilkki et al. (1991) found that the onset of LHON is determined by genes linked to the DXS7 region of chromosome X. The similar study performed by Hudson et al. (2005) in 100 European LHON families identified that the DXS8090 (166)-DXS1068 (258) region of chromosome X is linked to the onset of LHON. Also a study in a Brazilian LHON family found that region Xq25-Xp27.2 of chromosome X confers susceptibility to LHON (Shankar et al. 2008). There were relatively few studies performed in Chinese patients. Recently, Ji et al. (2010) analyzed 12 microsatellite markers and 4 single nucleotide polymorphisms (SNPs) in chromosome X in Chinese male LHON patients and controls and identified two microsatellites (DXS6803 and DXS984) that are associated with LHON. However, there were negative results reporting no association between chromosome X and LHON (Man et al. 2002a; Oostra et al. 1996; Pegoraro et al. 1996; Petruzzella et al. 2007). How genes in chromosome X affect LHON is still a mystery. The recent study performed by Hudson et al. (2007a) indicated that the oestrogen receptor-related genes in chromosome X may be susceptible genes for LHON. These results need to be validated, and further functional characterization should be carried out to elucidate the cause of the male-biased prevalence of LHON.

5.2 Other Nuclear Factors

A nuclear genetic effect has been thought to account for the puzzle posed by the incomplete penetrance of LHON, and identification of nuclear modifier(s) has received wide attention from researchers in the field. A previous study focusing on oxidative stress and apoptosis pathways identified *EPHX1* and *TP53* genes as candidate nuclear genes associated with earlier onset of LHON (Ishikawa et al. 2005). Recent genome-wide expression and genome-wide linkage studies have further explored the potential nuclear genes that may play a modifying role in LHON. In the genome-wide linkage study performed by Phasukkijwatana et al. (2010) in patients from Thailand, chromosome region 3q26.2-3q28 was found to be linked to LHON. This genomic region contains six genes, including *PARL*, *OPA1*

and others. Further analysis of SNPs in the *PARL* gene indicated that SNPs rs3749446 and rs1402000 conferred a susceptibility to LHON (Phasukkijwatana et al. 2010). However, the association between *PARL* SNPs and LHON could not be replicated in the Han Chinese population in our validation study (Zhang et al. 2010). The *OPA1* gene is a pathogenic gene for autosomal dominant optic atrophy (ADOA), which presents many overlapping clinical features with LHON (Carelli et al. 2004). Previous studies have revealed that the OPA1 protein is located in the mitochondrial inner membrane and plays important roles in mitochondrial fusion and apoptosis (Olichon et al. 2003). Interestingly, PARL is also a mitochondrial protein and was recently found to regulate OPA1 in the apoptosis pathway (Cipolat et al. 2006). In the genome-wide expression assay in LHON patients and controls performed by Amero-Abu et al. (2010), 137 up-regulated and 152 down-regulated genes were identified, in which the *OPA1* gene was found to be significantly down-regulated in LHON patients. Whether the OPA1-PARL pathway plays a key role in the onset of optic atrophy, from either LHON or ADOA, is an important and interesting question remaining to be answered.

A recent profiling of the mitochondrial proteome of fibroblasts from LHON patients with m.11778G>A showed a down-regulation of bioenergetics and mitochondrial protein quality control pathways (Tun et al. 2014). Coincidentally, Giordano et al. (2014) found that unaffected LHON mutation carriers had a significantly higher mtDNA copy number and mitochondrial mass and higher capacity for activating mitochondrial biogenesis under metabolic demand compared with their affected maternally related relatives and healthy individuals. These findings suggest that increased mitochondrial biogenesis in LHON mutation carriers may overcome some of the pathogenic effect of the LHON mutation, and this may account for the incomplete penetrance in LHON (Giordano et al. 2014). These efforts aiming at identifying nuclear modifiers in LHON have provided some helpful information for uncovering the puzzle of LHON, although we suggest that more studies are essential to define the modifier genes and their regulatory mechanisms in biochemical pathways.

6 Environmental Factors

The gender bias and incomplete penetrance of LHON strongly suggest that environmental factors which are more specific to males than to females, such as smoking and alcohol, may contribute to the increased disease risk of LHON in males (Charlmers and Harding 1996; Kerrison et al. 2000; Kirkman et al. 2009). A study by Charlmers and Harding (1996) showed that alcohol, but not smoking, may significantly increase the penetrance of LHON. However, different observations were obtained in a recent large-scale epidemiological study by Kirkman et al. (2009), which showed that smoking is significantly linked to the penetrance of LHON, independent of gender and alcohol intake, and excessive drinking would lead to increased risk of LHON. Furthermore, a recent study found that oestrogens could rescue the mitochondrial dysfunction caused by LHON primary mutations

(Giordano et al. 2011), which may underline the potential reason for the gender bias of LHON. There are some anecdotal reports for the effect of exposure to toxic substances or medicines, such as carbon monoxide poisoning (Hwang and Park 1996) and anti-tuberculosis medication including ethambutol (Seo et al. 2010), to increase the clinical expression of LHON.

7 Biochemical Effects of mtDNA Mutations and Their Implication for LHON Treatment

7.1 Biochemical Effect of mtDNA Mutations

It is of note that almost all the primary mutations and mutational hotspots for LHON are located in genes that encode the subunits of complex I in the mitochondrial respiration chain. For instance, mutations m.3460G>A (p.A52T) and m.3635G>A (p.S110N) occur in the MT-ND1 subunit, mutation m.11778G>A (p.R340H) is located in the MT-ND4 subunit and mutation m.14484T>C (p.M64V) is located in the MT-ND6 subunit, whereas mutation m.10680G>A (p.A71T) is located in the MT-ND4L subunit. All these mutations change the amino acids in different complex I subunits and were considered to cause LHON through impairing the function of complex I (Fig. 3) (Yen et al. 2006). Mitochondrial complex I, also known as NADH dehydrogenase, is crucial for cellular metabolism and ATP production (Hirst 2013). In addition, complex I is the major source of reactive oxygen species (ROS) (Hirst 2013). A large number of studies have showed significantly decreased complex I activity and respiration rate, as well as ATP level, in cybrids, fibroblasts or lymphoblasts with LHON pathogenic mutations (Baracca et al. 2005; Brown et al. 2000; Cock et al. 1999; Hirst 2013; Hofhaus et al. 1996; Lin et al. 2012; Majander et al. 1996; Wong et al. 2002). It is suggested that the increased oxidative stress caused by complex I dysfunction (Beretta et al. 2004; Wong et al. 2002) leads to a series of mitochondrial defects and further triggers the clearance of damaged mitochondria, which is also known as mitophagy (Melser et al. 2015). The excess of mitophagy might be the major reason for the decreased level of mitochondrial mass, as well as ATP production observed in LHON (Giordano et al. 2014). In this way complex I dysfunction may eventually lead to increased apoptosis and cell death (Hofhaus et al. 1996; Wong et al. 2002; Zanna et al. 2003) and be the potential reason for the optic neuron loss observed in LHON patients and animal models (Fig. 3). Consistent with this hypothesis, the chemically induced mouse model initiated by the use of rotenone, an inhibitor of complex I, presented similar degeneration of retinal ganglion cells as in LHON (Zhang et al. 2002). Therefore, complex I dysfunction induced by LHON primary mutations may well be a key pathogenic cause for the onset of LHON symptoms.

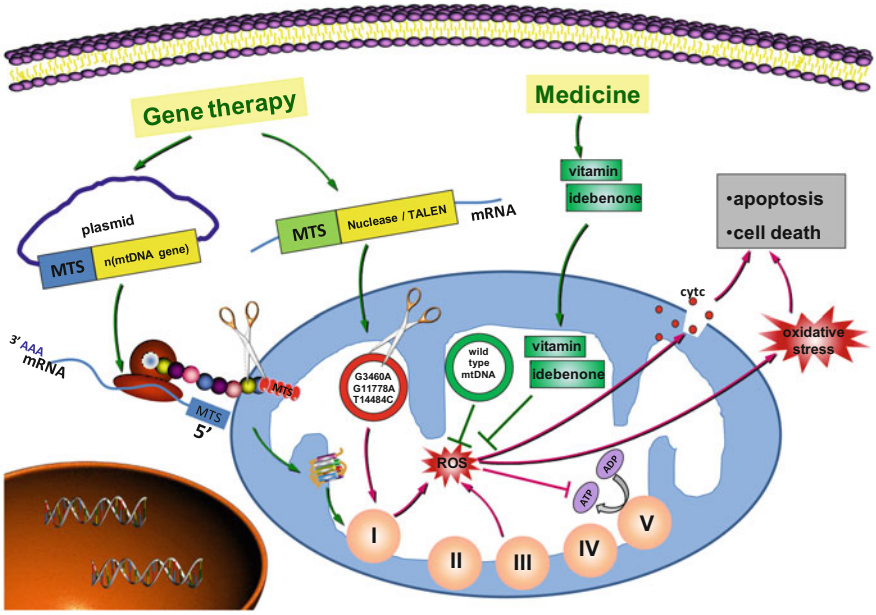

Fig. 3 Potential pathogenic role of LHON mutations in the cell and treatment strategy. The dysfunction of complex I due to LHON mutations leads to the overproduction of ROS, and the significantly increased oxidative stress triggers a series of mitochondrial-related dysfunctions and increased apoptosis and cell death (Yen et al. 2006). Antioxidants like idebenone might therefore be potentially effective medicines (Mashima et al. 2000). Gene therapy through allotopic expression of mitochondrial genes (Guy et al. 2002) or genome-editing technique using mitochondria-targeted restriction endonucleases or TALENs (Reddy et al. 2015) may also increase the percentage of wild-type mtDNA and prevent the germline transmission of pathogenic mtDNA mutations and is, therefore, another promising treatment for LHON symptoms

7.2 Functional Characterization of the LHON Primary Mutations

The pathogenicity of the LHON primary mutations needs to be proven by the use of functional assays (Bandelt et al. 2009). One feasible approach is to develop cell cybrids using blood platelets as these cells do not have nuclei (King and Attardi 1989), and this is now the most frequently used method to study the direct association between mtDNA variants and alterations in mitochondrial function. Early studies of the cybrids with LHON primary mutation m.11778G>A showed a significantly decreased oxygen consumption, demonstrating a pathogenic role of this mutation in impairing cell respiration (Vergani et al. 1995). A later study, however, showed mutations m.3460G>A and m.11778G>A could result in complex I defects, whereas m.14484T>C caused only a much milder biochemical defect (Brown et al. 2000). In addition, LHON primary mutations were found to disrupt glutamate transport in cybrid cell lines (Beretta et al. 2004).

Besides the trans-mitochondrial cybrids, the allotopic expression system, in which the mtDNA gene was converted to a nuclear-encoded version and the protein

was first expressed in the cytosol and then imported into mitochondria with the direction of mitochondrial targeting peptide (Fig. 3) (Manfredi et al. 2002), has also been demonstrated to be efficient for the functional characterization of certain mtDNA mutation(s) in our recent study (Bi et al. 2015) and others (Guy et al. 2002; Manfredi et al. 2002). Through allotopic expression of wild-type *ND4* gene in cybrids with m.11778G>A, Guy et al. (2002) found that this method could efficiently rescue the defective complex I-dependent ATP production and cell growth in galactose medium. However, because of the high hydrophobicity of mtDNA-encoded protein, the efficiency of this method was questioned in many studies (Figueroa-Martinez et al. 2010; Oca-Cossio et al. 2003). Kaltimbacher et al. (2006) optimized the allotopic expression system, in which the mRNA was first targeted to the mitochondrial surface and then the protein translation was coupled with protein transport. Allotopic expression of wild-type *ND1* and *ND4* genes in fibroblasts containing m.3460G>A and m.11778G>A using this optimized method successfully restored the complex I activity and mitochondrial dysfunction induced by the LHON primary mutations (Bonnet et al. 2008). Using the same method, Ellouze et al. (2008) introduced the *ND4* gene harbouring the m.11778G>A mutation into rat eyes by in vivo electroporation and developed an animal model that mimics the essential aspects of LHON, including degeneration of retinal ganglion cells and decline in visual performance. Further overexpression of the wild-type *ND4* gene in these LHON models could improve these signs.

7.3 New Avenues of Research: LHON Gene Therapy and Genome Editing

In order to accomplish a more efficient gene delivery, the allotopic expression method was further developed by injecting adeno-associated virus (AAV) with mtDNA genes into eyes (Chadderton et al. 2013; Koilkonda et al. 2014; Yu et al. 2012). Koilkonda et al. (2014) estimated the safety and effect of this AAV mediate allotopic expression system in non-human primate and ex vivo human eyes and found that the allotopic expression was efficient and long lasting with no serious adverse reactions in most retinal ganglion cells. These studies indicated that the gene therapy through allotopic expression of mitochondrial genes may be a promising method in the treatment of LHON (Fig. 3). Also, Iyer et al. (2012) had success by using healthy donor mtDNAs complexed with recombinant human mitochondrial transcription factor A (TFAM) to improve respiration and biogenesis in a LHON cell line. Recently, further work of this type has been reported from France (Cwerman-Thibault et al. 2014; Cwerman-Thibault et al. 2015), and a trial in carriers of the mutation m.11778G>A has been initiated. However, evidently, there is still a long way to go before this method is fully accepted as a therapy.

Another promising approach for LHON therapy is mitochondrial replacement, which involves spindle, pronuclear or polar body genome transfer into healthy enucleated donor oocytes or embryos, and this approach has been successfully used to eliminate pathogenic mtDNA variant(s) and to prevent the transmission of

mtDNA disease (Craven et al. 2010; Paull et al. 2013; Tachibana et al. 2009, 2013; Wang et al. 2014). More recently, Reddy and colleagues (2015) took advantage of the recently advanced genome-editing technique and selectively prevented the germline transmission of a mtDNA haplotype using either mitochondria-targeted restriction endonucleases or transcription activator-like effector nucleases (TALENs). They successfully reduced LHON mutation level in mammalian oocytes using mitochondria-targeted TALENs (mito-TALENs). We believe that the most recently available CRISPR/Cas system, which is very powerful for genome editing (Cong et al. 2013), will also be a workable method for mtDNA genome editing in the future. The safety and efficacy of this mtDNA genome-editing approach open a new avenue for LHON therapy (Fig. 3). However, there are also potential concerns about the use of these techniques in a clinical setting (Craven et al. 2011). Besides the complex technical issues, all these techniques still need to be confirmed as being safe for use in humans.

7.4 Implications for Medical Therapy for LHON

There is currently no effective treatment that can be proven to prevent, or restore, the visual loss in LHON (La Morgia et al. 2014; Yu-Wai-Man et al. 2014). But several different approaches and therapies have been suggested. LHON mutation carriers may have their symptoms precipitated by vitamin B12 deficiency, and known carriers should have an adequate dietary intake of vitamin B12 (Pott and Wong 2006). Spontaneous visual recovery is observed in some LHON patients, and idebenone in combination with vitamin B2 and vitamin C has been suggested to speed up this recovery (Fig. 3) (Mashima et al. 2000). Idebenone is described as an antioxidant (Mordente et al. 1998) and transports electrons bypassing complex I to complex III and thereby partly restores ATP production in cases where there is a complex I defect (Giorgio et al. 2011; Haefeli et al. 2011). Pharmacological study in a LHON mouse model with complex I dysfunction induced by rotenone indicated that idebenone can restore the loss of retinal ganglion cells and vision defects in a LHON mouse model (Heitz et al. 2012). In recent years, more evidence has shown that LHON patients might benefit from idebenone treatment (Carelli et al. 2011; Klopstock et al. 2011, 2013), suggesting that agents active against complex I dysfunction and oxidative stress may be an efficient treatment for LHON in the future (Fig. 3). However, the question of whether or not a new LHON patient should be offered treatment with idebenone is still unproven.

Another approach to therapy for LHON is promising but still very much at the theoretical stage and involves considering how to control the balance between mitochondrial biogenesis and clearance (mitophagy). Mitochondrial biogenesis is regulated by a complex system of transcriptional regulator networks of mitochondrial proteins (Vega et al. 2015). Furthermore, it was reported that the mitochondrial biogenesis and mitophagy could be regulated through the post-translational modification of mitochondrial proteins (i.e. lysine acetylation), which was found to be modified by the nutritional homeostasis (Webster et al. 2014). There are many

agents, chemicals and medicines that are known to reduce mitochondrial biogenesis, such as antibiotics targeting mitochondria like erythromycin, tetracycline, chloramphenicol and glycylcyclines (Lamb et al. 2015). Recent studies have identified several potentially therapeutic agents that could stimulate key regulators of mitochondrial biogenesis (Sanchis-Gomar et al. 2014; Valero 2014), including activators of the PPAR-PGC1α axis, bezafibrate, thiazolidinediones, pioglitazone, rosiglitazone and fenofibrate (Uittenbogaard and Chiaramello 2014; Wu et al. 2014; Yadav et al. 2014; Zamora and Villena 2014); agonists of AMPK, resveratrol, AICAR and metformin (Uittenbogaard and Chiaramello 2014; Wu et al. 2014; Zamora and Villena 2014); triggering factors of SIRT1, SRT1720, quercetin, resveratrol and several isoflavone-derived compounds (Uittenbogaard and Chiaramello 2014; Wu et al. 2014; Zamora and Villena 2014); and the agonists of Nrf2, triterpenoids and *Bacopa monnieri* (Yadav et al. 2014). However, the hypothesis to increase ATP production at times of stress through increasing mitochondrial mass has not been clinically proved. But it is an interesting line of research that may be of significance to carriers of LHON mutations, as the possibility of taking a simple medication to prevent the onset of symptoms is a pleasing prospect.

8 Conclusion and Future Perspective

LHON, the most extensively studied mitochondrial disease, predominantly causes blindness in young males and brings a lot of suffering to the patients and their families. Although many primary mtDNA mutations have been identified, the phenotypic expression of these primary mutations is very complex. Incomplete disease penetrance, male-biased onset, involvement of additional genetic factors (secondary mutations, mtDNA background, nuclear genetic modifiers, epigenetic changes) or environmental factors (smoking, alcohol, toxic substance) are all involved in the pathogenesis of the disorder; but their relevance has not been fully understood. Currently, there is no effective treatment for LHON. Studies in the past two decades have shown us that complex I dysfunction is the main biochemical effect of the pathogenic mutations and gene therapy or medicine aimed against complex I dysfunction are promising treatments for LHON. The new techniques of genome editing also offer the possibility of a successful treatment for LHON. Further studies focusing on the underlying mechanism of LHON will be of importance in elucidating the pathophysiology of retinal neuron loss as well as in searching for new clues that may finally prevent the visual loss caused by LHON.

References

Abu-Amero KK, Jaber M, Hellani A, Bosley TM (2010) Genome-wide expression profile of LHON patients with the 11778 mutation. Br J Ophthalmol 94:256–259

Achilli A, Perego UA, Bravi CM, Coble MD, Kong QP, Woodward SR, Salas A, Torroni A, Bandelt HJ (2008) The phylogeny of the four pan-American MtDNA haplogroups: implications for evolutionary and disease studies. PLoS One 3:e1764

Achilli A, Iommarini L, Olivieri A, Pala M, Hooshiar Kashani B, Reynier P, La Morgia C, Valentino ML, Liguori R, Pizza F, Barboni P, Sadun F, De Negri AM, Zeviani M, Dollfus H, Moulignier A, Ducos G, Orssaud C, Bonneau D, Procaccio V, Leo-Kottler B, Fauser S, Wissinger B, Amati-Bonneau P, Torroni A, Carelli V (2012) Rare primary mitochondrial DNA mutations and probable synergistic variants in Leber's hereditary optic neuropathy. PLoS One 7:e42242

Bandelt HJ, Salas A, Taylor RW, Yao YG (2009) Exaggerated status of "novel" and "pathogenic" mtDNA sequence variants due to inadequate database searches. Hum Mutat 30:191–196

Baracca A, Solaini G, Sgarbi G, Lenaz G, Baruzzi A, Schapira AH, Martinuzzi A, Carelli V (2005) Severe impairment of complex I-driven adenosine triphosphate synthesis in Leber hereditary optic neuropathy cybrids. Arch Neurol 62:730–736

Beretta S, Mattavelli L, Sala G, Tremolizzo L, Schapira AH, Martinuzzi A, Carelli V, Ferrarese C (2004) Leber hereditary optic neuropathy mtDNA mutations disrupt glutamate transport in cybrid cell lines. Brain 127:2183–2192

Bi R, Zhang AM, Yu D, Chen D, Yao YG (2010) Screening the three LHON primary mutations in the general Chinese population by using an optimized multiplex allele-specific PCR. Clin Chim Acta 411:1671–1674

Bi R, Zhang AM, Jia X, Zhang Q, Yao YG (2012) Complete mitochondrial DNA genome sequence variation of Chinese families with mutation m.3635G>A and Leber hereditary optic neuropathy. Mol Vis 18:3087–3094

Bi R, Zhang W, Yu D, Li X, Wang HZ, Hu QX, Zhang C, Lu W, Ni J, Fang Y, Li T, Yao YG (2015) Mitochondrial DNA haplogroup B5 confers genetic susceptibility to Alzheimer's disease in Han Chinese. Neurobiol Aging 36:1604.e7

Black GC, Morten K, Laborde A, Poulton J (1996) Leber's hereditary optic neuropathy: heteroplasmy is likely to be significant in the expression of LHON in families with the 3460 ND1 mutation. Br J Ophthalmol 80:915–917

Bonnet C, Augustin S, Ellouze S, Benit P, Bouaita A, Rustin P, Sahel JA, Corral-Debrinski M (2008) The optimized allotopic expression of ND1 or ND4 genes restores respiratory chain complex I activity in fibroblasts harboring mutations in these genes. Biochim Biophys Acta 1783:1707–1717

Brown MD, Voljavec AS, Lott MT, Torroni A, Yang CC, Wallace DC (1992) Mitochondrial DNA complex I and III mutations associated with Leber's hereditary optic neuropathy. Genetics 130:163–173

Brown MD, Trounce IA, Jun AS, Allen JC, Wallace DC (2000) Functional analysis of lymphoblast and cybrid mitochondria containing the 3460, 11778, or 14484 Leber's hereditary optic neuropathy mitochondrial DNA mutation. J Biol Chem 275:39831–39836

Brown MD, Allen JC, Van Stavern GP, Newman NJ, Wallace DC (2001a) Clinical, genetic, and biochemical characterization of a Leber hereditary optic neuropathy family containing both the 11778 and 14484 primary mutations. Am J Med Genet 104:331–338

Brown MD, Zhadanov S, Allen JC, Hosseini S, Newman NJ, Atamonov VV, Mikhailovskaya IE, Sukernik RI, Wallace DC (2001b) Novel mtDNA mutations and oxidative phosphorylation dysfunction in Russian LHON families. Hum Genet 109:33–39

Bu XD, Rotter JI (1991) X chromosome-linked and mitochondrial gene control of Leber hereditary optic neuropathy: evidence from segregation analysis for dependence on X chromosome inactivation. Proc Natl Acad Sci U S A 88:8198–8202

Bu X, Rotter JI (1992) Leber hereditary optic neuropathy: estimation of number of embryonic precursor cells and disease threshold in heterozygous affected females at the X-linked locus. Clin Genet 42:143–148

Carelli V, Vergani L, Bernazzi B, Zampieron C, Bucchi L, Valentino M, Rengo C, Torroni A, Martinuzzi A (2002) Respiratory function in cybrid cell lines carrying European mtDNA

haplogroups: implications for Leber's hereditary optic neuropathy. Biochim Biophys Acta 1588:7–14

Carelli V, Ross-Cisneros FN, Sadun AA (2004) Mitochondrial dysfunction as a cause of optic neuropathies. Prog Retin Eye Res 23:53–89

Carelli V, La Morgia C, Valentino ML, Rizzo G, Carbonelli M, De Negri AM, Sadun F, Carta A, Guerriero S, Simonelli F, Sadun AA, Aggarwal D, Liguori R, Avoni P, Baruzzi A, Zeviani M, Montagna P, Barboni P (2011) Idebenone treatment in Leber's hereditary optic neuropathy. Brain 134:e188

Chadderton N, Palfi A, Millington-Ward S, Gobbo O, Overlack N, Carrigan M, O'Reilly M, Campbell M, Ehrhardt C, Wolfrum U, Humphries P, Kenna PF, Farrar GJ (2013) Intravitreal delivery of AAV-NDI1 provides functional benefit in a murine model of Leber hereditary optic neuropathy. Eur J Hum Genet 21:62–68

Charlmers RM, Harding AE (1996) A case-control study of Leber's hereditary optic neuropathy. Brain 119(Pt 5):1481–1486

Chinnery PF, Johnson MA, Wardell TM, Singh-Kler R, Hayes C, Brown DT, Taylor RW, Bindoff LA, Turnbull DM (2000) The epidemiology of pathogenic mitochondrial DNA mutations. Ann Neurol 48:188–193

Chinnery PF, Andrews RM, Turnbull DM, Howell NN (2001a) Leber hereditary optic neuropathy: does heteroplasmy influence the inheritance and expression of the G11778A mitochondrial DNA mutation? Am J Med Genet 98:235–243

Chinnery PF, Brown DT, Andrews RM, Singh-Kler R, Riordan-Eva P, Lindley J, Applegarth DA, Turnbull DM, Howell N (2001b) The mitochondrial ND6 gene is a hot spot for mutations that cause Leber's hereditary optic neuropathy. Brain 124:209–218

Cipolat S, Rudka T, Hartmann D, Costa V, Serneels L, Craessaerts K, Metzger K, Frezza C, Annaert W, D'Adamio L, Derks C, Dejaegere T, Pellegrini L, D'Hooge R, Scorrano L, De Strooper B (2006) Mitochondrial rhomboid PARL regulates cytochrome c release during apoptosis via OPA1-dependent cristae remodeling. Cell 126:163–175

Cock HR, Cooper JM, Schapira AH (1999) Functional consequences of the 3460-bp mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. J Neurol Sci 165:10–17

Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F (2013) Multiplex genome engineering using CRISPR/Cas systems. Science 339:819–823

Craven L, Tuppen HA, Greggains GD, Harbottle SJ, Murphy JL, Cree LM, Murdoch AP, Chinnery PF, Taylor RW, Lightowlers RN, Herbert M, Turnbull DM (2010) Pronuclear transfer in human embryos to prevent transmission of mitochondrial DNA disease. Nature 465:82–85

Craven L, Elson JL, Irving L, Tuppen HA, Lister LM, Greggains GD, Byerley S, Murdoch AP, Herbert M, Turnbull D (2011) Mitochondrial DNA disease: new options for prevention. Hum Mol Genet 20:R168–R174

Cwerman-Thibault H, Augustin S, Ellouze S, Sahel JA, Corral-Debrinski M (2014) Gene therapy for mitochondrial diseases: Leber hereditary optic neuropathy as the first candidate for a clinical trial. C R Biol 337:193–206

Cwerman-Thibault H, Augustin S, Lechauve C, Ayache J, Ellouze S, Sahel JA, Corral-Debrinski M (2015) Nuclear expression of mitochondrial ND4 leads to the protein assembling in complex I and prevents optic atrophy and visual loss. Mol Ther Methods Clin Dev 2:15003

DiMauro S, Schon EA (2003) Mitochondrial respiratory-chain diseases. N Engl J Med 348:2656–2668

Elliott HR, Samuels DC, Eden JA, Relton CL, Chinnery PF (2008) Pathogenic mitochondrial DNA mutations are common in the general population. Am J Hum Genet 83:254–260

Ellouze S, Augustin S, Bouaita A, Bonnet C, Simonutti M, Forster V, Picaud S, Sahel JA, Corral-Debrinski M (2008) Optimized allotopic expression of the human mitochondrial ND4 prevents blindness in a rat model of mitochondrial dysfunction. Am J Hum Genet 83:373–387

Erickson RP (1972) Leber's optic atrophy, a possible example of maternal inheritance. Am J Hum Genet 24:348–349

Fauser S, Leo-Kottler B, Besch D, Luberichs J (2002a) Confirmation of the 14568 mutation in the mitochondrial ND6 gene as causative in Leber's hereditary optic neuropathy. Ophthalmic Genet 23:191–197

Fauser S, Luberichs J, Besch D, Leo-Kottler B (2002b) Sequence analysis of the complete mitochondrial genome in patients with Leber's hereditary optic neuropathy lacking the three most common pathogenic DNA mutations. Biochem Biophys Res Commun 295:342–347

Figueroa-Martinez F, Vazquez-Acevedo M, Cortes-Hernandez P, Garcia-Trejo JJ, Davidson E, King MP, Gonzalez-Halphen D (2010) What limits the allotopic expression of nucleus-encoded mitochondrial genes? The case of the chimeric Cox3 and Atp6 genes. Mitochondrion 11:147–154

Ghelli A, Porcelli AM, Zanna C, Vidoni S, Mattioli S, Barbieri A, Iommarini L, Pala M, Achilli A, Torroni A, Rugolo M, Carelli V (2009) The background of mitochondrial DNA haplogroup J increases the sensitivity of Leber's hereditary optic neuropathy cells to 2,5-hexanedione toxicity. PLoS One 4:e7922

Giordano C, Montopoli M, Perli E, Orlandi M, Fantin M, Ross-Cisneros FN, Caparrotta L, Martinuzzi A, Ragazzi E, Ghelli A, Sadun AA, d'Amati G, Carelli V (2011) Oestrogens ameliorate mitochondrial dysfunction in Leber's hereditary optic neuropathy. Brain 134:220–234

Giordano C, Iommarini L, Giordano L, Maresca A, Pisano A, Valentino ML, Caporali L, Liguori R, Deceglie S, Roberti M, Fanelli F, Fracasso F, Ross-Cisneros FN, D'Adamo P, Hudson G, Pyle A, Yu-Wai-Man P, Chinnery PF, Zeviani M, Salomao SR, Berezovsky A, Belfort R Jr, Ventura DF, Moraes M, Moraes Filho M, Barboni P, Sadun F, De Negri A, Sadun AA, Tancredi A, Mancini M, d'Amati G, Loguercio Polosa P, Cantatore P, Carelli V (2014) Efficient mitochondrial biogenesis drives incomplete penetrance in Leber's hereditary optic neuropathy. Brain 137:335–353

Giorgio V, Petronilli V, Ghelli A, Carelli V, Rugolo M, Lenaz G, Bernardi P (2011) The effects of idebenone on mitochondrial bioenergetics. Biochim Biophys Acta 1817:363–369

Gropman A, Chen TJ, Perng CL, Krasnewich D, Chernoff E, Tifft C, Wong LJ (2004) Variable clinical manifestation of homoplasmic G14459A mitochondrial DNA mutation. Am J Med Genet A 124A:377–382

Guan MX (2011) Mitochondrial 12S rRNA mutations associated with aminoglycoside ototoxicity. Mitochondrion 11:237–245

Guy J, Qi X, Pallotti F, Schon EA, Manfredi G, Carelli V, Martinuzzi A, Hauswirth WW, Lewin AS (2002) Rescue of a mitochondrial deficiency causing Leber hereditary optic neuropathy. Ann Neurol 52:534–542

Haefeli RH, Erb M, Gemperli AC, Robay D, Courdier Fruh I, Anklin C, Dallmann R, Gueven N (2011) NQO1-dependent redox cycling of idebenone: effects on cellular redox potential and energy levels. PLoS One 6:e17963

Harding AE, Sweeney MG, Govan GG, Riordan-Eva P (1995) Pedigree analysis in Leber hereditary optic neuropathy families with a pathogenic mtDNA mutation. Am J Hum Genet 57:77–86

Heitz FD, Erb M, Anklin C, Robay D, Pernet V, Gueven N (2012) Idebenone protects against retinal damage and loss of vision in a mouse model of Leber's hereditary optic neuropathy. PLoS One 7:e45182

Hirst J (2013) Mitochondrial complex I. Annu Rev Biochem 82:551–575

Hofhaus G, Johns DR, Hurko O, Attardi G, Chomyn A (1996) Respiration and growth defects in transmitochondrial cell lines carrying the 11778 mutation associated with Leber's hereditary optic neuropathy. J Biol Chem 271:13155–13161

Horvath J, Horvath R, Karcagi V, Komoly S, Johns DR (2002) Sequence analysis of Hungarian LHON patients not carrying the common primary mutations. J Inherit Metab Dis 25:323–324

Howell N, Bindoff LA, McCullough DA, Kubacka I, Poulton J, Mackey D, Taylor L, Turnbull DM (1991) Leber hereditary optic neuropathy: identification of the same mitochondrial ND1 mutation in six pedigrees. Am J Hum Genet 49:939–950

Hudson G, Keers S, Yu Wai Man P, Griffiths P, Huoponen K, Savontaus ML, Nikoskelainen E, Zeviani M, Carrara F, Horvath R, Karcagi V, Spruijt L, de Coo IF, Smeets HJ, Chinnery PF (2005) Identification of an X-chromosomal locus and haplotype modulating the phenotype of a mitochondrial DNA disorder. Am J Hum Genet 77:1086–1091

Hudson G, Carelli V, Horvath R, Zeviani M, Smeets HJ, Chinnery PF (2007a) X-Inactivation patterns in females harboring mtDNA mutations that cause Leber hereditary optic neuropathy. Mol Vis 13:2339–2343

Hudson G, Carelli V, Spruijt L, Gerards M, Mowbray C, Achilli A, Pyle A, Elson J, Howell N, La Morgia C, Valentino ML, Huoponen K, Savontaus ML, Nikoskelainen E, Sadun AA, Salomao SR, Belfort R Jr, Griffiths P, Man PY, de Coo RF, Horvath R, Zeviani M, Smeets HJ, Torroni A, Chinnery PF (2007b) Clinical expression of Leber hereditary optic neuropathy is affected by the mitochondrial DNA-haplogroup background. Am J Hum Genet 81:228–233

Huoponen K, Vilkki J, Aula P, Nikoskelainen EK, Savontaus ML (1991) A new mtDNA mutation associated with Leber hereditary optic neuroretinopathy. Am J Hum Genet 48:1147–1153

Huoponen K, Lamminen T, Juvonen V, Aula P, Nikoskelainen E, Savontaus ML (1993) The spectrum of mitochondrial DNA mutations in families with Leber hereditary optic neuroretinopathy. Hum Genet 92:379–384

Hwang JM, Park HW (1996) Carbon monoxide poisoning as an epigenetic factor for Leber's hereditary optic neuropathy. Korean J Ophthalmol 10:122–123

Ishikawa K, Funayama T, Ohde H, Inagaki Y, Mashima Y (2005) Genetic variants of TP53 and EPHX1 in Leber's hereditary optic neuropathy and their relationship to age at onset. Jpn J Ophthalmol 49:121–126

Iyer S, Bergquist K, Young K, Gnaiger E, Rao RR, Bennett JP Jr (2012) Mitochondrial gene therapy improves respiration, biogenesis, and transcription in G11778A Leber's hereditary optic neuropathy and T8993G Leigh's syndrome cells. Hum Gene Ther 23:647–657

Jacobi FK, Leo-Kottler B, Mittelviefhaus K, Zrenner E, Meyer J, Pusch CM, Wissinger B (2001) Segregation patterns and heteroplasmy prevalence in Leber's hereditary optic neuropathy. Invest Ophthalmol Vis Sci 42:1208–1214

Jaros E, Mahad DJ, Hudson G, Birchall D, Sawcer SJ, Griffiths PG, Sunter J, Compston DA, Perry RH, Chinnery PF (2007) Primary spinal cord neurodegeneration in Leber hereditary optic neuropathy. Neurology 69:214–216

Ji Y, Zhang AM, Jia X, Zhang YP, Xiao X, Li S, Guo X, Bandelt HJ, Zhang Q, Yao YG (2008) Mitochondrial DNA haplogroups M7b1′2 and M8a affect clinical expression of Leber hereditary optic neuropathy in Chinese families with the m.11778G>A mutation. Am J Hum Genet 83:760–768

Ji Y, Jia X, Li S, Xiao X, Guo X, Zhang Q (2010) Evaluation of the X-linked modifier loci for Leber hereditary optic neuropathy with the G11778A mutation in Chinese. Mol Vis 16:416–424

Jia X, Li S, Xiao X, Guo X, Zhang Q (2006) Molecular epidemiology of mtDNA mutations in 903 Chinese families suspected with Leber hereditary optic neuropathy. J Hum Genet 51:851–856

Jia X, Li S, Wang P, Guo X, Zhang Q (2010) mtDNA m.3635G>A may be classified as a common primary mutation for Leber hereditary optic neuropathy in the Chinese population. Biochem Biophys Res Commun 403:237–241

Kaewsutthi S, Phasukkijwatana N, Joyjinda Y, Chuenkongkaew W, Kunhapan B, Tun AW, Suktitipat B, Lertrit P (2011) Mitochondrial haplogroup background may influence Southeast Asian G11778A Leber hereditary optic neuropathy. Invest Ophthalmol Vis Sci 52:4742–4748

Kaltimbacher V, Bonnet C, Lecoeuvre G, Forster V, Sahel JA, Corral-Debrinski M (2006) mRNA localization to the mitochondrial surface allows the efficient translocation inside the organelle of a nuclear recoded ATP6 protein. RNA 12:1408–1417

Kerrison JB, Miller NR, Hsu F, Beaty TH, Maumenee IH, Smith KH, Savino PJ, Stone EM, Newman NJ (2000) A case-control study of tobacco and alcohol consumption in Leber hereditary optic neuropathy. Am J Ophthalmol 130:803–812

Khan NA, Govindaraj P, Jyothi V, Meena AK, Thangaraj K (2013) Co-occurrence of m.1555A>G and m.11778G>A mitochondrial DNA mutations in two Indian families with strikingly different clinical penetrance of Leber hereditary optic neuropathy. Mol Vis 19:1282–1289

Kim JY, Hwang JM, Park SS (2002) Mitochondrial DNA C4171A/ND1 is a novel primary causative mutation of Leber's hereditary optic neuropathy with a good prognosis. Ann Neurol 51:630–634

King MP, Attardi G (1989) Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. Science 246:500–503

Kirkman MA, Yu-Wai-Man P, Korsten A, Leonhardt M, Dimitriadis K, De Coo IF, Klopstock T, Chinnery PF (2009) Gene-environment interactions in Leber hereditary optic neuropathy. Brain 132:2317–2326

Klopstock T, Yu-Wai-Man P, Dimitriadis K, Rouleau J, Heck S, Bailie M, Atawan A, Chattopadhyay S, Schubert M, Garip A, Kernt M, Petraki D, Rummey C, Leinonen M, Metz G, Griffiths PG, Meier T, Chinnery PF (2011) A randomized placebo-controlled trial of idebenone in Leber's hereditary optic neuropathy. Brain 134:2677–2686

Klopstock T, Metz G, Yu-Wai-Man P, Buchner B, Gallenmuller C, Bailie M, Nwali N, Griffiths PG, von Livonius B, Reznicek L, Rouleau J, Coppard N, Meier T, Chinnery PF (2013) Persistence of the treatment effect of idebenone in Leber's hereditary optic neuropathy. Brain 136:e230

Kodron A, Krawczynski MR, Tonska K, Bartnik E (2014) m.3635G>A mutation as a cause of Leber hereditary optic neuropathy. J Clin Pathol 67:639–641

Koilkonda RD, Yu H, Chou TH, Feuer WJ, Ruggeri M, Porciatti V, Tse D, Hauswirth WW, Chiodo V, Boye SL, Lewin AS, Neuringer M, Renner L, Guy J (2014) Safety and effects of the vector for the Leber hereditary optic neuropathy gene therapy clinical trial. JAMA Ophthalmol 132:409–420

Kokotas H, Petersen MB, Willems PJ (2007) Mitochondrial deafness. Clin Genet 71:379–391

Kong QP, Bandelt HJ, Sun C, Yao YG, Salas A, Achilli A, Wang CY, Zhong L, Zhu CL, Wu SF, Torroni A, Zhang YP (2006) Updating the East Asian mtDNA phylogeny: a prerequisite for the identification of pathogenic mutations. Hum Mol Genet 15:2076–2086

Kong Q-P, Bandelt H-J, Zhao M, Zhang Y-P, Yao Y-G (2010) Reply to van Oven: suggestions and caveats for naming mtDNA haplogroup. Proc Natl Acad Sci U S A 107:E40–E41

La Morgia C, Achilli A, Iommarini L, Barboni P, Pala M, Olivieri A, Zanna C, Vidoni S, Tonon C, Lodi R, Vetrugno R, Mostacci B, Liguori R, Carroccia R, Montagna P, Rugolo M, Torroni A, Carelli V (2008) Rare mtDNA variants in Leber hereditary optic neuropathy families with recurrence of myoclonus. Neurology 70:762–770

La Morgia C, Carbonelli M, Barboni P, Sadun AA, Carelli V (2014) Medical management of hereditary optic neuropathies. Front Neurol 5:141

Lamb R, Ozsvari B, Lisanti CL, Tanowitz HB, Howell A, Martinez-Outschoorn UE, Sotgia F, Lisanti MP (2015) Antibiotics that target mitochondria effectively eradicate cancer stem cells, across multiple tumor types: treating cancer like an infectious disease. Oncotarget 6:4569–4584

Leber T (1871) Ueber hereditäreund congenital angelegte sehnervenleiden. Graefes Arch Clin Exp Ophthalmol 17:249–291

Lin CS, Sharpley MS, Fan W, Waymire KG, Sadun AA, Carelli V, Ross-Cisneros FN, Baciu P, Sung E, McManus MJ, Pan BX, Gil DW, Macgregor GR, Wallace DC (2012) Mouse mtDNA mutant model of Leber hereditary optic neuropathy. Proc Natl Acad Sci U S A 109:20065–20070

Mackey D, Howell N (1992) A variant of Leber hereditary optic neuropathy characterized by recovery of vision and by an unusual mitochondrial genetic etiology. Am J Hum Genet 51:1218–1228

Mackey DA, Oostra RJ, Rosenberg T, Nikoskelainen E, Bronte-Stewart J, Poulton J, Harding AE, Govan G, Bolhuis PA, Norby S (1996) Primary pathogenic mtDNA mutations in

multigeneration pedigrees with Leber hereditary optic neuropathy. Am J Hum Genet 59:481–485
Macmillan C, Kirkham T, Fu K, Allison V, Andermann E, Chitayat D, Fortier D, Gans M, Hare H, Quercia N, Zackon D, Shoubridge EA (1998) Pedigree analysis of French Canadian families with T14484C Leber's hereditary optic neuropathy. Neurology 50:417–422
Macmillan C, Johns TA, Fu K, Shoubridge EA (2000) Predominance of the T14484C mutation in French-Canadian families with Leber hereditary optic neuropathy is due to a founder effect. Am J Hum Genet 66:332–335
Majander A, Finel M, Savontaus ML, Nikoskelainen E, Wikstrom M (1996) Catalytic activity of complex I in cell lines that possess replacement mutations in the ND genes in Leber's hereditary optic neuropathy. Eur J Biochem 239:201–207
Man PY, Brown DT, Wehnert MS, Zeviani M, Carrara F, Turnbull DM, Chinnery PF (2002a) NDUFA-1 is not a nuclear modifier gene in Leber hereditary optic neuropathy. Neurology 58:1861–1862
Man PY, Turnbull DM, Chinnery PF (2002b) Leber hereditary optic neuropathy. J Med Genet 39:162–169
Man PY, Griffiths PG, Brown DT, Howell N, Turnbull DM, Chinnery PF (2003) The epidemiology of Leber hereditary optic neuropathy in the North East of England. Am J Hum Genet 72:333–339
Manfredi G, Fu J, Ojaimi J, Sadlock JE, Kwong JQ, Guy J, Schon EA (2002) Rescue of a deficiency in ATP synthesis by transfer of MTATP6, a mitochondrial DNA-encoded gene, to the nucleus. Nat Genet 30:394–399
Mashima Y, Kigasawa K, Wakakura M, Oguchi Y (2000) Do idebenone and vitamin therapy shorten the time to achieve visual recovery in Leber hereditary optic neuropathy? J Neuroophthalmol 20:166–170
Melser S, Lavie J, Benard G (2015) Mitochondrial degradation and energy metabolism. Biochim Biophys Acta 1853:2812–2821
Mimaki M, Ikota A, Sato A, Komaki H, Akanuma J, Nonaka I, Goto Y (2003) A double mutation (G11778A and G12192A) in mitochondrial DNA associated with Leber's hereditary optic neuropathy and cardiomyopathy. J Hum Genet 48:47–50
Mordente A, Martorana GE, Minotti G, Giardina B (1998) Antioxidant properties of 2,3-dimethoxy-5-methyl-6-(10-hydroxydecyl)-1,4-benzoquinone (idebenone). Chem Res Toxicol 11:54–63
Nikoskelainen EK, Huoponen K, Juvonen V, Lamminen T, Nummelin K, Savontaus ML (1996) Ophthalmologic findings in Leber hereditary optic neuropathy, with special reference to mtDNA mutations. Ophthalmology 103:504–514
Oca-Cossio J, Kenyon L, Hao H, Moraes CT (2003) Limitations of allotopic expression of mitochondrial genes in mammalian cells. Genetics 165:707–720
Olichon A, Baricault L, Gas N, Guillou E, Valette A, Belenguer P, Lenaers G (2003) Loss of OPA1 perturbates the mitochondrial inner membrane structure and integrity, leading to cytochrome c release and apoptosis. J Biol Chem 278:7743–7746
Oostra RJ, Kemp S, Bolhuis PA, Bleeker-Wagemakers EM (1996) No evidence for 'skewed' inactivation of the X-chromosome as cause of Leber's hereditary optic neuropathy in female carriers. Hum Genet 97:500–505
Pakendorf B, Stoneking M (2005) Mitochondrial DNA and human evolution. Annu Rev Genomics Hum Genet 6:165–183
Paull D, Emmanuele V, Weiss KA, Treff N, Stewart L, Hua H, Zimmer M, Kahler DJ, Goland RS, Noggle SA, Prosser R, Hirano M, Sauer MV, Egli D (2013) Nuclear genome transfer in human oocytes eliminates mitochondrial DNA variants. Nature 493:632–637
Pegoraro E, Carelli V, Zeviani M, Cortelli P, Montagna P, Barboni P, Angelini C, Hoffman EP (1996) X-inactivation patterns in female Leber's hereditary optic neuropathy patients do not support a strong X-linked determinant. Am J Med Genet 61:356–362

Petit C, Levilliers J, Hardelin JP (2001) Molecular genetics of hearing loss. Annu Rev Genet 35:589–646

Petruzzella V, Tessa A, Torraco A, Fattori F, Dotti MT, Bruno C, Cardaioli E, Papa S, Federico A, Santorelli FM (2007) The NDUFB11 gene is not a modifier in Leber hereditary optic neuropathy. Biochem Biophys Res Commun 355:181–187

Phasukkijwatana N, Chuenkongkaew WL, Suphavilai R, Suktitipat B, Pingsuthiwong S, Ruangvaravate N, Atchaneeyasakul LO, Warrasak S, Poonyathalang A, Sura T, Lertrit P (2006) The unique characteristics of Thai Leber hereditary optic neuropathy: analysis of 30 G11778A pedigrees. J Hum Genet 51:298–304

Phasukkijwatana N, Kunhapan B, Stankovich J, Chuenkongkaew WL, Thomson R, Thornton T, Bahlo M, Mushiroda T, Nakamura Y, Mahasirimongkol S, Tun AW, Srisawat C, Limwongse C, Peerapittayamongkol C, Sura T, Suthammarak W, Lertrit P (2010) Genome-wide linkage scan and association study of PARL to the expression of LHON families in Thailand. Hum Genet 128:39–49

Pott JW, Wong KH (2006) Leber's hereditary optic neuropathy and vitamin B12 deficiency. Graefes Arch Clin Exp Ophthalmol 244:1357–1359

Puomila A, Hamalainen P, Kivioja S, Savontaus ML, Koivumaki S, Huoponen K, Nikoskelainen E (2007) Epidemiology and penetrance of Leber hereditary optic neuropathy in Finland. Eur J Hum Genet 15:1079–1089

Reddy P, Ocampo A, Suzuki K, Luo J, Bacman SR, Williams SL, Sugawara A, Okamura D, Tsunekawa Y, Wu J, Lam D, Xiong X, Montserrat N, Esteban CR, Liu GH, Sancho-Martinez I, Manau D, Civico S, Cardellach F, Del Mar O'Callaghan M, Campistol J, Zhao H, Campistol JM, Moraes CT, Izpisua Belmonte JC (2015) Selective elimination of mitochondrial mutations in the germline by genome editing. Cell 161:459–469

Riordan-Eva P, Harding AE (1995) Leber's hereditary optic neuropathy: the clinical relevance of different mitochondrial DNA mutations. J Med Genet 32:81–87

Sadun AA, Carelli V, Salomao SR, Berezovsky A, Quiros P, Sadun F, DeNegri AM, Andrade R, Schein S, Belfort R (2002) A very large Brazilian pedigree with 11778 Leber's hereditary optic neuropathy. Trans Am Ophthalmol Soc 100:169–178

Sanchis-Gomar F, Garcia-Gimenez JL, Gomez-Cabrera MC, Pallardo FV (2014) Mitochondrial biogenesis in health and disease. Molecular and therapeutic approaches. Curr Pharm Des 20:5619–5633

Seedorff T (1985) The inheritance of Leber's disease. A genealogical follow-up study. Acta Ophthalmol (Copenh) 63:135–145

Seo JH, Hwang JM, Park SS (2010) Antituberculosis medication as a possible epigenetic factor of Leber's hereditary optic neuropathy. Clin Experiment Ophthalmol 38:363–366

Shafa Shariat Panahi M, Houshmand M, Tabassi AR (2006) Mitochondrial D-loop variation in Leber hereditary neuropathy patients harboring primary G11778A, G3460A, T14484C mutations: J and W haplogroups as high-risk factors. Arch Med Res 37:1028–1033

Shankar SP, Fingert JH, Carelli V, Valentino ML, King TM, Daiger SP, Salomao SR, Berezovsky A, Belfort R Jr, Braun TA, Sheffield VC, Sadun AA, Stone EM (2008) Evidence for a novel x-linked modifier locus for Leber hereditary optic neuropathy. Ophthalmic Genet 29:17–24

Shoffner JM, Brown MD, Stugard C, Jun AS, Pollock S, Haas RH, Kaufman A, Koontz D, Kim Y, Graham JR et al (1995) Leber's hereditary optic neuropathy plus dystonia is caused by a mitochondrial DNA point mutation. Ann Neurol 38:163–169

Sorajja P, Sweeney MG, Chalmers R, Sachdev B, Syrris P, Hanna M, Wood ND, McKenna WJ, Elliott PM (2003) Cardiac abnormalities in patients with Leber's hereditary optic neuropathy. Heart 89:791–792

Spruijt L, Kolbach DN, de Coo RF, Plomp AS, Bauer NJ, Smeets HJ, de Die-Smulders CE (2006) Influence of mutation type on clinical expression of Leber hereditary optic neuropathy. Am J Ophthalmol 141:676–682

Suissa S, Wang Z, Poole J, Wittkopp S, Feder J, Shutt TE, Wallace DC, Shadel GS, Mishmar D (2009) Ancient mtDNA genetic variants modulate mtDNA transcription and replication. PLoS Genet 5:e1000474

Tachibana M, Sparman M, Sritanaudomchai H, Ma H, Clepper L, Woodward J, Li Y, Ramsey C, Kolotushkina O, Mitalipov S (2009) Mitochondrial gene replacement in primate offspring and embryonic stem cells. Nature 461:367–372

Tachibana M, Amato P, Sparman M, Woodward J, Sanchis DM, Ma H, Gutierrez NM, Tippner-Hedges R, Kang E, Lee HS, Ramsey C, Masterson K, Battaglia D, Lee D, Wu D, Jensen J, Patton P, Gokhale S, Stouffer R, Mitalipov S (2013) Towards germline gene therapy of inherited mitochondrial diseases. Nature 493:627–631

Tonska K, Kurzawa M, Ambroziak AM, Korwin-Rujna M, Szaflik JP, Grabowska E, Szaflik J, Bartnik E (2008) A family with 3460G>A and 11778G>A mutations and haplogroup analysis of Polish Leber hereditary optic neuropathy patients. Mitochondrion 8:383–388

Torroni A, Petrozzi M, D'Urbano L, Sellitto D, Zeviani M, Carrara F, Carducci C, Leuzzi V, Carelli V, Barboni P, De Negri A, Scozzari R (1997) Haplotype and phylogenetic analyses suggest that one European-specific mtDNA background plays a role in the expression of Leber hereditary optic neuropathy by increasing the penetrance of the primary mutations 11778 and 14484. Am J Hum Genet 60:1107–1121

Torroni A, Richards M, Macaulay V, Forster P, Villems R, Norby S, Savontaus ML, Huoponen K, Scozzari R, Bandelt HJ (2000) mtDNA haplogroups and frequency patterns in Europe. Am J Hum Genet 66:1173–1177

Tun AW, Chaiyarit S, Kaewsutthi S, Katanyoo W, Chuenkongkaew W, Kuwano M, Tomonaga T, Peerapittayamongkol C, Thongboonkerd V, Lertrit P (2014) Profiling the mitochondrial proteome of Leber's Hereditary Optic Neuropathy (LHON) in Thailand: down-regulation of bioenergetics and mitochondrial protein quality control pathways in fibroblasts with the 11778G>A mutation. PLoS One 9:e106779

Uittenbogaard M, Chiaramello A (2014) Mitochondrial biogenesis: a therapeutic target for neurodevelopmental disorders and neurodegenerative diseases. Curr Pharm Des 20:5574–5593

Valentino ML, Barboni P, Ghelli A, Bucchi L, Rengo C, Achilli A, Torroni A, Lugaresi A, Lodi R, Barbiroli B, Dotti M, Federico A, Baruzzi A, Carelli V (2004) The ND1 gene of complex I is a mutational hot spot for Leber's hereditary optic neuropathy. Ann Neurol 56:631–641

Valero T (2014) Mitochondrial biogenesis: pharmacological approaches. Curr Pharm Des 20:5507–5509

van Oven M, Kayser M (2009) Updated comprehensive phylogenetic tree of global human mitochondrial DNA variation. Hum Mutat 30:E386–E394

Vega RB, Horton JL, Kelly DP (2015) Maintaining ancient organelles: mitochondrial biogenesis and maturation. Circ Res 116:1820–1834

Vergani L, Martinuzzi A, Carelli V, Cortelli P, Montagna P, Schievano G, Carrozzo R, Angelini C, Lugaresi E (1995) MtDNA mutations associated with Leber's hereditary optic neuropathy: studies on cytoplasmic hybrid (cybrid) cells. Biochem Biophys Res Commun 210:880–888

Vilkki J, Ott J, Savontaus ML, Aula P, Nikoskelainen EK (1991) Optic atrophy in Leber hereditary optic neuroretinopathy is probably determined by an X-chromosomal gene closely linked to DXS7. Am J Hum Genet 48:486–491

Wallace DC, Singh G, Lott MT, Hodge JA, Schurr TG, Lezza AM, Elsas LJ 2nd, Nikoskelainen EK (1988) Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. Science 242:1427–1430

Wang HW, Jia X, Ji Y, Kong QP, Zhang Q, Yao YG, Zhang YP (2008) Strikingly different penetrance of LHON in two Chinese families with primary mutation G11778A is independent of mtDNA haplogroup background and secondary mutation G13708A. Mutat Res 643:48–53

Wang T, Sha H, Ji D, Zhang HL, Chen D, Cao Y, Zhu J (2014) Polar body genome transfer for preventing the transmission of inherited mitochondrial diseases. Cell 157:1591–1604

Webster BR, Scott I, Traba J, Han K, Sack MN (2014) Regulation of autophagy and mitophagy by nutrient availability and acetylation. Biochim Biophys Acta 1841:525–534

Wissinger B, Besch D, Baumann B, Fauser S, Christ-Adler M, Jurklies B, Zrenner E, Leo-Kottler B (1997) Mutation analysis of the ND6 gene in patients with Lebers hereditary optic neuropathy. Biochem Biophys Res Commun 234:511–515

Wong A, Cavelier L, Collins-Schramm HE, Seldin MF, McGrogan M, Savontaus ML, Cortopassi GA (2002) Differentiation-specific effects of LHON mutations introduced into neuronal NT2 cells. Hum Mol Genet 11:431–438

Wu YT, Wu SB, Wei YH (2014) Metabolic reprogramming of human cells in response to oxidative stress: implications in the pathophysiology and therapy of mitochondrial diseases. Curr Pharm Des 20:5510–5526

Yadav A, Agarwal S, Tiwari SK, Chaturvedi RK (2014) Mitochondria: prospective targets for neuroprotection in Parkinson's disease. Curr Pharm Des 20:5558–5573

Yang J, Zhu Y, Tong Y, Chen L, Liu L, Zhang Z, Wang X, Huang D, Qiu W, Zhuang S, Ma X (2009a) Confirmation of the mitochondrial ND1 gene mutation G3635A as a primary LHON mutation. Biochem Biophys Res Commun 386:50–54

Yang J, Zhu Y, Tong Y, Zhang Z, Chen L, Chen S, Cao Z, Liu C, Xu J, Ma X (2009b) The novel G10680A mutation is associated with complete penetrance of the LHON/T14484C family. Mitochondrion 9:273–278

Yao YG, Kajigaya S, Young NS (2015) Mitochondrial DNA mutations in single human blood cells. Mutat Res 779:68–77

Yen MY, Wang AG, Wei YH (2006) Leber's hereditary optic neuropathy: a multifactorial disease. Prog Retin Eye Res 25:381–396

Yu D, Jia X, Zhang AM, Guo X, Zhang YP, Zhang Q, Yao YG (2010a) Molecular characterization of six Chinese families with m.3460G>A and Leber hereditary optic neuropathy. Neurogenetics 11:349–356

Yu D, Jia X, Zhang AM, Li S, Zou Y, Zhang Q, Yao YG (2010b) Mitochondrial DNA sequence variation and haplogroup distribution in Chinese patients with LHON and m.14484T>C. PLoS One 5:e13426

Yu H, Koilkonda RD, Chou TH, Porciatti V, Ozdemir SS, Chiodo V, Boye SL, Boye SE, Hauswirth WW, Lewin AS, Guy J (2012) Gene delivery to mitochondria by targeting modified adenoassociated virus suppresses Leber's hereditary optic neuropathy in a mouse model. Proc Natl Acad Sci U S A 109:E1238–E1247

Yu-Wai-Man P, Griffiths PG, Hudson G, Chinnery PF (2009) Inherited mitochondrial optic neuropathies. J Med Genet 46:145–158

Yu-Wai-Man P, Votruba M, Moore AT, Chinnery PF (2014) Treatment strategies for inherited optic neuropathies: past, present and future. Eye (Lond) 28:521–537

Zamora M, Villena JA (2014) Targeting mitochondrial biogenesis to treat insulin resistance. Curr Pharm Des 20:5527–5557

Zanna C, Ghelli A, Porcelli AM, Carelli V, Martinuzzi A, Rugolo M (2003) Apoptotic cell death of cybrid cells bearing Leber's hereditary optic neuropathy mutations is caspase independent. Ann N Y Acad Sci 1010:213–217

Zhang X, Jones D, Gonzalez-Lima F (2002) Mouse model of optic neuropathy caused by mitochondrial complex I dysfunction. Neurosci Lett 326:97–100

Zhang AM, Jia X, Yao YG, Zhang Q (2008) Co-occurrence of A1555G and G11778A in a Chinese family with high penetrance of Leber's hereditary optic neuropathy. Biochem Biophys Res Commun 376:221–224

Zhang AM, Zou Y, Guo X, Jia X, Zhang Q, Yao YG (2009) Mitochondrial DNA mutation m.3635G>A may be associated with Leber hereditary optic neuropathy in Chinese. Biochem Biophys Res Commun 386:392–395

Zhang AM, Jia X, Zhang Q, Yao YG (2010) No association between the SNPs (rs3749446 and rs1402000) in the PARL gene and LHON in Chinese patients with m.11778G>A. Hum Genet 128:465–468

Zhang AM, Bandelt HJ, Jia X, Zhang W, Li S, Yu D, Wang D, Zhuang XY, Zhang Q, Yao YG (2011a) Is mitochondrial tRNA(phe) variant m.593T>C a synergistically pathogenic mutation in Chinese LHON families with m.11778G>A? PLoS One 6:e26511

Zhang AM, Jia X, Bi R, Salas A, Li S, Xiao X, Wang P, Guo X, Kong QP, Zhang Q, Yao YG (2011b) Mitochondrial DNA haplogroup background affects LHON, but not suspected LHON, in Chinese patients. PLoS One 6:e27750

Zhang AM, Jia X, Guo X, Zhang Q, Yao YG (2012) Mitochondrial DNA mutation m.10680G>A is associated with Leber hereditary optic neuropathy in Chinese patients. J Transl Med 10:43

Zhang J, Jiang P, Jin X, Liu X, Zhang M, Xie S, Gao M, Zhang S, Sun YH, Zhu J, Ji Y, Wei QP, Tong Y, Guan MX (2014) Leber's hereditary optic neuropathy caused by the homoplasmic ND1 m.3635G>A mutation in nine Han Chinese families. Mitochondrion 18:18–26

Zou Y, Jia X, Zhang AM, Wang WZ, Li S, Guo X, Kong QP, Zhang Q, Yao YG (2010) The MT-ND1 and MT-ND5 genes are mutational hotspots for Chinese families with clinical features of LHON but lacking the three primary mutations. Biochem Biophys Res Commun 399:179–185

Leber Hereditary Optic Neuropathy: Exemplar of an mtDNA Disease

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Handbook of Experimental Pharmacology 240, DOI 10.1007/164_2017_2

Abstract

The report in 1988 that Leber Hereditary Optic Neuropathy (LHON) was the product of mitochondrial DNA (mtDNA) mutations provided the first demonstration of the clinical relevance of inherited mtDNA variation. From LHON studies, the medical importance was demonstrated for the mtDNA showing its coding for the most important energy genes, its maternal inheritance, its high mutation rate, its presence in hundreds to thousands of copies per cell, its quantitatively segregation of biallelic genotypes during both mitosis and meiosis, its preferential effect on the most energetic tissues including the eye and brain, its wide range of functional polymorphisms that predispose to common diseases, and its accumulation of mutations within somatic tissues providing the aging clock. These features of mtDNA genetics, in combination with the genetics of the 1–2000 nuclear DNA (nDNA) coded mitochondrial genes, is not only explaining the genetics of LHON but also providing a model for understanding the complexity of many common diseases. With the maturation of LHON biology and genetics, novel animal models for complex disease have been developed and new therapeutic targets and strategies envisioned, both pharmacological and genetic. Multiple somatic gene therapy approaches are being developed for LHON which are applicable to other mtDNA diseases. Moreover, the unique cytoplasmic genetics of the mtDNA has permitted the first successful human germline gene therapy via spindle nDNA transfer from mtDNA mutant oocytes to enucleated normal mtDNA oocytes. Such LHON lessons are actively being applied to common ophthalmological diseases like glaucoma and neurological diseases like Parkinsonism.

Keywords

Animal models • Estrogen receptor beta • Gene therapy • Haplogroup • Leber's hereditary optic neuropathy • LHON • Mitochondrial disease • Mitochondrial DNA • Mitochondrial physiology • mtDNA copy number • Oxidative stress • Retinal ganglion cells • Transmitochondrial cybrids

1 Leber Hereditary Optic Neuropathy (LHON), Window into Rare and Common Diseases

Leber Hereditary Optic Neuropathy (LHON) was the first disease reported to be caused by a maternally inherited mitochondrial DNA (mtDNA) mutation (Wallace et al. 1988a). Since publication of the first LHON report in 1988, hundreds of mtDNA missense and protein synthesis base substitution mutations have been reported in the mtDNA associated with a plethora of clinical manifestations (MITOMAP 2017). Yet even now, a quarter of a century later, there are still mysteries about the pathobiology of LHON.

Affected LHON patients develop a mid-life, acute-onset, central-vision loss resulting in central scotoma due to the death of the retinal ganglion cells. Generally, all maternal relatives have the same mtDNA genotype, yet only some maternal

relatives go blind, with males being about four times more likely to go blind than females (Carelli et al. 2004; Yu-Wai-Man 2015; Yu-Wai-Man and Chinnery 1993).

Like LHON, delayed onset and variable expressivity are characteristics of a range of common metabolic and degenerative diseases, and a male bias is characteristic of other neuropsychiatric disorders including autism (Developmental Disabilities Monitoring Network Surveillance Year 2010 Principal Investigators, Centers for Disease Control and Prevention (CDC) 2014) and Parkinson disease (Nicoletti et al. 2016). Moreover, mild mitochondrial dysfunction frequently affects retinal function, with glaucoma affecting the retinal ganglion cells (Lopez Sanchez et al. 2016; Chrysostomou et al. 2013) and retinitis pigmentosa and age-related macular degeneration affecting the retinal pigment epithelial cells (Ortiz et al. 1993; Udar et al. 2009). Consequently, the methodical elucidation of the factors leading to mtDNA mutation-induced blindness is providing insight into the etiology of a variety of common ophthalmological and neurological diseases.

The mitochondrial genome consists of 1–2,000 nuclear DNA (nDNA) genes plus hundreds to thousands of copies of the maternally inherited mtDNA per cell. The mtDNA codes for the 13 most important polypeptides of the mitochondrial energy generating process, oxidative phosphorylation (OXPHOS), plus the 12S and 16S rRNA and 22 tRNAs required for mitochondrial protein synthesis to translate these mRNAs. The additional approximately 80 OXPHOS polypeptides are coded in the nucleus. Thus complex I (NADH:ubiquinone oxidoreductase) is composed of ~45 polypeptides, 7 (*ND1, 2, 3, 4, 4L, 5, 6*) coded by the mtDNA; complex II (succinate: ubiquinone oxidoreductase) is composed of four nDNA coded subunits; complex III (bc1 complex) is composed of 11 polypeptides, cytochrome b coded by the mtDNA; complex IV (cytochrome c oxidase) is composed of 13 polypeptides, three (*COI-III*) coded in the mtDNA; and complex V (ATP synthase) is composed of 18 polypeptides, two (*ATP6&8*) coded in the mtDNA. All of the remaining mitochondrial polypeptides are coded in the nucleus.

In Europeans pathogenic mtDNA disease mutations have an estimated frequency of greater than one in 5000 (Schaefer et al. 2008; Chinnery et al. 2000; Cree et al. 2009). Furthermore, about 1 in 200 cord bloods have been observed to harbor one of the ten most common mtDNA mutations implying a high de novo mtDNA mutation rate (Elliott et al. 2008). Numerous mutations in nDNA coded mitochondrial genes have also been associated with disease (Koopman et al. 2012). One of these is autosomal dominant optic atrophy (DOA) caused my mutations in the *OPA1* gene (Alexander et al. 2000; Delettre et al. 2000). DOA has clinical manifestations similar to those of LHON, though with an earlier onset and progressive course, demonstrating that mutations in either mtDNA or nDNA coded mitochondrial genes can produce similar symptoms if they perturb the same mitochondrial physiological parameters.

The existence of "mitochondrial diseases" caused by high impact single mitochondrial gene mutations is now well established. These have been designated "primary mitochondrial diseases." However, there is rapidly growing evidence that many of the common "complex" diseases have an underlying mitochondrial etiology (Picard et al. 2016). Females harboring LHON mtDNA mutations have an increased predilection to a multiple sclerosis-like syndrome (Harding et al. 1992;

Palace 2009; Pfeffer et al. 2013; Matthews et al. 2015) as do *OPA1* mutation patients (Yu-Wai-Man et al. 2016). Individuals harboring the more deleterious mtDNA LHON mutations can present with other neurological diseases such as generalized dystonia at higher heteroplasmy levels (Jun et al. 1994a, b). Finally, LHON and DOA share the clinical and cellular pathological features of Primary Open Angle Glaucoma (POAG) (Lopez Sanchez et al. 2016).

In addition to the production of ATP by the process of oxidative phosphorylation (OXPHOS), the mitochondria regulate a broad range of cellular processes. These include production of reactive oxygen species (ROS) and regulation of cellular oxidation-reduction (REDOX) status, uptake of cytosolic Ca^{++} thus regulating cellular Ca^{++} concentrations, the control of cell death (apoptosis) by activation of the mitochondrial permeability transition pore (mtPTP), and the production of high energy intermediates that modulate cytosolic signal transduction and nuclear epigenomic pathways. Hence, mitochondrial function is intimately interdigitated with essentially every cellular process and thus has sweeping implications for the cellular pathology (Picard et al. 2014; Wallace and Fan 2010; Wallace et al. 2010; Wallace 2013a).

Given the prevalence and importance of mitochondrial dysfunction to a wide range of common diseases (Wallace 2013a), it is surprising how little is known about the mitochondrion and its role in human health. Even more problematic is that very limited attention has been paid to developing mitochondrial therapeutic interventions. This lack of attention to the mitochondrion may be an important factor in our limited capacity to generate effective therapies for the common metabolic and degenerative diseases (Wallace 2013b). To begin rectifying these limitations, we need to develop an in-depth understanding of this representative "primary" mitochondrial disease, LHON (Carelli et al. 2004).

2 LHON Genetics and Biology

The incidence of LHON is estimated at >1 in 7000 (Sadun et al. 2011). Most European LHON mutations occur in the mtDNA OXPHOS complex I (NADH: ubiquinone oxidoreductase or NADH dehydrogenase) genes. The three most common LHON mutations are the missense mutations in the *ND4* gene at nucleotide (nt) 11778G>A (R340H) (Wallace et al. 1988a), the *ND1* gene at nt 3460G>A (A52T) (Huoponen et al. 1991), and the *ND6* gene at nt 14484T>C (M64V) (Johns et al. 1992) (Table 1). Milder LHON mutations are generally homoplasmic (all cellular mtDNAs contain the mutation). More severe complex I mutations cause optic atrophy when heteroplasmic (a mixture of mutant and normal mtDNAs), but when homoplasmic, result in more severe phenotypes including basal ganglia degeneration, dystonia, and Leigh Syndrome. The *ND6* G14459A (A72V) (Jun et al. 1994a) and *ND6* G14600A (P25L) (Malfatti et al. 2007) mutations are examples of the latter mutations (Table 1).

LHON presents as unilateral acute or subacute optic atrophy in the second or third decade of life, followed within a year of optic atrophy in the contralateral eye. The visual impairment is associated with a central scotoma and pseudopapilledema of the optic disc with retinal nerve fiber loss. Within the optic nerve the central

Table 1 Top Leber hereditary optic neuropathy (LHON) disease mutations from www.mitomap.org

Mutation	Nt Δ	AA Δ	AA Cons [a]	% Patients	Het.[b]	Penetrance[c] % relatives	Penetrance[c] % males	% Recovery[d]
MTND4*LHON11778A	**G-A**	**R340H**	**H**	**69**	+/−	**33–60**	**82**	**4**
MTND1*LHON3460A	**G-A**	**A52T**	**M**	**13**	+/−	**14–75**	**40–80**	**22**
MTND6*LHON14484C	**T-C**	**M64V**	**L**	**14**	+/−	**27–80**	**68**	**37–65**
MTND1*LHON-MELAS3376A	G-A	E24K	H	Rare	+/−	NA	NA	NA
MTND1*LHON3635A	G-A	S110N	H	Rare	+/−	29 (range 11–64)	54 (range 25–100)	Low
MTND1*LDYT3697A	G-A	G131S	H	Rare	+/−	NA	NA	NA
MTND1*LHON3700A	G-A	A112T	H	Rare	−	NA	NA	UN
MTND1*LHON3733A	G-A	E143K	H	Rare	+/−	24–30	36–44	Yes
MTND1*LHON4171A	C-A	L289M	H	Rare	+/−	46	47	Yes
MTND3*LDYT10197A	G-A	A47T	H	Rare	+/−	NA	NA	NA
MTND4L*LHON10663C	T-C	V65A	L	Rare	+/−	56	60	UN
MTND5*LHON13051A	G-A	G239S	H	Rare	−	56	63	UN
MTND6*LDYT14459A	G-A	A72V	M	Rare	+	NA	NA	Low
MTND6*LHON14482A		M64I	L	Rare	+/−	NA	89	Yes

(continued)

Table 1 (continued)

Mutation	Nt Δ	AA Δ	AA Cons [a]	% Patients	Het.[b]	Penetrance[c] % relatives	Penetrance[c] % males	% Recovery[d]
	C-A							
MTND6*LHON14482G	C-G	M64I	L	Rare	–	NA	NA	UN
MTND6*LHON14495G	A-G	L60S	H	Rare	+	NA	NA	Low
MTND6*LHON14502C	T-C	I58V	H	Rare	–	14502:10% 14502 + 11778:37%	14502:11% 14502 + 11778:47%	UN
MTND6*LHON14568T	C-T	G36S	M	Rare	–	NA	NA	UN

Top 18″ primary LHON mutations

The first three mutations listed (in boldface) represent approximately 95% of all cases. The remaining mutations are listed in nucleotide order

[a]*H* high amino acid conservation, *M* moderate *L* low, *NA* not applicable, *Ter* termination codon

[b]*Het*. Heteroplasmy, + = detected, − = not detected

[c]*NA* not applicable, *UN* unknown; penetrance values are rough estimates

[d]Low = anecdotal low degree of vision recovery; Yes = anecdotal moderate to high degree of vision recovery; *UN* unknown, *NA* not applicable

small caliber fibers of the papillomacular bundle are preferentially affected while the larger caliber peripheral fibers are preserved. The preferential involvement of the optic nerve has been attributed to the high energy demand placed on the unmyelinated portion of the optic nerve fibers anterior to the lamina cribrosa, an area rich in mitochondria (Carelli et al. 2004; Yu-Wai-Man 2015; Yu-Wai-Man and Chinnery 1993; Lopez Sanchez et al. 2016; Sadun et al. 2011).

For homoplasmic LHON mutations, the penetrance of acute onset central scotoma is remarkably variable with males being 2–6 times more likely to go blind than females, and with the percentage of central scotoma patients varying markedly between pedigrees (Sadun et al. 2011). Maternal relatives that have not progressed to subacute optic atrophy still show signs of visual impairment including dyschromatopsia, which implies cone dysfunction, alterations in electroretinographic (ERG) responses (Salomao et al. 2004; Shibata et al. 1999), and retinal nerve fiber layer thickening as detected by optical coherence tomography (OCT) (Ramos et al. 2009; Ventura et al. 2007). Many outstanding questions regarding LHON remain. How does a systemic mtDNA defect only affect the visual system? What is responsible for the delayed onset and variable penetrance of the blindness? Why are males preferentially affected? Most importantly how can we treat or prevent LHON? (Yu-Wai-Man 2015; Yu-Wai-Man and Chinnery 1993; Sadun et al. 2006, 2011; Ventura et al. 2007).

Both common (*ND4* 11778A, *ND1* 3460A, *ND6* 14484C) and rare (*ND6* 14459A, *ND4L* 10663C (V65A) (Brown et al. 2002), *ND1* at 3394C (Y30H) (Brown et al. 1995; Puomila et al. 2007; Zhang et al. 2010; Liang et al. 2009) complex I mutations have been associated with LHON (Table 1). Numerous other rare mtDNA mutations have also been reported affecting virtually every mtDNA gene (Wallace et al. 2007; Koilkonda and Guy 2011; Ji et al. 2016). These are reported in MITOMAP (MITOMAP 2017).

The transmitochondrial cybrid technique (Bunn et al. 1974; Wallace et al. 1975; Wallace 1981, 1982a; Trounce et al. 1996) has been used to confirm that the mtDNA variants associated with LHON cause the mitochondrial and cellular defects associated with the disease. LHON mtDNA mutations can be transferred into established human cell lines via fusion of patient blood platelets or cytoplasts derived from patient fibroblasts or lymphoblasts to established human cells which lack mtDNA (ρ^0 cells) (King and Attardi 1989). Three human ρ^0 cell lines have been used in these cybrid studies: the lymphoblastoid cell line WAL2A (Brown et al. 2000), the osteosarcoma cell line 143B(TK^-) (Vergani et al. 1995; Danielson et al. 2002), and the neuroblastoma Ntera2/D1 which can be differentiated in vitro into neurons with retinoic acid (Wong et al. 2002). These transmitochondrial cybrids have been analyzed for biochemical effects associated with the mtDNA mutations (Trounce et al. 1996) and have revealed that the common LHON mutations cause partial complex I and site I respiration defects, reduce ATP production, altered excitatory glutamate transport, increase mitochondrial reactive oxygen species (ROS) production, and sensitize the mitochondrial permeability transition pore (mtPTP) to predispose to apoptosis (Jun et al. 1994a; Brown et al. 2000, 2001; Danielson et al. 2002; Carelli et al. 1997, 1999; Baracca et al. 2005; Beretta et al. 2004, 2006; Floreani et al. 2005; Ghelli et al. 2008; Sala et al. 2008).

In neurons prepared by induced differentiation of Ntera2D1 11778 or 3460 cybrids, the mtDNA/nDNA ratio declined and the mitochondrial ROS production increased markedly, indicating that elevated mitochondrial ROS production may be an important feature of the neuronal pathophysiology of LHON (Wong et al. 2002).

3 LHON Penetrance and mtDNA Background

The penetrance of the milder LHON mutations (11778A, 14484C, 10366C, etc.) is markedly affected by the mtDNA background, or haplogroup. Because the mtDNA is exclusively maternally inherited, it can only change by the accumulation of sequential mutations along radiating maternal lineages. The establishment of a new mtDNA mutation creates a branch in the mtDNA mutational tree. Hence, all extant mtDNA variation can be traced back to a single mtDNA which arose in Africa about 150,000–200,000 years before present (YBP) (Cann et al. 1987; Johnson et al. 1983; Merriwether et al. 1991). From that origin, mtDNA sequences radiated in Africa until approximately 65,000 YBP generating the African "L" mtDNA lineages. At that point, two mtDNAs (M and N) succeeded in leaving Africa and colonizing the rest of the world. Derivatives of N radiated in Europe giving rise to haplogroups H, I, J, K(Uk), T, U, V, W, and X while both M and N colonized and radiated in Asian generating a plethora of haplogroups. Of all of the Eurasian mtDNA lineages, only haplogroups A, B, C, D, and X colonized the Americas starting about 20,000 YBP (Brown et al. 1998; Wallace 2015).

As women colonized the globe, occasional mutations arose that were beneficial, subtly altering cellular mitochondrial metabolism in ways that were optimal in the local environment. These functional mutations conferred an advantage to the local population permitting the descendants to proliferate resulting in regional groups of related mtDNA haplotypes, termed haplogroups. These haplogroups can be beneficial in the environment in which they arose, but may be maladaptive if placed in a different environment. Thus, mtDNA haplogroups can predispose to common diseases and also alter the expression of the milder LHON mutations which arise on that mtDNA background (Wallace 2015).

Evidence that mtDNA haplogroups can modulate the expressivity of primary mtDNA disease mutations was first documented in LHON. LHON mutations 11778, 14484, and 10663 that arise on European mtDNA haplogroup J have a significantly increased penetrance then when the same mutation arises on haplogroup H mtDNA (Brown et al. 1995, 1997, 2002; Torroni et al. 1997), and cybrids harboring the 11778A (340H) mutation on the J background have lower respiration rates than 11778A haplogroup H cybrids (Vergani et al. 1995). The largest LHON *ND4* nt 11778G>A (R340H) pedigree which connects patients between Italy and Brazil arose on the haplogroup J background (Sadun et al. 2003).

European haplogroup J is a derivative of macrohaplogroup N, which encompasses the *ND3* 10398A>G (A114T) and *ATP6* 8701A>G (A59T) polypeptide variants. J is defined by the reversion of the *ND3* variant 10398G>A (T114A) plus the acquisition of a new variant, *ND5* 13708G>A (A458T). J encompasses two major subclades, J1c and J2b, which harbor cytochrome b mutations at nucleotides

14798T>C (F18L) and 15257G>A (D171N). These J subclades increase the penetrance of the milder LHON mutations (Mishmar et al. 2006; Ruiz-Pesini and Wallace 2006; Kazuno et al. 2006; Gomez-Duran et al. 2010; Carelli et al. 2006).

The contextual importance for mtDNA variant phenotypes is demonstrated by the *ND1* at 3394C (Y30H) variant. *ND1* Y30H is associated with LHON when it occurs on macrohaplogroup N. However, this same variant is nonpathogenic and even enriched in high altitude Tibetans when it occurs on macrohaplogroup M mtDNAs, especially M9. On the N background the 3394C variant reduces complex I activity, yet on the M9 background it is associated with the highest complex I activity observed (Ji et al. 2012). Hence, mtDNA and presumably nDNA-coded mitochondrial gene variants (Strauss et al. 2013) can have marked differential effects on the expressivity of LHON mtDNA variants.

4 LHON Penetrance and X-Linked Chromosomal Modifier Genes

Since mtDNA background is established as a modulator of primary LHON mutation expressivity, it follows that nDNA variants in mitochondrial genes might have a similar effect. For example, polymorphisms in an OXPHOS X-chromosomal gene might account for the increased penetrance of LHON in males. Reported associations have included the DXS7 marker on Xp (Vilkki et al. 1991), a locus at Xp21.1-q21.2 (Hudson et al. 2005), and a locus at Xq25-27.2 in the large Brazilian 11778A plus J pedigree (Shankar et al. 2008). The Xp21.1-q21.2 association has been confirmed in a Chinese LHON study (Ji et al. 2010), but these associations remain controversial (Chalmers et al. 1996; Handoko et al. 1998; Sweeney et al. 1992; Petruzzella et al. 2007; Qu et al. 2010). Autosomal nDNA loci have also been associated with LHON penetrance (Phasukkijwatana et al. 2010; Ishikawa et al. 2005).

5 Estrogen Modulation of Mitochondrial Function and the Male Bias in Expressivity

Another explanation for the male bias in LHON could be that the female hormone, estrogen, may be protective of mitochondrial dysfunction through increasing mitochondrial antioxidant defenses. It has been shown that about 20% of the estrogen receptor β (ERβ) is located in the mitochondrial matrix, and treatment of cells with estradiol doubles the mitochondrial Mn superoxide dismutase (MnSOD) activity within 60 min, thus decreasing mitochondrial ROS production. Mitochondrial ROS activates c-jun N-terminal kinase (JNK) and protein kinase Cδ which activates BAX to translocate to the mitochondrion where it stimulates the release of cytochrome c and activates the intrinsic pathway of apoptosis. By inducing MnSOD, estradiol removes mitochondrial ROS and blocks this pathway (Pedram et al. 2006). Pre-menopausal women are thus partially protected from chronic mitochondrial oxidative stress by the cyclic induction of estrogen.

ERβ has been shown to be localized within the mitochondria of retinal ganglion cells (RGCs). In studies of cybrids harboring the *ND4* nt 11778G>A mutation, phytoestrogens have been found to strongly reduce mitochondrial ROS production, induce mitochondrial biogenesis, and reduce predilection to apoptosis. The combination of phytoestrogens, genistein + daidzein + equol (G + D + E), preferentially bind to ERβ but not ERα thus activating mitochondrial function without activating pathways leading to feminization. While LHON cybrids grow poorly in galactose and have an increased predilection to apoptosis, G + D + E renders the LHON cybrid growth comparable to controls. Physiologically, G + D + E increases the oxygen consumption rate of the LHON cybrids to that of normal controls and elevates the oxygen consumption of the controls above the untreated level. Finally, the elevated LHON cybrid ROS production is reduced to normal by G + D + E in association with the up-regulation SIRT1, PGC-1α, NRF1, and Tfam (Pisano et al. 2015).

6 Environmental Modifiers of LHON

Environmental factors that impair mitochondrial function also increase the penetrance of primary LHON mutations. Smoking and excessive alcohol consumption, which create oxidative stress, increase the penetrance of the common LHON mutations (Sadun et al. 2011; Amaral-Fernandes et al. 2011; Giordano et al. 2015; Carelli et al. 2016). Similarly, the antibiotics erythromycin and ethambutol, a treatment for tuberculosis, have been reported to precipitate optic atrophy in patents harboring the *ND4* 11778A>G mutation (Yu-Wai-Man et al. 2011).

7 Mitochondrial Biogenesis and mtDNA Copy Number as Modifiers of LHON Mutant Expressivity

The fact that many individuals that are homoplasmic for a primary mtDNA LHON mutation do not go blind has led to a nomenclature in which individuals with an LHON mutation that go blind being designated "affected" while those with the mutation that do not go blind designated "carriers." But what is the difference?

Analysis of the mtDNA copy number in white blood cells of individuals harboring mtDNA LHON mutations who are affected or are carriers has revealed that carriers have a higher mtDNA copy number and mitochondrial quantity than affected individuals. This trend has been confirmed in skeletal muscle and retinal ganglion cells. Affected individuals have higher mtDNA copy numbers than controls, suggesting that the partial mitochondrial defect caused by the LHON mutations induces a compensatory induction of mitochondrial biogenesis, with carriers able to mount a more robust compensatory response than affected individuals. The physiological significance of this has been confirmed by growth of subject fibroblasts in galactose. Controls grew best, affected grew poorly, but carriers grew in galactose only slightly less well than controls. These data indicated that carriers must have additional genetic factors, other than mtDNA variants, that

permit them to more robustly compensate for the LHON mtDNA genetic defect (Giordano et al. 2014).

LHON patients that smoke have been found to have decreased mtDNA copy numbers. Exposure of cells to cigarette condensate reduced the mtDNA copy number in cells from affecteds and carriers of LHON mutations, but also controls. Cigarette condensate also reduced the specific activity of complexes I and IV, increased carbonylated proteins, and induced antioxidant defenses including MnSOD. Presumably in LHON carriers, heavy smoking may offset the beneficial effects of higher mtDNA copy, resulting in late-onset blindness (Giordano et al. 2015; Carelli et al. 2016).

8 Animal Models of LHON

8.1 Tissue Models of LHON

Mammalian models of mtDNA LHON are essential if we are to fully elucidate the genetics and pathophysiology of LHON. Only mammals can permit determination of how systemic mitochondrial defects cause tissue-specific disease and thus can provide meaningful preclinical models for therapeutic testing.

An LHON-like phenotype can be induced in rodents by respiratory poisons. The most common approach is chronic treatment with the complex I inhibitor rotenone (Mansergh et al. 2014; Marella et al. 2010). However, these models do not permit investigation of the complex genetic interactions that lead to the variable penetrance of blindness.

LHON-like phenotypes can also be produced by transient genetic modifications. Import into retinal cells of siRNAs that inhibit the translation of the nDNA coded mitochondrial genes for the complex I polypeptide, NDUFA1 (Qi et al. 2003a), or the mitochondrial antioxidant polypeptide, MnSOD (Qi et al. 2003b) have been used.

A common transient genetic approach to produce an LHON-like phenotype has been to import a mutant mitochondrial polypeptide into the mitochondrion via "allotropic" expression of the mutant mtDNA gene within the nucleus. The most common case involves the mutant mtDNA *ND4* nt 11778A (codon 340 H) gene. The mtDNA mutant gene is cloned, the mtDNA genetic code altered to be compatible with the nDNA genetic code, the recoded mtDNA gene is fused to a mitochondrial targeting peptide, and the modified gene combined with the appropriate nDNA promoter-enhancer and poly-A addition signals. This gene cassette is then inserted into an Adeno-Associated Vector (AAV) and the AAV-borne allotropic gene construct used to transduce cultured cells or retinal ganglion cells. Once in the nucleus, the allotropic *ND4* gene is transcribed, the mRNA translated in the cytosol, and the mutant polypeptide imported into the mitochondrion. Allotopically expressed *ND4* mutant protein has been reported to be incorporated into complex I to generate the LHON defect and LHON-like phenotype (Koilkonda and Guy 2011; Cwerman-Thibault et al. 2011).

8.2 Germline Models of LHON

While the somatic tissue models of LHON are useful for investigating somatic gene therapy approaches, investigation of the pathophysiology of LHON mutations requires mouse germline mutations. One novel approach has been to introduce a plasmid bearing the mutant mtDNA gene into the mitochondrion. AAV has been produced with a mitochondrial targeting peptide attached to the capsid core protein. The AAV capsid was used to package a human mtDNA *ND4* gene bearing the common nt 11778A 340H LHON mutation integrated into a plasmid containing the mtDNA H-strand promoter. This construct was cloned into a self-complementary AAV (scAAV) backbone and the AAV construct injected into fertilized oocytes. The resulting animals harbored the human mtDNA *ND4* mutant gene in a plasmid within the mitochondrion. The human *ND4* mutant gene was present at 20% of the normal mouse *ND4* gene. These mice developed an optic atrophy comparable to that of LHON, and the phenotype could be ameliorated by allotopically expressed normal human *ND4* protein (Yu et al. 2015).

While both the tissue-specific models and the mitochondrial plasmid model introduce the mutant mtDNA polypeptide into the mitochondrion, in all cases the resulting cell or animal harbors a mixture of introduced mutant and endogenous normal mtDNA genes and proteins. Yet most LHON families are homoplasmic mutant and heteroplasmy has been found to be strongly protective of the onset of optic atrophy. Since the endogenous normal gene and protein would likely be in excess, it seems possible that the ophthalmological pathology resulting from mixtures of mutant and normal proteins may be the result of some factor other than the mutant *ND4* protein. One possibility might be the perturbation of the normal stoichiometry of assemble of the polypeptides of complex I.

8.3 The mtDNA ND6 nt 13997G>A P25A Mutant Mouse

To generate a homoplasmic LHON mtDNA mutant mouse, an alternative approach is required. One such mouse has been generated which harbors the same *ND6* gene missense mutation as found in the family in which heteroplasmic individuals presented with optic atrophy and cerebellar ataxia while homoplasmic individuals died of multisystem diseases including a Leigh Syndrome presentation (Malfatti et al. 2007). Because mice live only 2 years and must develop the phenotype within approximately 1 year, while people live 80 years and develop the phenotypes in about 25 years, it follows that mice may need to harbor more severe mutations to get the same neurological phenotype as humans. Consequently, creating a mouse harboring the *ND6* P25L mutation seemed likely to generate a credible model of LHON.

The mouse mutation equivalent of the human the *ND6* 14600G>A (P25L) mutation is the *ND6* 13997G>A (P25L). To generate the *ND6* G13997A P25L missense mutation, cultured LM(TK$^-$) mouse cells were mutagenized with trimethyl psoralen and UV mutagenesis, mutants enriched by ethidium bromide

depletion-re-amplification, and partially respiratory deficient cells identified by impaired growth on galactose (Acin-Perez et al. 2004). The mtDNAs of the resulting mutant clones were sequenced and one clone was identified, LT13, that was homoplasmic for the ND6 gene mutation at nt 13997A (Lin et al. 2012). Biochemical characterization of the mutant cell line revealed that it has a 23% reduction in complex I, a 24% reduction in NADH-linked mitochondrial respiration but normal succinate respiration, a 65% reduction in ATP synthesis rate, and a 48% increase in mitochondrial ROS production. Hence, this mutation had the biochemical profile consistent with legitimate LHON mutations.

This mutation was transferred into the mouse germline using the female embryonic stem cell line (CC9.3.1) (MacGregor et al. 2006). The resident mtDNAs of CC9.3.1 cells were removed with rhodamine 6G and replaced with the LT13 cell line *ND6* 13997A 25L mutant mtDNAs by fusion to the CC9.3.1 cells with enucleated LT13 cell cytoplasts. The resulting pluripotent stem cell cybrids were then injected into blastocysts, the blastocysts introduced into a foster mother, and chimeric female progeny bred to transmit the mutant mtDNA through the maternal lineage. This process resulted in homoplasmic ND6 13997A 25L mutant mice that are viable and fertile and have been backcrossed onto the C57BL/6J background. Characterization of the mtDNA ND6 13997A 25L mutant mouse revealed that it exhibits all of the features of LHON possible given the anatomical differences between the eyes of mice and men.

Reduced Retinal Response in ND6 P25L Mice Electroretinogram (ERG) analysis of 14-month-old *ND6* 13997A 25L mice revealed a 17–26% reduction in the amplitudes of all ERG parameters tested including oscillatory potentials (OP) which interrogate multiple retinal cell types. Yet the mice remain responsive to light by optokinetic analysis. The overall retinal function of these mice also declined further by 23–24 months. Such partial retinal deficiencies including reduced ERG cone responses and altered color vision are seen in both LHON asymptomatic carriers and LHON patients (Salomao et al. 2004; Shibata et al. 1999).

RGC Axonal Swelling and Preferential Loss of Smallest Fibers in the ND6 P25L Mice Transmission electron microscopic analysis of RGC axons revealed that the *ND6* P25L eyes exhibited axonal swelling in both 14- and 24-month mutant optic nerves (Fig. 1a, b) associated with demyelination and proliferation of abnormal mitochondria (Fig. 1c).

The average axonal diameter was 0.67 ± 0.01 μm in wild-type and 0.80 ± 0.04 μm ($P < 0.0001$) in *ND6* 25L mutant 14-month-old mice and 0.73 ± 0.01 μm in wild-type and 0.85 ± 0.05 μm in *ND6* 25L mutant mice at 24 month ($P < 0.0001$) (Fig. 1d). Moreover, the distribution of the axonal diameters revealed an increased number of large fibers and a marked loss of small axonal fibers (≤0.5 μm) in 14-month-old *ND6* mutant, and this was more pronounced in 24-months (Fig. 1e). Thus there were fewer small and medium axons (≤0.8 μm) and more swollen axonal fibers with diameter larger than 1 μm in the *ND6* 25L mice

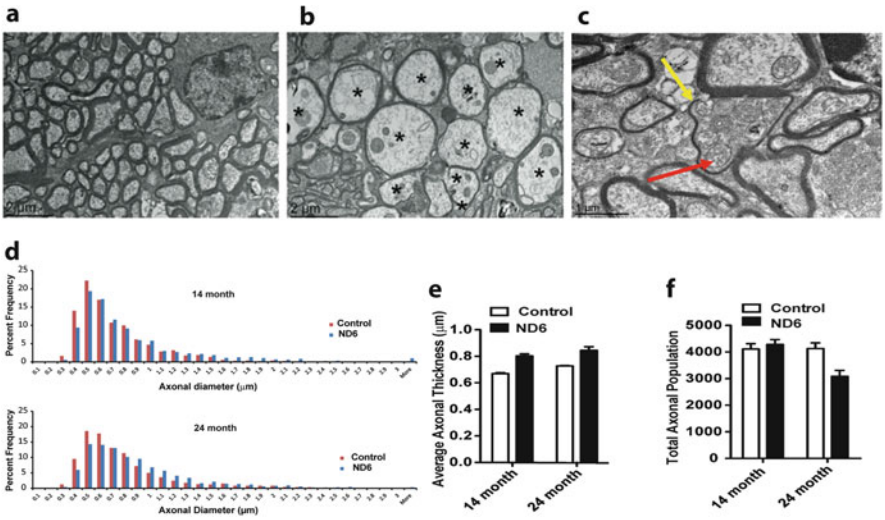

Fig. 1 RGC axonal swelling and loss examined by electron microscopy. Plastic embedded thin sections of cross-sectional profiles of 14 month (**a**) wild-type and (**b**) 14 month *ND6* mutant mouse retrobulbar optic nerves at 2000× magnification. Several swollen axons with thin myelin (*asterisks*) could be seen adjacent to normal caliber axons (*lower left*) from a 14-month *ND6* mutant mouse. (**c**) Sections showing demyelination (*yellow arrow*), abnormal mitochondria (*red arrow*) and mitochondrial proliferation. (**d**) The average axonal diameter in the 14-month and 24-month control and ND6 mutant mice ($n = 4$). (**e**) Histograms of percent distribution of optic nerve axon diameters in both 14- and 24-month wild-type and *ND6* mutant mice (bin size = 0.1 μm). The distribution of axonal diameters in the 14-month age group showed a shift in the *ND6* mutant subset to larger diameter axons, while those smaller than 0.5 μm in diameter were reduced in number. This trend was augmented in 24-month age group as the shift drifted further towards large axons to 0.8 μm. (**f**) Reduction of axonal number in 24-month *ND6* P25L mutant optic nerves

(Fig. 1e) and these adverse effects were most apparent in the region of the smallest fibers at the central and temporal regions of the mouse optic nerve.

The number of axons in the optic nerves regardless of axonal diameters also declined 30% by 24 months ($P < 0.0001$) (Fig. 1f). Thus, the observed shift towards larger axons is initially (14 months) due to swelling of medium axons and later (24 months) the loss of small axons. Examination of the spatial distribution of axons in a cross-sectional view of the optic nerve of the mutant mice revealed that axonal changes were primarily central and eccentric toward the temporal region, corresponding to the human temporal region characteristically affected in LHON pathology.

The optic tract axons of 14-month-old *ND6* 25L mice had a 58% increase in mitochondria ($P = 0.0036$) while those at 24 months had a 94% increase ($P = 0.064$) with 31.5% more of the *ND6* 25L mitochondria being abnormal at 14 months ($P = 0.023$) and 56% more at 24 months ($P = 0.03$).

Reduced Complex/Activity and Increased Forward but Inhibited Reverse ROS Production in ND6 25L Brain Synaptosomes and Mitochondria The complex I activity in *ND6* 25L liver mitochondria was decreased by 29% ($P = 0.0119$), equivalent to the reduction seen in the LT13 cell line. However, the NADH: ferricyanide oxidoreductase was unaltered indicating that the decrease in activity was not due to a lower abundance of complex I. The *ND6* mutation also caused a 25% decrease in mitochondrial oxygen consumption ($P = 0.0152$).

Analysis of liver sub-mitochondrial particle (SMP) H_2O_2 production when metabolizing NADH revealed significantly increased in H_2O_2 production during forward electron transfer. However, when mitochondrial complex I H_2O_2 production was measured during reverse electron flow driven by succinate plus oligomycin the *ND6* 25L mutation had an almost complete absence of ROS production. Hence, the *ND6* 25L mutation increased ROS production in the forward electron flow direction, while completely blocking ROS production by reverse electron flow. This is a completely novel finding, since reverse electron flow generally generates the maximum ROS.

Recently, it has been proposed that in ischemia-reperfusion, the buildup of succinate in the mitochondrion in the absence of oxygen results in increased ROS production on reperfusion, by reverse electron flow. The increased ROS then oxidizes the Cys39 thiol on complex I subunit ND3 inactivating complex I and blocking reverse electron flow induced ROS. Upon oxidation of the excess succinate, the Cys39 can be reduced and complex I reactivated (Chouchani et al. 2014, 2016). Though *ND6* 25L mice are resistant to reverse electron flow ROS production, their mitochondria constitutively generate more ROS by forward electron flow. If this is a general feature of LHON mutations, it suggests that the primary pathophysiology of LHON may be ROS toxicity to retinal ganglion cells.

To evaluate the consequences of the *ND6* 25L mutation on neuronal cells, mitochondrial function was examined in isolated synaptosomes (Fig. 2a). *ND6* 25L mutant synaptosomes had reduced oxygen consumption under all conditions examined. However, this reduction was greatest under low turnover conditions (basal respiration or respiration in the presence of oligomycin) while the deficit was reduced as the mitochondrial membrane potential was reduced and respiration rate increased, due to either uncoupling with FCCP or activation of the plasma membrane sodium ion channel with veratridine, which increases ATP consumption by the Na^+-K^+ ATPase (Fig. 2b). In contrast to cultured cell mitochondria in which ATP production was reduced, in situ ATP levels in the synaptosomes were maintained under various energetically demanding conditions including incubation with 4-aminopyridine, a potassium ion channel inhibitor; a high concentration of KCl that depolarizes synaptosome; and veratridine (Fig. 2b). The mutant synaptosomes did display a slight but significant decrease in ATP levels when partially inhibited by rotenone (9.7% less than controls, $P = 0.0173$), a phenomenon previously reported for cybrid cell lines harboring LHON mtDNA mutations (Beretta et al. 2006; Marella et al. 2010; Cock et al. 1999; Silva et al. 2009), but in the absence of rotenone the *ND6* 25L synaptosome ATP levels were equal to

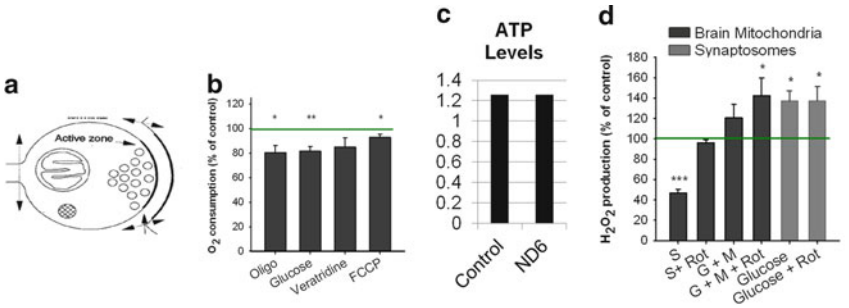

Fig. 2 Mitochondrial physiology of the *ND6* 25L mouse brain synaptosomes. (**a**) Brain synaptosome used to analyze neuronal function. (**b**) Oxygen consumption of synaptosomes at various levels of synaptosome energetic demand. (**c**) Relative synaptosome ATP levels. (**d**) Mitochondrial ROS production by synaptosomes (*black*) and brain mitochondria (*grey*) in various metabolic states determined by substrates and inhibitors. *Oligo* Oligomycin, *S* succinate, *ROT* rotenone, *G* + *M* glutamate + malate

controls (Fig. 2c). This again suggests that the primary pathophysiology of LHON is oxidative stress, not ATP deficiency.

Analysis of brain mitochondrial H_2O_2 production confirmed that the *ND6* P25L mutation caused elevated ROS production during forward complex I electron transport but marked suppression of ROS production during reverse electron transport (Fig. 2d). Rotenone increased the rate of H_2O_2 production during forward electron transfer in both wild-type (2.3-fold increase) and *ND6* P25L (2.7-fold increase). Therefore, the *ND6* 25L brain mitochondria have an increased rate of H_2O_2 generation even when electron transfer to ubiquinone is fully inhibited by rotenone (Fig. 2d). ND6 25L synaptosomes respiring using glucose as a substrate also had increased forward electron transfer H_2O_2 production (37% increase, $P = 0.015$) and this increase was preserved in the presence of rotenone (37% increase, $P = 0.0462$) (Fig. 2d). Together these data suggest that the *ND6* 25L mutation acts "up-stream" of the rotenone binding site, likely at the FNM redox site. Finally, the increased ROS production has cellular consequences since 3-nitrotyrosine was increased more than twofold (236%, $P < 0.0001$) and glial fibrillary acid protein (GFAP) was increased by 65.5% ($P = 0.0059$) in *ND6* P25L whole brain lysates.

In summary, the mouse mtDNA *ND6* P25L mutation model approximates phenotype of LHON as closely as is possible given the anatomical differences between man and mouse. These include reduced light response, swelling and loss of optic nerve fibers, preferential loss of the small caliber central optic nerve fibers with sparing of the larger caliber peripheral fibers, and accumulation of highly abnormal mitochondria. Given that the mtDNA *ND6* 25L mouse is a valid representation of LHON, it suggests some interesting conclusions about the pathophysiology of LHON. The most striking observation is that the biochemical defect of the neuronal cells, as indicated by the synaptosome studies, is not about ATP deficiency but rather about chronic oxidative stress. Since the *ND6* 25L mutation blocks ROS production by reverse electron flow, this suggests that the mutation partially inhibits

the transfer of electrons from complex I iron-sulfur center (N2) to coenzyme Q (CoQ). This would mean that reverse electron flow would not be able to bridge the increased energetic gap between CoQ and N2. Moreover, the forward electron flow rate would be diminished resulting in the chronic reduction of all upstream complex I electron carriers including FMN which is thought to be an important site of complex I ROS production (Pryde and Hirst 2011; Murphy 2009; Brand 2010). Since the block is partial, under high electron flux, the differential electron transfer rate is less significant and ATP levels are maintained but the production of ROS would increase as energetic demands on the neurons increases. It is possible that this type of complex I defect may be a common feature of LHON mutations since the *ND6* 14459G>A (A72V) mutation also retards electron flow to CoQ (Jun et al. 1996). Therefore, the pathophysiology of LHON may not be energy deficiency but rather chronic ROS production and oxidative stress.

9 Possible Therapeutic Approaches to LHON and Mitochondrial Diseases

Efforts to treat LHON have involved both metabolic-pharmacological and genetic approaches. Metabolic interventions with nutraceuticals have been of limited effect. However, some success has been achieved with redox pharmaceuticals. Until now, gene therapy approaches have emphasized AAV transduction of allotropically expressed normal mtDNA genes. However, considerable recent interest has developed around germline gene therapy.

9.1 Nutraceutical and Pharmacological Treatment of LHON

While administration of Coenzyme Q (CoQ) and various antioxidant and vitamin nutraceuticals are commonly used in LHON therapy, there is little empirical evidence that this is beneficial (Pfeffer et al. 2012). Redox pharmaceuticals have shown some benefit. The CoQ analogue, idebenone (2.3-dimethoxy-5-methyl-6-(10-hydroxydecyl)-1,4-benzoquinone), has been reported to be beneficial in both open label studies (Mashima et al. 2000; Carelli et al. 2011) and a double blind trial (Klopstock et al. 2011). Idebenone treatment of 11778 and 3460 patient fibroblasts increased complex I activity 42% (Angebault et al. 2011), but may also activate the mtPTP (Giorgio et al. 2012).

The CoQ analogue, EPI-743, has also been used to treat both a range of mitochondrial disease patients (Enns et al. 2012) and LHON patients (Sadun et al. 2012). In open label studies most mitochondrial diseases patients reported beneficial effects (Enns et al. 2012; Sadun et al. 2012). The advantageous application of the antioxidant drugs such as Idebenone and EPI-743 would be consistent with the hypothesis that the primary pathophysiological etiology of LHON is chronic retinal ganglion cell oxidative stress.

Drugs that stabilize the mtPTP and inhibit apoptosis have also been proposed as potential therapies. However, a trial of brimonidine purite (0.15% eye drops 4 times per day), an α-2 agonist aimed at upregulating Bcl-2 and inhibiting the activation of the mtPTP, produced little beneficial effect (Newman et al. 2005).

Drugs that increase the number of mitochondria might provide protection from LHON mutation-induced blindness. This could be accomplished by activation of the peroxisome proliferator-activated receptors (PPARs) and the PPAR gamma coactivator-1 (PGC-1α) biosynthetic pathways with bezafibrate. This drug has been used successfully to treat Cox10 deficient mice and to increase lifespan, muscle strength, and skeletal muscle COX activity (Wenz et al. 2016, 2008). It should also be beneficial in the central nervous system since it has been reported to ameliorate symptoms in the Huntington Disease mouse model, R6/2. For R6/2 the drug restored depressed levels of PCG-1α, PPARα, PPARγ, PPARδ, cytochrome c, Tfam, and ATP synthase in brain, skeletal muscle, and brown fat in association with extending lifespan, grip strength, and activity levels (Wenz et al. 2008; Johri et al. 2012).

As mentioned, the resistance of females to succumbing to blindness suggests a hormonal agent may be protective of mitochondrial dysfunction. Consistent with this idea, the phytoestrogens genistein, daidzein, and equol which interact with the non-feminizing estrogen receptor β (ERβ) look particularly promising (Pisano et al. 2015).

9.2 Somatic Gene Therapy of the Retina

Direct somatic gene therapy of the retina currently appears to be the most promising approach to treating retinal diseases such as LHON. Leber Congenital Amaurosis resulting from genetic inactivation of the nDNA RP65 gene has been successfully treated by sub-retinal injection of AAV2-hRPE65v2 (Maguire et al. 2008, 2009; Bennicelli et al. 2008; Amado et al. 2010; Simonelli et al. 2010; Bennett et al. 2012). Since the retinal ganglion cells lie adjacent to the vitreous of the eye, they can be treated by injection of AAV viral vectors harboring the therapeutic transgene into the vitreous directly above the retinal ganglion cells.

Gene Therapy Using Allotopic Protein Expression Two research teams, Dr. John Guy (Koilkonda and Guy 2011) and Dr. Marisol Corral-Debrinski (Cwerman-Thibault et al. 2011), have been developing procedures for treating LHON by allotropic gene expression of a normal *ND4* nt 11778G (340R) gene. The mtDNA *ND4* gene is isolated, the genetic code changed to that of the cytosol, a mitochondrial targeting peptide added, nuclear gene expression elements added, and the allotropic gene inserted into the nDNA where the gene can be transcribed, the protein translated in the cytosol, and the normal protein imported into the mitochondrion.

The allotopic system was first used to correct the mtDNA *ATP6* 8993T>G defect in cultured cells (Manfredi et al. 2002) and this same construct was used to suppress the oncogenic phenotype of HeLa cells harboring the *ATP6* 8993T>G

mutant mtDNA (Shidara et al. 2005). Positive allotopic expression has been reported for *ATP8* (Oca-Cossio et al. 2003), *ATP6* (Bonnet et al. 2007), *ATP6*-coded oligomycin resistance (Zullo et al. 2005), *ND1* (Bonnet et al. 2008), and *ND4* (Bonnet et al. 2007, 2008; Guy et al. 2002; Ellouze et al. 2008) genes.

By contrast, efforts to allotopically introduce apocytochrome b and ND4 into cultured cell mitochondria were unsuccessful and resulted in the formation of cytoplasmic fiber-like aggregates (Oca-Cossio et al. 2003). Similarly, attempts to complement a frame shift mutation in the mouse mtDNA *ND6* gene (nt 13887delC) were unsuccessful (Perales-Clemente et al. 2011) as were efforts to complement the human *ATP6* nt 8993 T>G and a COXIII 15 base deletion mtDNA mutations in cultured cells using *Chlamydomonas reinhardtii*optimized *ATP6* and *COX3* genes (Figueroa-Martinez et al. 2011). These negative results have led to concern that allotopically expressed proteins may not enter the mitochondrion and/or assemble in the respiratory complex and perhaps even aggregate and be toxic to the retinal ganglion cells (Oca-Cossio et al. 2003; Perales-Clemente et al. 2011; Figueroa-Martinez et al. 2011). This might initiate a mitochondrial unfolded protein response (UPR^{MT}) (Houtkooper et al. 2013) which would transiently improve mitochondrial function without curing the disease.

Still, both the Guy and Corral-Debrinski laboratories have proceeded with clinical trials to use allotropic gene therapy to treat patients with the 11778 340H mutation using Adeno-Associated Virus (AAV) carrying an allotropic *ND4* gene injected into the vitreous of the eye (Cwerman-Thibault et al. 2011; Guy et al. 2009; Lam et al. 2010). Dr. Corral-Debrinski's laboratory also adds a 3′-UTR that causes the allotopic gene mRNA to be translated on mitochondrially bound polysomes. This increases the import of long, hydrophobic proteins into the mitochondrion (Koilkonda and Guy 2011; Cwerman-Thibault et al. 2011). Both groups have focused on allotopic expression of the mtDNA *ND4* gene since the 11778A mutation is the most common molecular cause of LHON.

Extensive research has been conducted by the Guy team in developing AAV delivery of mitochondrially targeted genes to the eye. This group prepared an allotopic *ND4* protein with the ATPase "c" protein mitochondrial targeting sequence and reported that it could complement the complex I defects in 11778A (340H) mtDNA-containing cybrids (Guy et al. 2002). MnSOD transduction also complements (Qi et al. 2007a). Since the Guy team lack a mouse harboring an optic atrophy producing mtDNA mutation, they created an allotopic *ND4* 11778A (340H) mutant construct and used AAV to transduce the retinal ganglion cells resulting in optic atrophy (Qi et al. 2007b). They then reported that the ND4 polypeptide entered the mitochondrial matrix and was incorporated into complex I (Guy et al. 2009). Additional AAV modifications have been made to increase transduction efficiency (Koilkonda et al. 2009, 2010).

Using 3′-UTR sequence that targets the mRNAs of nDNA coded mitochondrial proteins for translation on mitochondria-bound polysomes Dr. Corral-Debrinski enhances the co-translational import into the mitochondrion of hydrophobic proteins such as ND4 through the TOMM and TIMM complexes. Dr. Corral-Debrinski's allotopic constructs employ the COX10 mitochondrial targeting

sequence (21 amino acids) and COX10 3′-UTR (1,425 nt) to bracket the mtDNA derived gene, with the whole assembly transcribed from a CMC enhancer-promoter (Cwerman-Thibault et al. 2011; Sylvestre et al. 2003a, b). With these constructs the Corral-Debrinski team has reported the allotopic complementation of the mitochondrial defects of the NARP *ATP6* 8993T>G (L156R), LHON *ND4* 11778G>A (Bonnet et al. 2007) and LHON *ND1* 3460G>A (Bonnet et al. 2008) mutations in cultured cells. They have also transformed retinal ganglion cells with an allotopic *ND4* 11778A (340H) mutant gene and observed retinopathy (Ellouze et al. 2008). Finally, they have used AAV to transduce the apoptosis inducing factor (AIF) cDNA into Harlequin mutant mouse eyes and corrected the complex I defect stabilizing the retinas (Bouaita et al. 2012).

While these reports appear positive, the allotopic retinal gene therapy approach is untested in animal mtDNA mutant models of LHON. The creation of LHON animal models by allotopic expression of the mutant *ND4* 11778A 340H protein imported into the retinal ganglion cells may be problematic since individuals harboring heteroplasmic LHON mutations are generally unaffected, yet the AAV-*ND4* 11778A treated mice develop optic atrophy while retaining a full complement of normal mtDNAs.

The next generation somatic gene therapy proposed by the Guy laboratory is to introduce a plasmid containing the normal mtDNA gene into the mitochondrion. As used to generate a germline mutant model for LHON, AAV has been generated with a mitochondrial targeting peptide bound capside protein. The normal human *ND4* gene borne on a mitochondrial H-strand containing plasmid is inserted into an scAAV DNA. The concept is that the transducing virus will be injected into the vitreous of the eye, enter the retinal ganglion cells, enter the mitochondrion, the plasmid will be uncoated, and the *ND4* gene transcribed and translated to complement the LHON defect (Yu et al. 2012).

Gene Therapy Using Allotopic Expression of mRNA A main concern about allotopic protein therapy is that the cytosolic-translated mtDNA protein will aggregate in the cytosol outside the mitochondrion and become toxic. This is normally prevented for mtDNA encoded proteins, because the altered mtDNA genetic code blocks the cytosolic translation of mtDNA mRNAs (Wallace 1982b). This genetic code barrier would be retained if the natural mtDNA mRNA sequence is transcribed in the nucleus and the mRNAs imported into the mitochondrial matrix for translation. Such a system has been developed by Drs. Michael Teitell and Carla Koehler of UCLA drawing on the Teitell laboratory discovery that the mitochondrial RNA processing enzyme, polynucleotide phosphorylase or PNPASE, is located in the mitochondrial inter membrane space and functions as a chaperone for the import of nDNA coded RNAs into the mitochondria. The imported nuclear-coded RNAs include components of the RNase P and the MRP enzymes, the 5S rRNA, and perhaps tRNAs (Wang et al. 2010, 2012a). To be imported, the RNA needs to have a 5′ stem loop structure like that found on the H1 RNA component of the RNase P RNA (the "RP" targeting element) and the 3′-UTR element that directs mRNAs of the nDNA-coded mitochondrial proteins to bind to the outer mitochondrial

membrane. The Teitell laboratory is using the 154 nt 3′-UTR from the mitochondrial ribosomal protein MRPS12 for this purpose. With various arrangements of these RNA elements, the Teitell laboratory has imported tRNAs into the mitochondrion (Wang et al. 2012b) and complemented the $tRNA^{Lys}$ nt 8344A>G MERRF mutation (Wallace et al. 1988b; Shoffner et al. 1990) and the $tRNA^{Leu(UUR)}$ nt 3243A>G MELAS mutation (Goto et al. 1990). They have also flanked the human mtDNA COII mRNA with the RP and 3′-UTR sequences. This human mtDNA mRNA construct was successfully imported into the mitochondria of mouse embryonic fibroblasts where it was translated. While only about 5–10% of the mRNA is imported into the mitochondrion (Wang et al. 2012b) the extra allotopic mRNA cannot be translated on cytosolic ribosomes and is degraded. Since relatively low levels of heteroplasmy are sufficient to inhibit the development of optic atrophy in humans, the poor RNA uptake efficiency may not be problematic. This technology has been extended by Dr. Michael Paladino's laboratory to import antisense RNAs into the mitochondrion to modulate the expression of *the ATP6* and *COII* genes in *Drosophila* (Towheed et al. 2014).

Gene Therapy to Bypass Complex I and/or Reduce ROS Toxicity An alternative approach could be to bypass the complex I defect or to ameliorate the physiological defect. Defects in mitochondrial complex I could be by-passed by the transduction of the *Saccharomyces cerevisiae* single polypeptide complex I, Ndi1. Initial studies using viral transduction of Nde1 to bypass complex I inhibition in the substantia nigra have been explored for treating Parkinson disease (Seo et al. 1998, 2000, 2002, 2004, 2006; Marella et al. 2008). This approach has also been proposed for the treatment of the complex I defects on LHON (Park et al. 2007) and delivery of Ndi1 into the optic layer of the superior colliculus was able to effectively ameliorate the optic atrophy in a rotenone-induced rat model of LHON (Marella et al. 2010). An AAV-borne Ndi1 gene has been directly injected in to the eye vitreous to transduce the retinal ganglion cells and ameliorate the effects of rotenone induced LHON in rodent models (Mansergh et al. 2014; Chadderton et al. 2013).

Other interventions could attempt to mitigate the toxic physiological effects of the mtDNA mutations. Respiratory defects result in the buildup of NADH, thus increasing electron flux through complex I and complex I ROS production. The excess NADH could be reduced by introduction into the retinal ganglion cells of the water-forming NADH oxidase from *Lactobacillus brevis* (LbNOX) (Titov et al. 2016). Assuming the complex I ROS production is the primary mediator of retinal ganglion cell toxicity, this could be ameliorated by introduction of a catalytic antioxidant enzyme such as mCAT into the retinal ganglion cell mitochondrial matrix (Schriner et al. 2005).

Heteroplasmy Shifting Heteroplasmy shifting appears to have considerable promise in treating mitochondrial diseases resulting from severe heteroplasmic mutations (Santra et al. 2004; Srivastava and Moraes 2001; Bacman et al. 2007, 2010, 2013; Hashimoto et al. 2015; Reddy et al. 2015). Regrettably, however, most LHON mutations are homoplasmic so this approach will have limited utility.

9.3 Germline Therapy by Nuclear Transplantation

While the development of effective treatments of the retinal ganglion cells of LHON may ultimately be able to improve mitochondrial function sufficiently to prevent the onset of blindness, women harboring homoplasmic primary LHON mtDNA mutations will still have a 100% probability of transmitting predisposition to LHON to their children, putting their sons at significant risk of mid-life blindness. The maternal transmission of LHON risk could be obviated by replacing the maternal mutant mtDNA with normal mtDNAs via transfer of the maternal oocyte or zygote nucleus into an enucleate oocyte from a woman with normal mtDNAs (Wallace 1987).

Four research teams are actively pursuing this strategy: Drs. Douglass Turnbull, Mary Herbert, and associates in Newcastle (Craven et al. 2011), Dr. Shoukhrat Mitalipov in Oregon (Craven et al. 2011), Dr. Egli in New York (Paull et al. 2013; Yamada et al. 2016), and Dr. John Zhang in New York. Two major strategies have been developed. The first is fertilization of the maternal oocyte with the paternal sperm and then transfer the zygote nucleus into the recipient enucleated oocyte (Craven et al. 2011; Paull et al. 2013; Hyslop et al. 2016). The second is the transfer of the oocyte metaphase II (MII) spindle into the enucleate recipient oocyte followed by fertilization with the paternal sperm. This latter procedure has been used to generate rhesus macaque monkeys with little transfer of donor oocyte mitochondria and apparently normal development and phenotype (Paull et al. 2013; Tachibana et al. 2013; Lee et al. 2012; Kang et al. 2016).

Both the pronuclear and spindle transfer techniques have been refined to transfer a minute amount of nuclear donor mtDNA. While low level heteroplasmy of a pathogenic mutation would probably be masked by the predominance of normal mtDNAs, minimizing the risk of a clinical phenotype, there is a risk of incompatibility between the two mtDNA haplogroup lineages (Sharpley et al. 2012). In cases of incompatibility, there is the possibility that the mutant heteroplasmy may resurface in subsequent embryonic stem cells and thus in maternal offspring (Paull et al. 2013; Kang et al. 2016). This concern might be mitigated by prescreening potential oocyte mtDNA donor women for their mtDNA haplogroups and matching the mtDNA haplogroups of the nuclear donor mother with that of the oocyte recipient. That spindle transfer can be used to eliminate a maternally inherited, homoplasmic, mtDNA LHON mutation has been confirmed by the recent public announcement by Dr. Zhang that a normal child has been borne applying this technique to a mother who had previous children with Leigh syndrome due to high mtDNA heteroplasmy levels of the mtDNA *ATP6* nt 8993T>G (L156R) mutation https://www.newscientist.com/article/2107219-exclusive-worlds-first-baby-born-with-new-3-parent-technique/.

10 LHON, Entry to the Mitochondrial Etiology of Common Ophthalmological Diseases

The preferential sensitivity of retinal cells to partial mitochondrial dysfunction has spawned interest in the possibility that mitochondrial defects may be the pathophysiological mechanism for a range of common ophthalmological diseases from glaucoma to age-related macular degeneration (AMD). Glaucoma patient's fibroblasts and lymphoblastoid cell lines have been found to have partial complex I defects (Lopez Sanchez et al. 2016; Chrysostomou et al. 2013; Van Bergen et al. 2015) and mtDNA mutations have been identified in the mtDNAs of African-American glaucoma patients (Collins et al. 2016).

While LHON, DOA, and glaucoma all implicate mitochondrial dysfunction retinal ganglion cells dysfunction and death, mitochondrial dysfunction has also been implicated in other common retinal diseases including retinitis pigmentosa (Ortiz et al. 1993) and age-related macular degeneration (AMD) (Udar et al. 2009). AMD is the most common cause of age-related blindness and specific mtDNA haplogroups have been associated with increased AMD risk, specifically European haplogroups J, T, and U (Udar et al. 2009; Kenney et al. 2013a). Functional evaluation of cybrids prepare with haplogroups J mtDNAs in retinal pigmentary epithelial cells have been found to have mitochondrial physiological alterations and perturbed expression of the nDNA inflammatory genes that have been linked to AMD (Kenney et al. 2013b; Kenney et al. 2014; Nashine et al. 2016). Conversely, mutation in the complement factor H gene previously linked to AMD risk have been found to be associated with increases in retinal pigment epithelial mtDNA damage (Ferrington et al. 2016).

11 Conclusion

Many common diseases ranging from Type II diabetes to Parkinson and Alzheimer Disease have a delayed onset, progressive course, and highly variable penetrance. This phenomenon is not predicted under the deterministic Mendelian paradigm. The discovery that LHON is due primarily to mtDNA mutations has offered a new perspective on such common complex diseases. In many common diseases, multiple gene defects can produce the same phenotype. Since mitochondrial energy metabolism requires thousands of nDNA genes plus thousands of copies of the mtDNA, many different gene defects and combinations of gene variants can cause the same metabolic defects and associated phenotype (Wallace 2013a). The delayed onset and progressive course of the common degenerative diseases can be understood as a result of a partial mitochondrial defects predisposing to the disease which become exacerbated by the accumulation of somatic mtDNA mutation during the aging process (Corral-Debrinski et al. 1992). As the degree of mitochondrial dysfunction increases, mitochondrial signaling to the nuclear epigenome causes phased changes in nuclear gene expression and progression of disease phenotypes (Picard et al. 2014). Since energy production is central to an animal's interaction with the

environment (diet, oxygen tension, thermal stress, infection, toxins), the mitochondria are the environmental sensors (Wallace 2016). Finally, mitochondrial energetics must be attuned by flight-fight responses, activity levels, and reproduction so hormonal systems must also modulate the mitochondria. Hence, the studies of LHON have opened broad new vistas to our understanding of human health and disease.

Acknowledgements This work was supported by NIH grant 5R01-NS021328-30 awarded to DCW.

References

Acin-Perez R, Bayona-Bafaluy MP, Fernandez-Silva P, Moreno-Loshuertos R, Perez-Martos A, Bruno C, Moraes CT, Enriquez JA (2004) Respiratory complex III is required to maintain complex I in mammalian mitochondria. Mol Cell 13(6):805–815. PubMed PMID: 15053874

Alexander C, Votruba M, Pesch UEA, Thiselton DL, Mayer S, Moore A, Rodriquez M, Kellner U, Leo-Kottler B, Auburger G, Bhattcharya SS, Wissinger B (2000) OPA1, encoding a dynamin-related GTPase, is mutated in autosomal dominant optic atrophy linked to chromosome 3q28. Nat Genet 26(2):211–215. PubMed PMID: 11017080

Amado D, Mingozzi F, Hui D, Bennicelli JL, Wei Z, Chen Y, Bote E, Grant RL, Golden JA, Narfstrom K, Syed NA, Orlin SE, High KA, Maguire AM, Bennett J (2010) Safety and efficacy of subretinal readministration of a viral vector in large animals to treat congenital blindness. Sci Transl Med 2(21):21ra16. Epub 2010/04/09. doi: 10.1126/scitranslmed.3000659. PubMed PMID: 20374996

Amaral-Fernandes MS, Marcondes AM, Miranda PM, Maciel-Guerra AT, Sartorato EL (2011) Mutations for Leber hereditary optic neuropathy in patients with alcohol and tobacco optic neuropathy. Mol Vis 17:3175–3179. http://www.molvis.org/molvis/v17/a342/. Epub 2011/12/24. PubMed PMID: 22194643

Angebault C, Gueguen N, Desquiret-Dumas V, Chevrollier A, Guillet V, Verny C, Cassereau J, Ferre M, Milea D, Amati-Bonneau P, Bonneau D, Procaccio V, Reynier P, Loiseau D (2011) Idebenone increases mitochondrial complex I activity in fibroblasts from LHON patients while producing contradictory effects on respiration. BMC Res Notes 4(1):557. doi:10.1186/1756-0500-4-557. Epub 2011/12/24. PubMed PMID: 22192149

Bacman SR, Williams SL, Hernandez D, Moraes CT (2007) Modulating mtDNA heteroplasmy by mitochondria-targeted restriction endonucleases in a "differential multiple cleavage-site" model. Gene Ther 14(18):1309–1318. PubMed PMID: 17597792

Bacman SR, Williams SL, Garcia S, Moraes CT (2010) Organ-specific shifts in mtDNA heteroplasmy following systemic delivery of a mitochondria-targeted restriction endonuclease. Gene Ther 17(6):713–720. doi:10.1038/gt.2010.25. Epub 2010/03/12. PubMed PMID: 20220783

Bacman SR, Williams SL, Pinto M, Peralta S, Moraes CT (2013) Specific elimination of mutant mitochondrial genomes in patient-derived cells by mitoTALENs. Nat Med 19(9):1111–1113. Epub Aug 4. doi: 10.1038/nm.3261. PubMed PMID: 23913125

Baracca A, Solaini G, Sgarbi G, Lenaz G, Baruzzi A, Schapira AH, Martinuzzi A, Carelli V (2005) Severe impairment of complex I-driven adenosine triphosphate synthesis in Leber hereditary optic neuropathy cybrids. Arch Neurol 62(5):730–736. doi:10.1001/archneur.62.5.730. Epub 2005/05/11. PubMed PMID: 15883259

Bennett J, Ashtari M, Wellman J, Marshall KA, Cyckowski LL, Chung DC, McCague S, Pierce EA, Chen Y, Bennicelli JL, Zhu X, Ying GS, Sun J, Wright JF, Auricchio A, Simonelli F, Shindler KS, Mingozzi F, High KA, Maguire AM (2012) AAV2 gene therapy readministration

in three adults with congenital blindness. Sci Transl Med 4(120):120ra15. doi:10.1126/scitranslmed.3002865. Epub 2012/02/11. PubMed PMID: 22323828

Bennicelli J, Wright JF, Komaromy A, Jacobs JB, Hauck B, Zelenaia O, Mingozzi F, Hui D, Chung D, Rex TS, Wei Z, Qu G, Zhou S, Zeiss C, Arruda VR, Acland GM, Dell'Osso LF, High KA, Maguire AM, Bennett J (2008) Reversal of blindness in animal models of leber congenital amaurosis using optimized AAV2-mediated gene transfer. Mol Ther 16(3):458–465. doi:10.1038/sj.mt.6300389. Epub 2008/01/23. PubMed PMID: 18209734

Beretta S, Mattavelli L, Sala G, Tremolizzo L, Schapira AH, Martinuzzi A, Carelli V, Ferrarese C (2004) Leber hereditary optic neuropathy mtDNA mutations disrupt glutamate transport in cybrid cell lines. Brain 127(Pt 10):2183–2192. doi:10.1093/brain/awh258. Epub 2004/09/03. PubMed PMID: 15342361

Beretta S, Wood JP, Derham B, Sala G, Tremolizzo L, Ferrarese C, Osborne NN (2006) Partial mitochondrial complex I inhibition induces oxidative damage and perturbs glutamate transport in primary retinal cultures. Relevance to Leber hereditary optic neuropathy (LHON). Neurobiol Dis 24(2):308–317. doi:10.1016/j.nbd.2006.07.016. Epub 2006/09/09. PubMed PMID: 16959493

Bonnet C, Kaltimbacher V, Ellouze S, Augustin S, Benit P, Forster V, Rustin P, Sahel JA, Corral-Debrinski M (2007) Allotopic mRNA localization to the mitochondrial surface rescues respiratory chain defects in fibroblasts harboring mitochondrial DNA mutations affecting complex I or v subunits. Rejuvenation Res 10(2):127–144. PubMed PMID: 17518546

Bonnet C, Augustin S, Ellouze S, Benit P, Bouaita A, Rustin P, Sahel JA, Corral-Debrinski M (2008) The optimized allotopic expression of ND1 or ND4 genes restores respiratory chain complex I activity in fibroblasts harboring mutations in these genes. Biochim Biophys Acta 1783(10):1707–1717. PubMed PMID: 18513491

Bouaita A, Augustin S, Lechauve C, Cwerman-Thibault H, Benit P, Simonutti M, Paques M, Rustin P, Sahel JA, Corral-Debrinski M (2012) Downregulation of apoptosis-inducing factor in Harlequin mice induces progressive and severe optic atrophy which is durably prevented by AAV2-AIF1 gene therapy. Brain 135(Pt 1):35–52. doi:10.1093/brain/awr290. Epub 2011/11/29. PubMed PMID: 22120150

Brand MD (2010) The sites and topology of mitochondrial superoxide production. Exp Gerontol 45(7–8):466–472. doi:10.1016/j.exger.2010.01.003. Epub 2010/01/13. PubMed PMID: 20064600

Brown MD, Torroni A, Reckord CL, Wallace DC (1995) Phylogenetic analysis of Leber's hereditary optic neuropathy mitochondrial DNA's indicates multiple independent occurrences of the common mutations. Hum Mutat 6(4):311–325. doi:10.1002/humu.1380060405. PubMed PMID: 8680405

Brown MD, Sun F, Wallace DC (1997) Clustering of Caucasian Leber hereditary optic neuropathy patients containing the 11778 or 14484 mutations on an mtDNA lineage. Am J Hum Genet 60(2):381–387. PubMed PMID: 9012411

Brown MD, Hosseini SH, Torroni A, Bandelt HJ, Allen JC, Schurr TG, Scozzari R, Cruciani F, Wallace DC (1998) mtDNA Haplogroup X: an ancient link between Europe/Western Asia and North America? Am J Hum Genet 63(6):1852–1861. PubMed PMID: 9837837

Brown MD, Trounce IA, Jun AS, Allen JC, Wallace DC (2000) Functional analysis of lymphoblast and cybrid mitochondria containing the 3460, 11778, or 14484 Leber's hereditary optic neuropathy mtDNA mutation. J Biol Chem 275(51):39831–39836. PubMed PMID: 10976107

Brown MD, Allen JC, Van Stavern GP, Newman NJ, Wallace DC (2001) Clinical, genetic, and biochemical characterization of a Leber hereditary optic neuropathy family containing both the 11778 and 14484 primary mutations. Am J Med Genet 104(4):331–338. PubMed PMID: 11754070

Brown MD, Starikovskaya E, Derbeneva O, Hosseini S, Allen JC, Mikhailovskaya IE, Sukernik RI, Wallace DC (2002) The role of mtDNA background in disease expression: a new primary LHON mutation associated with Western Eurasian haplogroup. J Hum Genet 110(2):130–138. PubMed PMID: 11935318

Bunn CL, Wallace DC, Eisenstadt JM (1974) Cytoplasmic inheritance of chloramphenicol resistance in mouse tissue culture cells. Proc Natl Acad Sci U S A 71(5):1681–1685. PubMed PMID: 4525288

Cann RL, Stoneking M, Wilson AC (1987) Mitochondrial DNA and human evolution. Nature 325(6099):31–36. PubMed PMID: 3025745

Carelli V, Ghelli A, Ratta M, Bacchilega E, Sangiorgi S, Mancini R, Leuzzi V, Cortelli P, Montagna P, Lugaresi E, Degli EM (1997) Leber's hereditary optic neuropathy: biochemical effect of 11778/ND4 and 3460/ND1 mutations and correlation with the mitochondrial genotype. Neurology 48(6):1623–1632

Carelli V, Ghelli A, Bucchi L, Montagna P, De Negri A, Leuzzi V, Carducci C, Lenaz G, Lugaresi E, Degli Esposti M (1999) Biochemical features of mtDNA 14484 (ND6/M64V) point mutation associated with Leber's hereditary optic neuropathy. Ann Neurol 45 (3):320–328

Carelli V, Ross-Cisneros FN, Sadun AA (2004) Mitochondrial dysfunction as a cause of optic neuropathies. Prog Retin Eye Res 23(1):53–89. PubMed PMID: 14766317

Carelli V, Achilli A, Valentino ML, Rengo C, Semino O, Pala M, Olivieri A, Mattiazzi M, Pallotti F, Carrara F, Zeviani M, Leuzzi V, Carducci C, Valle G, Simionati B, Mendieta L, Salomao S, Belfort R, Sadun AA, Torroni A (2006) Haplogroup effects and recombination of mitochondrial DNA: novel clues from the analysis of Leber hereditary optic neuropathy pedigrees. Am J Hum Genet 78(4):564–574. PubMed PMID: 16532388

Carelli V, La Morgia C, Valentino ML, Rizzo G, Carbonelli M, De Negri AM, Sadun F, Carta A, Guerriero S, Simonelli F, Sadun AA, Aggarwal D, Liguori R, Avoni P, Baruzzi A, Zeviani M, Montagna P, Barboni P (2011) Idebenone treatment In Leber's hereditary optic neuropathy. Brain 134(9):e188. doi:10.1093/brain/awr180. Epub 2011/08/04. PubMed PMID: 21810891

Carelli V, d'Adamo P, Valentino ML, La Morgia C, Ross-Cisneros FN, Caporali L, Maresca A, Loguercio Polosa P, Barboni P, De Negri A, Sadun F, Karanjia R, Salomao SR, Berezovsky A, Chicani F, Moraes M, Moraes Filho M, Belfort R Jr, Sadun AA (2016) Parsing the differences in affected with LHON: genetic versus environmental triggers of disease conversion. Brain 139(Pt 3):e17. doi:10.1093/brain/awv339. PubMed PMID: 26657166

Chadderton N, Palfi A, Millington-Ward S, Gobbo O, Overlack N, Carrigan M, O'Reilly M, Campbell M, Ehrhardt C, Wolfrum U, Humphries P, Kenna PF, Farrar GJ (2013) Intravitreal delivery of AAV-NDI1 provides functional benefit in a murine model of Leber hereditary optic neuropathy. Eur J Hum Genet 21(1):62–68. doi:10.1038/ejhg.2012.112. PubMed PMID: 22669418; PMCID: PMC3522193

Chalmers RM, Davis MB, Sweeney MG, Wood NW, Harding AE (1996) Evidence against an X-linked visual loss susceptibility locus in Leber hereditary optic neuropathy. Am J Hum Genet 59(1):103–108

Chinnery PF, Johnson MA, Wardell TM, Singh-Kler R, Hayes C, Brown DT, Taylor RW, Bindoff LA, Turnbull DM (2000) The epidemiology of pathogenic mitochondrial DNA mutations. Ann Neurol 48(2):188–193. PubMed PMID: 10939569

Chouchani ET, Pell VR, Gaude E, Aksentijevic D, Sundier SY, Robb EL, Logan A, Nadtochiy SM, Ord EN, Smith AC, Eyassu F, Shirley R, Hu CH, Dare AJ, James AM, Rogatti S, Hartley RC, Eaton S, Costa AS, Brookes PS, Davidson SM, Duchen MR, Saeb-Parsy K, Shattock MJ, Robinson AJ, Work LM, Frezza C, Krieg T, Murphy MP (2014) Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. Nature 515(7527):431–435. doi:10.1038/nature13909. PubMed PMID: 25383517

Chouchani ET, Pell VR, James AM, Work LM, Saeb-Parsy K, Frezza C, Krieg T, Murphy MP (2016) A unifying mechanism for mitochondrial superoxide production during ischemia-reperfusion injury. Cell Metab 23(2):254–263. doi:10.1016/j.cmet.2015.12.009. PubMed PMID: 26777689

Chrysostomou V, Rezania F, Trounce IA, Crowston JG (2013) Oxidative stress and mitochondrial dysfunction in glaucoma. Curr Opin Pharmacol 13(1):12–15. doi:10.1016/j.coph.2012.09.008. PubMed PMID: 23069478

Cock HR, Cooper JM, Schapira AH (1999) Functional consequences of the 3460-bp mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. J Neurol Sci 165(1): 10–17. PubMed PMID: 0010426140

Collins DW, Gudiseva HV, Trachtman B, Bowman AS, Sagaser A, Sankar P, Miller-Ellis E, Lehman A, Addis V, O'Brien JM (2016) Association of primary open-angle glaucoma with mitochondrial variants and haplogroups common in African Americans. Mol Vis 22:454–471. PubMed PMID: 27217714; PMCID: PMC4872278

Corral-Debrinski M, Horton T, Lott MT, Shoffner JM, Beal MF, Wallace DC (1992) Mitochondrial DNA deletions in human brain: regional variability and increase with advanced age. Nat Genet 2(4):324–329. PubMed PMID: 1303288

Craven L, Elson JL, Irving L, Tuppen HA, Lister LM, Greggains GD, Byerley S, Murdoch AP, Herbert M, Turnbull D (2011) Mitochondrial DNA disease: new options for prevention. Hum Mol Genet 20(R2):R168–R174. doi:10.1093/hmg/ddr373. Epub 2011/08/20. PubMed PMID: 21852248

Cree LM, Samuels DC, Chinnery PF (2009) The inheritance of pathogenic mitochondrial DNA mutations. Biochim Biophys Acta 1792(12):1097–1102. doi:10.1016/j.bbadis.2009.03.002. PubMed PMID: 19303927

Cwerman-Thibault H, Sahel JA, Corral-Debrinski M (2011) Mitochondrial medicine: to a new era of gene therapy for mitochondrial DNA mutations. J Inherit Metab Dis 34(2):327–344. doi:10.1007/s10545-010-9131-5. Epub 2010/06/24. PubMed PMID: 20571866

Danielson SR, Wong A, Carelli V, Martinuzzi A, Schapira AHV, Cortopassi GA (2002) Cells bearing mutation causing Leber's hereditary optic neuropathy are sensitized to Fas-induced apoptosis. J Biol Chem 277(8):5810–5815. PubMed PMID: 11741983

Delettre C, Lenaers G, Griffoin JM, Gigarel N, Lorenzo C, Belenguer P, Pelloquin L, Grosgeorge J, Turc-Carel C, Perret E, Astarie-Dequeker C, Lasquellec L, Arnaud B, Ducommun B, Kaplan J, Hamel CP (2000) Nuclear gene OPA1, encoding a mitochondrial dynamin-related protein, is mutated in dominant optic atrophy. Nat Genet 26(2):207–210. PubMed PMID: 11017079

Developmental Disabilities Monitoring Network Surveillance Year 2010 Principal Investigators, Centers for Disease Control and Prevention (CDC) (2014) Prevalence of autism spectrum disorder among children aged 8 years – autism and developmental disabilities monitoring network, 11 sites, United States, 2010. MMWR Surveill Summ 63(2):1–21. doi: 00000-open online access at http://www.cdc.gov/mmwr/preview/mmwrhtml/ss6302a1.htm. PubMed PMID: 24670961

Elliott HR, Samuels DC, Eden JA, Relton CL, Chinnery PF (2008) Pathogenic mitochondrial DNA mutations are common in the general population. Am J Hum Genet 83(2):254–260. PubMed PMID: 18674747

Ellouze S, Augustin S, Bouaita A, Bonnet C, Simonutti M, Forster V, Picaud S, Sahel JA, Corral-Debrinski M (2008) Optimized allotopic expression of the human mitochondrial ND4 prevents blindness in a rat model of mitochondrial dysfunction. Am J Hum Genet 83(3):373–387. PubMed PMID: 18771762

Enns GM, Kinsman SL, Perlman SL, Spicer KM, Abdenur JE, Cohen BH, Amagata A, Barnes A, Kheifets V, Shrader WD, Thoolen M, Blankenberg F, Miller G (2012) Initial experience in the treatment of inherited mitochondrial disease with EPI-743. Mol Genet Metab 105(1):91–102. doi:10.1016/j.ymgme.2011.10.009. Epub 2011/11/26. PubMed PMID: 22115768

Ferrington DA, Kapphahn RJ, Leary MM, Atilano SR, Terluk MR, Karunadharma P, Chen GK, Ratnapriya R, Swaroop A, Montezuma SR, Kenney MC (2016) Increased retinal mtDNA damage in the CFH variant associated with age-related macular degeneration. Exp Eye Res 145:269–277. doi: 10.1016/j.exer.2016.01.018. PubMed PMID: 26854823; PMCID: PMC4842097

Figueroa-Martinez F, Vazquez-Acevedo M, Cortes-Hernandez P, Garcia-Trejo JJ, Davidson E, King MP, Gonzalez-Halphen D (2011) What limits the allotopic expression of nucleus-

encoded mitochondrial genes? The case of the chimeric Cox3 and Atp6 genes. Mitochondrion 11(1):147–154. doi:10.1016/j.mito.2010.09.003. Epub 2010/09/22. PubMed PMID: 20854934

Floreani M, Napoli E, Martinuzzi A, Pantano G, De Riva V, Trevisan R, Bisetto E, Valente L, Carelli V, Dabbeni-Sala F (2005) Antioxidant defences in cybrids harboring mtDNA mutations associated with Leber's hereditary optic neuropathy. FEBS J 272(5):1124–1135. doi:10.1111/j.1742-4658.2004.04542.x. Epub 2005/02/22. PubMed PMID: 15720387

Ghelli A, Porcelli AM, Zanna C, Martinuzzi A, Carelli V, Rugolo M (2008) Protection against oxidant-induced apoptosis by exogenous glutathione in Leber hereditary optic neuropathy cybrids. Invest Ophthalmol Vis Sci 49(2):671–676. doi:10.1167/iovs.07-0880. Epub 2008/02/01. PubMed PMID: 18235013

Giordano C, Iommarini L, Giordano L, Maresca A, Pisano A, Valentino ML, Caporali L, Liguori R, Deceglie S, Roberti M, Fanelli F, Fracasso F, Ross-Cisneros FN, D'Adamo P, Hudson G, Pyle A, Yu-Wai-Man P, Chinnery PF, Zeviani M, Salomao SR, Berezovsky A, Belfort R Jr, Ventura DF, Moraes M, Moraes Filho M, Barboni P, Sadun F, De Negri A, Sadun AA, Tancredi A, Mancini M, d'Amati G, Loguercio Polosa P, Cantatore P, Carelli V (2014) Efficient mitochondrial biogenesis drives incomplete penetrance in Leber's hereditary optic neuropathy. Brain 137(Pt 2):335–353. doi:10.1093/brain/awt343. Epub 2013/12/27. PubMed PMID: 24369379

Giordano L, Deceglie S, d'Adamo P, Valentino ML, La Morgia C, Fracasso F, Roberti M, Cappellari M, Petrosillo G, Ciaravolo S, Parente D, Giordano C, Maresca A, Iommarini L, Del Dotto V, Ghelli AM, Salomao SR, Berezovsky A, Belfort R Jr, Sadun AA, Carelli V, Loguercio Polosa P, Cantatore P (2015) Cigarette toxicity triggers Leber's hereditary optic neuropathy by affecting mtDNA copy number, oxidative phosphorylation and ROS detoxification pathways. Cell Death Dis 6:e2021. doi:10.1038/cddis.2015.364. PubMed PMID: 26673666; PMCID: PMC4720897

Giorgio V, Petronilli V, Ghelli A, Carelli V, Rugolo M, Lenaz G, Bernardi P (2012) The effects of idebenone on mitochondrial bioenergetics. Biochim Biophys Acta 1817(2):363–369. doi:10.1016/j.bbabio.2011.10.012. Epub 2011/11/17. PubMed PMID: 22086148

Gomez-Duran A, Pacheu-Grau D, Lopez-Gallardo E, Diez-Sanchez C, Montoya J, Lopez-Perez MJ, Ruiz-Pesini E (2010) Unmasking the causes of multifactorial disorders: OXPHOS differences between mitochondrial haplogroups. Hum Mol Genet 19(17):3343–3353. doi:10.1093/hmg/ddq246. Epub 2010/06/23. PubMed PMID: 20566709

Goto Y, Nonaka I, Horai S (1990) A mutation in the $tRNA^{Leu(UUR)}$ gene associated with the MELAS subgroup of mitochondrial encephalomyopathies. Nature 348(6302):651–653. PubMed PMID: 2102678

Guy J, Qi X, Pallotti F, Schon EA, Manfredi G, Carelli V, Martinuzzi A, Hauswirth WW, Lewin AS (2002) Rescue of a mitochondrial deficiency causing Leber hereditary optic neuropathy. Ann Neurol 52(5):534–542. PubMed PMID: 12402249

Guy J, Qi X, Koilkonda RD, Arguello T, Chou TH, Ruggeri M, Porciatti V, Lewin AS, Hauswirth WW (2009) Efficiency and safety of AAV-mediated gene delivery of the human ND4 complex I subunit in the mouse visual system. Invest Ophthalmol Vis Sci 50(9):4205–4214. doi:10.1167/iovs.08-3214. Epub 2009/04/24. PubMed PMID: 19387075

Handoko HY, Wirapati PJ, Sudoyo HA, Sitepu M, Marzuki S (1998) Meiotic breakpoint mapping of a proposed X linked visual loss susceptibility locus in Leber's hereditary optic neuropathy. J Med Genet 35(8):668–671. Epub 1998/08/27. PubMed PMID: 9719375

Harding AE, Sweeney MG, Miller DH, Mumford CJ, Kellar-Wood H, Menard D, McDonald WI, Compston DAS (1992) Occurrence of a multiple sclerosis-like illness in women who have a Leber's heditary optic neuropathy mitochondrial DNA mutation. Brain 115:979–989

Hashimoto M, Bacman SR, Peralta S, Falk MJ, Chomyn A, Chan DC, Williams SL, Moraes CT (2015) MitoTALEN: a general approach to reduce mutant mtDNA loads and restore oxidative phosphorylation function in mitochondrial diseases. Mol Ther 23(10):1592–1599. doi:10.1038/mt.2015.126. PubMed PMID: 26159306; PMCID: PMC4817924

Houtkooper RH, Mouchiroud L, Ryu D, Moullan N, Katsyuba E, Knott G, Williams RW, Auwerx J (2013) Mitonuclear protein imbalance as a conserved longevity mechanism. Nature 497 (7450):451–457. doi:10.1038/nature12188. Epub 2013/05/24. PubMed PMID: 23698443

Hudson G, Keers S, Yu Wai Man P, Griffiths P, Huoponen K, Savontaus ML, Nikoskelainen E, Zeviani M, Carrara F, Horvath R, Karcagi V, Spruijt L, de Coo IF, Smeets HJ, Chinnery PF (2005) Identification of an X-chromosomal locus and haplotype modulating the phenotype of a mitochondrial DNA disorder. Am J Hum Genet 77(6):1086–1091. PubMed PMID: 16380918

Huoponen K, Vilkki J, Aula P, Nikoskelainen EK, Savontaus ML (1991) A new mtDNA mutation associated with Leber hereditary optic neuroretinopathy. Am J Hum Genet 48(6):1147–1153. PubMed PMID: 1674640

Hyslop LA, Blakeley P, Craven L, Richardson J, Fogarty NM, Fragouli E, Lamb M, Wamaitha SE, Prathalingam N, Zhang Q, O'Keefe H, Takeda Y, Arizzi L, Alfarawati S, Tuppen HA, Irving L, Kalleas D, Choudhary M, Wells D, Murdoch AP, Turnbull DM, Niakan KK, Herbert M (2016) Towards clinical application of pronuclear transfer to prevent mitochondrial DNA disease. Nature 534(7607):383–386. doi:10.1038/nature18303. PubMed PMID: 27281217; PMCID: PMC5131843

Ishikawa K, Funayama T, Ohde H, Inagaki Y, Mashima Y (2005) Genetic variants of TP53 and EPHX1 in Leber's hereditary optic neuropathy and their relationship to age at onset. Jpn J Ophthalmol 49(2):121–126. PubMed PMID: 15838728

Ji Y, Jia X, Li S, Xiao X, Guo X, Zhang Q (2010) Evaluation of the X-linked modifier loci for Leber hereditary optic neuropathy with the G11778A mutation in Chinese. Mol Vis 16: 416–424. http://www.molvis.org/molvis/v16/a47/. PubMed PMID: 20300564

Ji F, Sharpley MS, Derbeneva O, Alves LS, Qian P, Wang Y, Chalkia D, Lvova M, Xu J, Yao W, Simon M, Platt J, Xu S, Angelin A, Davila A, Huang T, Wang PH, Chuang LM, Moore LG, Qian G, Wallace DC (2012) Mitochondrial DNA variant associated with Leber hereditary optic neuropathy and high-altitude Tibetans. Proc Natl Acad Sci U S A 109(19):7391–7396. doi:10.1073/pnas.1202484109. PubMed PMID: 22517755

Ji Y, Liang M, Zhang J, Zhu L, Zhang Z, Fu R, Liu X, Zhang M, Fu Q, Zhao F, Tong Y, Sun Y, Jiang P, Guan MX (2016) Mitochondrial ND1 variants in 1281 Chinese subjects with Leber's hereditary optic neuropathy. Invest Ophthalmol Vis Sci 57(6):2377–2389. doi:10.1167/iovs.16-19243. PubMed PMID: 27177320

Johns DR, Neufeld MJ, Park RD (1992) An ND-6 mitochondrial DNA mutation associated with Leber hereditary optic neuropathy. Biochem Biophys Res Commun 187(3):1551–1557. PubMed PMID: 1417830

Johnson MJ, Wallace DC, Ferris SD, Rattazzi MC, Cavalli-Sforza LL (1983) Radiation of human mitochondria DNA types analyzed by restriction endonuclease cleavage patterns. J Mol Evol 19(3–4):255–271. PubMed PMID: 6310133

Johri A, Calingasan NY, Hennessey TM, Sharma A, Yang L, Wille E, Chandra A, Beal MF (2012) Pharmacologic activation of mitochondrial biogenesis exerts widespread beneficial effects in a transgenic mouse model of Huntington's disease. Hum Mol Genet 21(5):1124–1137. doi:10.1093/hmg/ddr541. Epub 2011/11/19. PubMed PMID: 22095692

Jun AS, Brown MD, Wallace DC (1994a) A mitochondrial DNA mutation at np 14459 of the ND6 gene associated with maternally inherited Leber's hereditary optic neuropathy and dystonia. Proc Natl Acad Sci U S A 91(13):6206–6210. PubMed PMID: 8016139

Jun AS, Trounce IA, Brown MD, Shoffner JM, Kim YL, Wallace DC (1994b) Marked complex I deficiency in patients harboring the np14459 ND6 mitochondrial DNA mutation associated with Leber hereditary optic neuropathy and dystonia. (Abstract1311). Am J Hum Genet 55(Supplement):A225. PubMed PMID: Not in PubMed

Jun AS, Trounce IA, Brown MD, Shoffner JM, Wallace DC (1996) Use of transmitochondrial cybrids to assign a complex I defect to the mitochondrial DNA-encoded NADH dehydrogenase subunit 6 gene mutation at nucleotide pair 14459 that causes Leber hereditary optic neuropathy and dystonia. Mol Cell Biol 16(3):771–777. PubMed PMID: 8622678

Kang E, Wu J, Gutierrez NM, Koski A, Tippner-Hedges R, Agaronyan K, Platero-Luengo A, Martinez-Redondo P, Ma H, Lee Y, Hayama T, Van Dyken C, Wang X, Luo S, Ahmed R, Li Y, Ji D, Kayali R, Cinnioglu C, Olson S, Jensen J, Battaglia D, Lee D, Wu D, Huang T, Wolf DP, Temiakov D, Belmonte JC, Amato P, Mitalipov S (2016) Mitochondrial replacement in human oocytes carrying pathogenic mitochondrial DNA mutations. Nature 540(7632):270–275. doi:10.1038/nature20592. PubMed PMID: 27919073

Kazuno AA, Munakata K, Nagai T, Shimozono S, Tanaka M, Yoneda M, Kato N, Miyawaki A, Kato T (2006) Identification of mitochondrial DNA polymorphisms that alter mitochondrial matrix pH and intracellular calcium dynamics. PLoS Genet 2(8):e128. PubMed PMID: 16895436

Kenney MC, Hertzog D, Chak G, Atilano SR, Khatibi N, Soe K, Nobe A, Yang E, Chwa M, Zhu F, Memarzadeh M, King J, Langberg J, Small K, Nesburn AB, Boyer DS, Udar N (2013a) Mitochondrial DNA haplogroups confer differences in risk for age-related macular degeneration: a case control study. BMC Med Genet 14:4. doi:10.1186/1471-2350-14-4. PubMed PMID: 23302509

Kenney MC, Chwa M, Atilano SR, Pavlis JM, Falatoonzadeh P, Ramirez C, Malik D, Hsu T, Woo G, Soe K, Nesburn AB, Boyer DS, Kuppermann BD, Jazwinski SM, Miceli MV, Wallace DC, Udar N (2013b) Mitochondrial DNA variants mediate energy production and expression levels for CFH, C3 and EFEMP1 genes: implications for age-related macular degeneration. PLoS One 8(1):e54339. doi:10.1371/journal.pone.0054339. PubMed PMID: 23365660

Kenney MC, Chwa M, Atilano SR, Falatoonzadeh P, Ramirez C, Malik D, Tarek M, Caceres-Del-Carpio J, Nesburn AB, Boyer DS, Kuppermann BD, Vawter M, Michal Jazwinski S, Miceli M, Wallace DC, Udar N (2014) Inherited mitochondrial DNA variants can affect complement, inflammation and apoptosis pathways: insights into mitochondrial-nuclear interactions. Hum Mol Genet 23(13):3537–3551. doi:10.1093/hmg/ddu065. Epub 2014/03/04. PubMed PMID: 24584571 http://dx.doi.org/10.1093/hmg/ddu065

King MP, Attardi G (1989) Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. Science 246(4929):500–503. PubMed PMID: 2814477

Klopstock T, Yu-Wai-Man P, Dimitriadis K, Rouleau J, Heck S, Bailie M, Atawan A, Chattopadhyay S, Schubert M, Garip A, Kernt M, Petraki D, Rummey C, Leinonen M, Metz G, Griffiths PG, Meier T, Chinnery PF (2011) A randomized placebo-controlled trial of idebenone in Leber's hereditary optic neuropathy. Brain 134(9):2677–2686. doi:10.1093/brain/awr170. Epub 2011/07/27. PubMed PMID: 21788663

Koilkonda RD, Guy J (2011) Leber's hereditary optic neuropathy – gene therapy: from benchtop to bedside. J Ophthalmol 2011:179412. doi:10.1155/2011/179412. Epub 2011/01/22. PubMed PMID: 21253496

Koilkonda RD, Hauswirth WW, Guy J (2009) Efficient expression of self-complementary AAV in ganglion cells of the ex vivo primate retina. Mol Vis 15:2796–2802. doi: 00000 – open online access at http://www.molvis.org/molvis/v15/a295/. Epub 2009/12/19. PubMed PMID: 20019878

Koilkonda RD, Chou TH, Porciatti V, Hauswirth WW, Guy J (2010) Induction of rapid and highly efficient expression of the human ND4 complex I subunit in the mouse visual system by self-complementary adeno-associated virus. Arch Ophthalmol 128(7):876–883. doi:10.1001/archophthalmol.2010.135. Epub 2010/07/14. PubMed PMID: 20625049

Koopman WJ, Willems PH, Smeitink JA (2012) Monogenic mitochondrial disorders. N Engl J Med 366(12):1132–1141. doi:10.1056/NEJMra1012478. Epub 2012/03/23. PubMed PMID: 22435372

Lam BL, Feuer WJ, Abukhalil F, Porciatti V, Hauswirth WW, Guy J (2010) Leber hereditary optic neuropathy gene therapy clinical trial recruitment: year 1. Arch Ophthalmol 128(9):1129–1135. doi:10.1001/archophthalmol.2010.201. Epub 2010/09/15. PubMed PMID: 20837795

Lee HS, Ma H, Juanes RC, Tachibana M, Sparman M, Woodward J, Ramsey C, Xu J, Kang EJ, Amato P, Mair G, Steinborn R, Mitalipov S (2012) Rapid mitochondrial DNA segregation in

primate preimplantation embryos precedes somatic and germline bottleneck. Cell Rep 1 (5):506–515. doi:10.1016/j.celrep.2012.03.011. Epub 2012/06/16. PubMed PMID: 22701816

Liang M, Guan M, Zhao F, Zhou X, Yuan M, Tong Y, Yang L, Wei QP, Sun YH, Lu F, Qu J, Guan MX (2009) Leber's hereditary optic neuropathy is associated with mitochondrial ND1 T3394C mutation. Biochem Biophys Res Commun 383(3):286–292. doi:10.1016/j.bbrc.2009.03.097. Epub 2009/03/28. PubMed PMID: 19324017

Lin CS, Sharpley MS, Fan W, Waymire KG, Sadun A, Carelli V, Ross-Cisneros FN, Baciu P, Sung E, McManus MJ, Pan BX, Gil DW, MacGregor GR, Wallace DC (2012) A mouse mtDNA mutant model of Leber's hereditary optic neuropathy. Proc Natl Acad Sci U S A 109(49):20065–20070. doi:10.1073/pnas.1217113109. PubMed PMID: 23129651

Lopez Sanchez MI, Crowston JG, Mackey DA, Trounce IA (2016) Emerging mitochondrial therapeutic targets in optic neuropathies. Pharmacol Ther 165:132–152. doi:10.1016/j.pharmthera.2016.06.004. PubMed PMID: 27288727

MacGregor GR, Fan WW, Waymire KG, Wallace DC (2006) Generating animal models of human mitochondrial genetic disease using mouse ES cells. In: Notarianni E, Evans MJ (eds) Embryonic stem cells. Oxford University Press, New York, NY, pp 72–104

Maguire AM, Simonelli F, Pierce EA, Pugh EN Jr, Mingozzi F, Bennicelli J, Banfi S, Marshall KA, Testa F, Surace EM, Rossi S, Lyubarsky A, Arruda VR, Konkle B, Stone E, Sun J, Jacobs J, Dell'Osso L, Hertle R, Ma JX, Redmond TM, Zhu X, Hauck B, Zelenaia O, Shindler KS, Maguire MG, Wright JF, Volpe NJ, McDonnell JW, Auricchio A, High KA, Bennett J (2008) Safety and efficacy of gene transfer for Leber's congenital amaurosis. N Engl J Med 358 (21):2240–2248. doi:10.1056/NEJMoa0802315. Epub 2008/04/29. PubMed PMID: 18441370

Maguire AM, High KA, Auricchio A, Wright JF, Pierce EA, Testa F, Mingozzi F, Bennicelli JL, Ying GS, Rossi S, Fulton A, Marshall KA, Banfi S, Chung DC, Morgan JI, Hauck B, Zelenaia O, Zhu X, Raffini L, Coppieters F, De Baere E, Shindler KS, Volpe NJ, Surace EM, Acerra C, Lyubarsky A, Redmond TM, Stone E, Sun J, McDonnell JW, Leroy BP, Simonelli F, Bennett J (2009) Age-dependent effects of RPE65 gene therapy for Leber's congenital amaurosis: a phase 1 dose-escalation trial. Lancet 374(9701):1597–1605. doi:10.1016/S0140-6736(09)61836-5. PubMed PMID: 19854499. Epub 2009/10/27

Malfatti E, Bugiani M, Invernizzi F, de Souza CF, Farina L, Carrara F, Lamantea E, Antozzi C, Confalonieri P, Sanseverino MT, Giugliani R, Uziel G, Zeviani M (2007) Novel mutations of ND genes in complex I deficiency associated with mitochondrial encephalopathy. Brain 130(Pt 7):1894–1904. PubMed PMID: 17535832

Manfredi G, Fu J, Ojaimi J, Sadlock JE, Kwong JQ, Guy J, Schon EA (2002) Rescue of a deficiency in ATP synthesis by transfer of MTATP6, a mitochondrial DNA-encoded gene, to the nucleus. Nat Genet 30(4):394–399. PubMed PMID: 11925565

Mansergh FC, Chadderton N, Kenna PF, Gobbo OL, Farrar GJ (2014) Cell therapy using retinal progenitor cells shows therapeutic effect in a chemically-induced rotenone mouse model of Leber hereditary optic neuropathy. Eur J Hum Genet 22(11):1314–1320. doi:10.1038/ejhg.2014.26. PubMed PMID: 24569607; PMCID: PMC4200438

Marella M, Seo BB, Nakamaru-Ogiso E, Greenamyre JT, Matsuno-Yagi A, Yagi T (2008) Protection by the NDI1 gene against neurodegeneration in a rotenone rat model of Parkinson's disease. PLoS One 3(1):e1433. PubMed PMID: 18197244

Marella M, Seo BB, Thomas BB, Matsuno-Yagi A, Yagi T (2010) Successful amelioration of mitochondrial optic neuropathy using the yeast NDI1 gene in a rat animal model. PLoS One 5 (7):e11472. doi:10.1371/journal.pone.0011472. Epub 2010/07/16. PubMed PMID: 20628600

Mashima Y, Kigasawa K, Wakakura M, Oguchi Y (2000) Do idebenone and vitamin therapy shorten the time to achieve visual recovery in Leber hereditary optic neuropathy? J Neuroophthalmol 20(3):166–170. Epub 2000/09/23. PubMed PMID: 11001192

Matthews L, Enzinger C, Fazekas F, Rovira A, Ciccarelli O, Dotti MT, Filippi M, Frederiksen JL, Giorgio A, Kuker W, Lukas C, Rocca MA, De Stefano N, Toosy A, Yousry T, Palace J, on behalf of the Magnims network (2015) MRI in Leber's hereditary optic neuropathy:

the relationship to multiple sclerosis. J Neurol Neurosurg Psychiatry 86(5):537–542. doi:10.1136/jnnp-2014-308186. PubMed PMID: 25053773

Merriwether DA, Clark AG, Ballinger SW, Schurr TG, Soodyall H, Jenkins T, Sherry ST, Wallace DC (1991) The structure of human mitochondrial DNA variation. J Mol Evol 33(6):543–555. PubMed PMID: 1685753

Mishmar D, Ruiz-Pesini E, Mondragon-Palomino M, Procaccio V, Gaut B, Wallace DC (2006) Adaptive selection of mitochondrial complex I subunits during primate radiation. Gene 378: 11–18. PubMed PMID: 16828987

MITOMAP (2017) A human mitochondrial genome database. http://www.mitomap.org

Murphy MP (2009) How mitochondria produce reactive oxygen species. Biochem J 417(1):1–13. doi:10.1042/BJ20081386. Epub 2008/12/09. PubMed PMID: 19061483

Nashine S, Chwa M, Kazemian M, Thaker K, Lu S, Nesburn A, Kuppermann BD, Kenney MC (2016) Differential expression of complement markers in normal and AMD transmitochondrial cybrids. PLoS One 11(8):e0159828. doi: 10.1371/journal.pone.0159828. PubMed PMID: 27486856; PMCID: PMC4972370

Newman NJ, Biousse V, David R, Bhatti MT, Hamilton SR, Farris BK, Lesser RL, Newman SA, Turbin RE, Chen K, Keaney RP (2005) Prophylaxis for second eye involvement in leber hereditary optic neuropathy: an open-labeled, nonrandomized multicenter trial of topical brimonidine purite. Am J Ophthalmol 140(3):407–415. doi:10.1016/j.ajo.2005.03.058. Epub 2005/08/09. PubMed PMID: 16083844

Nicoletti A, Vasta R, Mostile G, Nicoletti G, Arabia G, Iliceto G, Lamberti P, Marconi R, Morgante L, Barone P, Quattrone A, Zappia M (2016) Gender effect on non-motor symptoms in Parkinson's disease: are men more at risk? Parkinsonism Relat Disord. ePub ahead of print. http://dx.doi.org/10.1016/j.parkreldis.2016.12.008 doi: 10.1016/j.parkreldis.2016.12.008. PubMed PMID: 28017549

Oca-Cossio J, Kenyon L, Hao H, Moraes CT (2003) Limitations of allotopic expression of mitochondrial genes in mammalian cells. Genetics 165(2):707–720. PubMed PMID: 14573482

Ortiz RG, Newman NJ, Shoffner JM, Kaufman AE, Koontz DA, Wallace DC (1993) Variable retinal and neurologic manifestations in patients harboring the mitochondrial DNA 8993 mutation. Arch Ophthalmol 111(11):1525–1530. PubMed PMID: 8240109

Palace J (2009) Multiple sclerosis associated with Leber's hereditary optic neuropathy. J Neurol Sci 286(1–2):24–27. doi:10.1016/j.jns.2009.09.009. Epub 2009/10/06. PubMed PMID: 19800080

Park JS, Li YF, Bai Y (2007) Yeast NDI1 improves oxidative phosphorylation capacity and increases protection against oxidative stress and cell death in cells carrying a Leber's hereditary optic neuropathy mutation. Biochim Biophys Acta 1772(5):533–542. Epub 2007/02/27. PubMed PMID: 17320357. doi: S0925-4439(07)00029-4 [pii] 10.1016/j.bbadis.2007.01.009

Paull D, Emmanuele V, Weiss KA, Treff N, Stewart L, Hua H, Zimmer M, Kahler DJ, Goland RS, Noggle SA, Prosser R, Hirano M, Sauer MV, Egli D (2013) Nuclear genome transfer in human oocytes eliminates mitochondrial DNA variants. Nature 493(7434):632–637. doi:10.1038/nature11800. Epub 2012/12/21. PubMed PMID: 23254936

Pedram A, Razandi M, Wallace DC, Levin ER (2006) Functional estrogen receptors in the mitochondria of breast cancer cells. Mol Biol Cell 17(5):2125–2137. PubMed PMID: 16495339

Perales-Clemente E, Fernandez-Silva P, Acin-Perez R, Perez-Martos A, Enriquez JA (2011) Allotopic expression of mitochondrial-encoded genes in mammals: achieved goal, undemonstrated mechanism or impossible task? Nucleic Acids Res 39(1):225–234. doi:10.1093/nar/gkq769. Epub 2010/09/09. PubMed PMID: 20823090

Petruzzella V, Tessa A, Torraco A, Fattori F, Dotti MT, Bruno C, Cardaioli E, Papa S, Federico A, Santorelli FM (2007) The NDUFB11 gene is not a modifier in Leber hereditary optic neuropathy. Biochem Biophys Res Commun 355(1):181–187. PubMed PMID: 17292333

Pfeffer G, Majamaa K, Turnbull DM, Thorburn D, Chinnery PF (2012) Treatment for mitochondrial disorders. Cochrane Database Syst Rev 4:CD004426. doi:10.1002/14651858.CD004426.pub3. PubMed PMID: 22513923

Pfeffer G, Burke A, Yu-Wai-Man P, Compston DA, Chinnery PF (2013) Clinical features of MS associated with Leber hereditary optic neuropathy mtDNA mutations. Neurology 81(24): 2073–2781. doi:10.1212/01.wnl.0000437308.22603.43. PubMed PMID: 24198293; PMCID: PMC3863351

Phasukkijwatana N, Kunhapan B, Stankovich J, Chuenkongkaew WL, Thomson R, Thornton T, Bahlo M, Mushiroda T, Nakamura Y, Mahasirimongkol S, Tun AW, Srisawat C, Limwongse C, Peerapittayamongkol C, Sura T, Suthammarak W, Lertrit P (2010) Genome-wide linkage scan and association study of PARL to the expression of LHON families in Thailand. Hum Genet 128(1):39–49. doi:10.1007/s00439-010-0821-8. PubMed PMID: 20407791

Picard M, Zhang J, Hancock S, Derbeneva O, Golhar R, Golik P, O'Hearn S, Levy S, Potluri P, Lvova M, Davila A, Lin CS, Perin JC, Rappaport EF, Hakonarson H, Trounce IA, Procaccio V, Wallace DC (2014) Progressive increase in mtDNA 3243A>G heteroplasmy causes abrupt transcriptional reprogramming. Proc Natl Acad Sci U S A 111(38):E4033–E4E42. doi:10.1073/pnas.1414028111. PubMed PMID: 25192935

Picard M, Wallace DC, Burelle Y (2016) The rise of mitochondria in medicine. Mitochondrion 30: 105–116. doi:10.1016/j.mito.2016.07.003. PubMed PMID: 27423788; PMCID: PMC5023480

Pisano A, Preziuso C, Iommarini L, Perli E, Grazioli P, Campese AF, Maresca A, Montopoli M, Masuelli L, Sadun AA, d'Amati G, Carelli V, Ghelli A, Giordano C (2015) Targeting estrogen receptor beta as preventive therapeutic strategy for Leber's hereditary optic neuropathy. Hum Mol Genet 24(24):6921–6931. doi:10.1093/hmg/ddv396. PubMed PMID: 26410888

Pryde KR, Hirst J (2011) Superoxide is produced by the reduced flavin in mitochondrial complex I: a single, unified mechanism that applies during both forward and reverse electron transfer. J Biol Chem 286(20):18056–18065. doi:10.1074/jbc.M110.186841. Epub 2011/03/12. PubMed PMID: 21393237

Puomila A, Hamalainen P, Kivioja S, Savontaus ML, Koivumaki S, Huoponen K, Nikoskelainen E (2007) Epidemiology and penetrance of Leber hereditary optic neuropathy in Finland. Eur J Hum Genet 15(10):1079–1089. PubMed PMID: 17406640

Qi X, Lewin AS, Hauswirth WW, Guy J (2003a) Suppression of complex I gene expression induces optic neuropathy. Ann Neurol 53(2):198–205. PubMed PMID: 12557286

Qi X, Lewin AS, Hauswirth WW, Guy J (2003b) Optic neuropathy induced by reductions in mitochondrial superoxide dismutase. Invest Ophthalmol Vis Sci 44(3):1088–1096. PubMed PMID: 12601034

Qi X, Sun L, Hauswirth WW, Lewin AS, Guy J (2007a) Use of mitochondrial antioxidant defenses for rescue of cells with a Leber hereditary optic neuropathy-causing mutation. Arch Ophthalmol 125(2):268–272. doi:10.1001/archopht.125.2.268. Epub 2007/02/14. PubMed PMID: 17296905

Qi X, Sun L, Lewin AS, Hauswirth WW, Guy J (2007b) The mutant human ND4 subunit of complex I induces optic neuropathy in the mouse. Invest Ophthalmol Vis Sci 48(1):1–10. doi:10.1167/iovs.06-0789. Epub 2007/01/02. PubMed PMID: 17197509

Qu J, Wang Y, Tong Y, Zhou X, Zhao F, Yang L, Zhang S, Zhang J, West CE, Guan MX (2010) Leber's hereditary optic neuropathy affects only female matrilineal relatives in two Chinese families. Invest Ophthalmol Vis Sci 51(10):4906–4912. doi:10.1167/iovs.09-5027. Epub 2010/05/04. PubMed PMID: 20435583

Ramos CVF, Bellusci C, Savini G, Carbonelli M, Berezovsky A, Tamaki C, Cinoto R, Sacai PY, Moraes-Filho MN, Miura HM, Valentino ML, Iommarini L, De Negri AM, Sadun F, Cortelli P, Montagna P, Salomao SR, Sadun AA, Carelli V, Barboni P (2009) Association of optic disc size with development and prognosis of Leber's hereditary optic neuropathy. Invest Oph-

thalmol Vis Sci 50(4):1666–1674. doi:10.1167/iovs.08-2695. Epub 2008/12/23. PubMed PMID: 19098324

Reddy P, Ocampo A, Suzuki K, Luo J, Bacman SR, Williams SL, Sugawara A, Okamura D, Tsunekawa Y, Wu J, Lam D, Xiong X, Montserrat N, Esteban CR, Liu GH, Sancho-Martinez I, Manau D, Civico S, Cardellach F, Del Mar O'CM, Campistol J, Zhao H, Campistol JM, Moraes CT, Izpisua Belmonte JC (2015) Selective elimination of mitochondrial mutations in the germline by genome editing. Cell 161(3):459–469. doi:10.1016/j.cell.2015.03.051. PubMed PMID: 25910206; PMCID: PMC4505837

Ruiz-Pesini E, Wallace DC (2006) Evidence for adaptive selection acting on the tRNA and rRNA genes of the human mitochondrial DNA. Hum Mutat 27(11):1072–1081. PubMed PMID: 16947981

Sadun AA, Carelli V, Salomao SR, Berezovsky A, Quiros PA, Sadun F, DeNegri AM, Andrade R, Moraes M, Passos A, Kjaer P, Pereira J, Valentino ML, Schein S, Belfort R (2003) Extensive investigation of a large Brazilian pedigree of 11778/haplogroup J Leber hereditary optic neuropathy. Am J Ophthalmol 136(2):231–238. Epub 2003/07/31. PubMed PMID: 12888043

Sadun AA, Salomao SR, Berezovsky A, Sadun F, Denegri AM, Quiros PA, Chicani F, Ventura D, Barboni P, Sherman J, Sutter E, Belfort R Jr, Carelli V (2006) Subclinical carriers and conversions in Leber hereditary optic neuropathy: a prospective psychophysical study. Trans Am Ophthalmol Soc 104:51–61. Epub 2007/05/02. PubMed PMID: 17471325

Sadun AA, La Morgia C, Carelli V (2011) Leber's hereditary optic neuropathy. Curr Treat Options Neurol 13(1):109–117. doi:10.1007/s11940-010-0100-y. Epub 2010/11/11. PubMed PMID: 21063922

Sadun AA, Chicani CF, Ross-Cisneros FN, Barboni P, Thoolen M, Shrader WD, Kubis K, Carelli V, Miller G (2012) Effect of EPI-743 on the clinical course of the mitochondrial disease Leber hereditary optic neuropathy. Arch Neurol 69(3):331–338. doi:10.1001/archneurol.2011.2972. PubMed PMID: 22410442

Sala G, Trombin F, Beretta S, Tremolizzo L, Presutto P, Montopoli M, Fantin M, Martinuzzi A, Carelli V, Ferrarese C (2008) Antioxidants partially restore glutamate transport defect in Leber hereditary optic neuropathy cybrids. J Neurosci Res 86(15):3331–3337. doi:10.1002/jnr.21773. Epub 2008/07/11. PubMed PMID: 18615737

Salomao SR, Berezovsky A, Andrade RE, Belfort R Jr, Carelli V, Sadun AA (2004) Visual electrophysiologic findings in patients from an extensive Brazilian family with Leber's hereditary optic neuropathy. Doc Ophthalmol 108(2):147–155. Epub 2004/10/01. PubMed PMID: 15455797

Santra S, Gilkerson RW, Davidson M, Schon EA (2004) Ketogenic treatment reduces deleted mitochondrial DNAs in cultured human cells. Ann Neurol 56(5):662–669. PubMed PMID: 15389892

Schaefer AM, McFarland R, Blakely EL, He L, Whittaker RG, Taylor RW, Chinnery PF, Turnbull DM (2008) Prevalence of mitochondrial DNA disease in adults. Ann Neurol 63(1):35–39. doi:10.1002/ana.21217. PubMed PMID: 17886296

Schriner SE, Linford NJ, Martin GM, Treuting P, Ogburn CE, Emond M, Coskun PE, Ladiges W, Wolf N, Van Remmen H, Wallace DC, Rabinovitch PS (2005) Extension of murine life span by overexpression of catalase targeted to mitochondria. Science 308(5730):1909–1911. PubMed PMID: 15879174

Seo BB, Kitajima-Ihara T, Chan EK, Scheffler IE, Matsuno-Yagi A, Yagi T (1998) Molecular remedy of complex I defects: rotenone-insensitive internal NADH-quinone oxidoreductase of *Saccharomyces cerevisiae* mitochondria restores the NADH oxidase activity of complex I-deficient mammalian cells. Proc Natl Acad Sci U S A 95(16):9167–9171. PubMed PMID: 9689052

Seo BB, Wang J, Flotte TR, Yagi T, Matsuno-Yagi A (2000) Use of the NADH-quinone oxidoreductase (NDI1) gene of *Saccharomyces cerevisiae* as a possible cure for complex I defects in human cells. J Biol Chem 275(48):37774–37778. PubMed PMID: 10982813

Seo BB, Nakamaru-Ogiso E, Flotte TR, Yagi T, Matsuno-Yagi A (2002) A single-subunit NADH-quinone oxidoreductase renders resistance to mammalian nerve cells against complex I inhibition. Mol Ther 6(3):336–341. PubMed PMID: 12231169

Seo BB, Nakamaru-Ogiso E, Cruz P, Flotte TR, Yagi T, Matsuno-Yagi A (2004) Functional expression of the single subunit NADH dehydrogenase in mitochondria in vivo: a potential therapy for complex I deficiencies. Hum Gene Ther 15(9):887–895. PubMed PMID: 15353043

Seo BB, Nakamaru-Ogiso E, Flotte TR, Matsuno-Yagi A, Yagi T (2006) In vivo complementation of complex I by the yeast Ndi1 enzyme. Possible application for treatment of Parkinson disease. J Biol Chem 281(20):14250–14255. PubMed PMID: 16543240

Shankar SP, Fingert JH, Carelli V, Valentino ML, King TM, Daiger SP, Salomao SR, Berezovsky A, Belfort R Jr, Braun TA, Sheffield VC, Sadun AA, Stone EM (2008) Evidence for a novel X-linked modifier locus for Leber hereditary optic neuropathy. Ophthalmic Genet 29(1):17–24. PubMed PMID: 18363168

Sharpley MS, Marciniak C, Eckel-Mahan K, McManus MJ, Crimi M, Waymire K, Lin CS, Masubuchi S, Friend N, Koike M, Chalkia D, MacGregor GR, Sassone-Corsi P, Wallace DC (2012) Heteroplasmy of mouse mtDNA Is genetically unstable and results in altered behavior and cognition. Cell 151(2):333–343. doi:10.1016/j.cell.2012.09.004. PubMed PMID: 23063123

Shibata K, Shibagaki Y, Nagai C, Iwata M (1999) Visual evoked potentials and electroretinograms in an early stage of Leber's hereditary optic neuropathy. J Neurol 246(9):847–849. doi:10.1007/s004150050468. Epub 1999/10/20. PubMed PMID: 10525988

Shidara Y, Yamagata K, Kanamori T, Nakano K, Kwong JQ, Manfredi G, Oda H, Ohta S (2005) Positive contribution of pathogenic mutations in the mitochondrial genome to the promotion of cancer by prevention from apoptosis. Cancer Res 65(5):1655–1663. doi:10.1158/0008-5472.CAN-04-2012. Epub 2005/03/09. PubMed PMID: 15753359

Shoffner JM, Lott MT, Lezza AM, Seibel P, Ballinger SW, Wallace DC (1990) Myoclonic epilepsy and ragged-red fiber disease (MERRF) is associated with a mitochondrial DNA tRNA^{Lys} mutation. Cell 61(6):931–937. PubMed PMID: 2112427

Silva JM, Wong A, Carelli V, Cortopassi GA (2009) Inhibition of mitochondrial function induces an integrated stress response in oligodendroglia. Neurobiol Dis 34(2):357–365. doi:10.1016/j.nbd.2009.02.005. Epub 2009/02/24. PubMed PMID: 19233273

Simonelli F, Maguire AM, Testa F, Pierce EA, Mingozzi F, Bennicelli JL, Rossi S, Marshall K, Banfi S, Surace EM, Sun J, Redmond TM, Zhu X, Shindler KS, Ying GS, Ziviello C, Acerra C, Wright JF, McDonnell JW, High KA, Bennett J, Auricchio A (2010) Gene therapy for Leber's congenital amaurosis is safe and effective through 1.5 years after vector administration. Mol Ther 18(3):643–650. doi:10.1038/mt.2009.277. Epub 2009/12/03. PubMed PMID: 19953081

Srivastava S, Moraes CT (2001) Manipulating mitochondrial DNA heteroplasmy by a mitochondrially targeted restriction endonuclease. Hum Mol Genet 10(26):3093–3099. Epub 2001/12/26. PubMed PMID: 11751691

Strauss KA, Dubiner L, Simon M, Zaragoza M, Sengupta PP, Li P, Narula N, Dreike S, Platt J, Procaccio V, Ortiz-Gonzalez XR, Puffenberger EG, Kelley RI, Morton DH, Narula J, Wallace DC (2013) Severity of cardiomyopathy associated with adenine nucleotide translocator-1 deficiency correlates with mtDNA haplogroup. Proc Natl Acad Sci U S A 110(9):3253–3458. doi:10.1073/pnas.1300690110. Epub 2013/02/13. PubMed PMID: 23401503

Sweeney MG, Davis MB, Lashwood A, Brockington M, Toscano A, Harding AE (1992) Evidence against an X-linked locus close to DXS7 determining visual loss susceptibility in British and Italian families with Leber hereditary optic neuropathy. Am J Hum Genet 51(4):741–748. Epub 1992/10/01. PubMed PMID: 1415219

Sylvestre J, Margeot A, Jacq C, Dujardin G, Corral-Debrinski M (2003a) The role of the 3′ untranslated region in mRNA sorting to the vicinity of mitochondria is conserved from yeast to human cells. Mol Biol Cell 14(9):3848–3856. doi: 10.1091/mbc.E03-02-0074. Epub 2003/09/16. PubMed PMID: 12972568

Sylvestre J, Vialette S, Corral Debrinski M, Jacq C (2003b) Long mRNAs coding for yeast mitochondrial proteins of prokaryotic origin preferentially localize to the vicinity of mitochondria. Genome Biol 4(7):R44. doi:10.1186/gb-2003-4-7-r44. Epub 2003/07/08. PubMed PMID: 12844360

Tachibana M, Amato P, Sparman M, Woodward J, Sanchis DM, Ma H, Gutierrez NM, Tippner-Hedges R, Kang E, Lee HS, Ramsey C, Masterson K, Battaglia D, Lee D, Wu D, Jensen J, Patton P, Gokhale S, Stouffer R, Mitalipov S (2013) Towards germline gene therapy of inherited mitochondrial diseases. Nature 493(7434):627–631. doi:10.1038/nature11647. Epub 2012/10/30. PubMed PMID: 23103867

Titov DV, Cracan V, Goodman RP, Peng J, Grabarek Z, Mootha VK (2016) Complementation of mitochondrial electron transport chain by manipulation of the NAD+/NADH ratio. Science 352(6282):231–235. doi:10.1126/science.aad4017. PubMed PMID: 27124460; PMCID: PMC4850741

Torroni A, Petrozzi M, D'Urbano L, Sellitto D, Zeviani M, Carrara F, Carducci C, Leuzzi V, Carelli V, Barboni P, De Negri A, Scozzari R (1997) Haplotype and phylogenetic analyses suggest that one European-specific mtDNA background plays a role in the expression of Leber hereditary optic neuropathy by increasing the penetrance of the primary mutations 11778 and 14484. Am J Hum Genet 60(5):1107–1121. PubMed PMID: 9150158

Towheed A, Markantone DM, Crain AT, Celotto AM, Palladino MJ (2014) Small mitochondrial-targeted RNAs modulate endogenous mitochondrial protein expression in vivo. Neurobiol Dis 69:15–22

Trounce IA, Kim YL, Jun AS, Wallace DC (1996) Assessment of mitochondrial oxidative phosphorylation in patient muscle biopsies, lymphoblasts, and transmitochondrial cell lines. Methods Enzymol 264:484–509. PubMed PMID: 8965721

Udar N, Atilano SR, Memarzadeh M, Boyer DS, Chwa M, Lu S, Maguen B, Langberg J, Coskun P, Wallace DC, Nesburn AB, Khatibi N, Hertzog D, Le K, Hwang D, Kenney MC (2009) Mitochondrial DNA haplogroups associated with age-related macular degeneration. Invest Ophthalmol Vis Sci 50(6):2966–2974. PubMed PMID: 19151382

Van Bergen NJ, Crowston JG, Craig JE, Burdon KP, Kearns LS, Sharma S, Hewitt AW, Mackey DA, Trounce IA (2015) Measurement of systemic mitochondrial function in advanced primary open-angle glaucoma and Leber hereditary optic neuropathy. PLoS One 10(10):e0140919. doi:10.1371/journal.pone.0140919. PubMed PMID: 26496696; PMCID: PMC4619697

Ventura DF, Gualtieri M, Oliveira AG, Costa MF, Quiros P, Sadun F, de Negri AM, Salomao SR, Berezovsky A, Sherman J, Sadun AA, Carelli V (2007) Male prevalence of acquired color vision defects in asymptomatic carriers of Leber's hereditary optic neuropathy. Invest Ophthalmol Vis Sci 48(5):2362–2370. PubMed PMID: 17460303

Vergani L, Martinuzzi A, Carelli V, Cortelli P, Montagna P, Schievano G, Carrozzo R, Angelini C, Lugaresi E (1995) MtDNA mutations associated with Leber's hereditary optic neuropathy: studies on cytoplasmic hybrid (cybrid) cells. Biochem Biophys Res Commun 210(3):880–888. PubMed PMID: 7763260

Vilkki J, Ott J, Savontaus ML, Aula P, Nikoskelainen EK (1991) Optic atrophy in Leber hereditary optic neuroretinopathy is probably determined by an X-chromosomal gene closely linked to DXS7. Am J Hum Genet 48(3):486–491. PubMed PMID: 1998335

Wallace DC (1981) Assignment of the chloramphenicol resistance gene to mitochondrial deoxyribonucelic acid and analysis of its expression in cultured human cells. Mol Cell Biol 1(8):697–710. PubMed PMID: 9279383

Wallace DC (1982a) Cytoplasmic inheritance of chloramphenicol resistance in mammalian cells. Chapter 12. In: Shay JW (ed) Techniques in somatic cell genetics. Plenum Press, New York, pp 159–187

Wallace DC (1982b) Structure and evolution of organelle genomes. Microbiol Rev 46(2): 208–240. PubMed PMID: 6750346

Wallace DC (1987) Maternal genes: mitochondrial diseases. In: McKusick VA, Roderick TH, Mori J, Paul MW (eds) Medical and experimental mammalian genetics: a perspective. A.R. Liss, Inc., for the March of Dimes Foundation, New York, pp 137–190

Wallace DC (2013a) Mitochondrial bioenergetic etiology of disease. J Clin Invest 123(4): 1405–1412. doi:10.1172/JCI61398. PubMed PMID: 23543062

Wallace DC (2013b) Bioenergetics in human evolution and disease:Implications for the origins of biological complexity and the missing genetic variation of common diseases. Philos Trans R Soc Lond B Biol Sci 368(1622):20120267. doi:10.1098/rstb.2012.0267. PubMed PMID: 23754818

Wallace DC (2015) Mitochondrial DNA variation in human radiation and disease. Cell 163(1): 33–38. doi:10.1016/j.cell.2015.08.067. PubMed PMID: 26406369

Wallace DC (2016) Genetics: mitochondrial DNA in evolution and disease. Nature 535(7613): 498–500. doi:10.1038/nature18902. PubMed PMID: 27383787

Wallace DC, Fan W (2010) Energetics, epigenetics, mitochondrial genetics. Mitochondrion 10(1): 12–31. doi:10.1016/j.mito.2009.09.006. PubMed PMID: 19796712

Wallace DC, Bunn CL, Eisenstadt JM (1975) Cytoplasmic transfer of chloramphenicol resistance in human tissue culture cells. J Cell Biol 67(1):174–188. PubMed PMID: 1176530

Wallace DC, Singh G, Lott MT, Hodge JA, Schurr TG, Lezza AM, Elsas LJ, Nikoskelainen EK (1988a) Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. Science 242(4884):1427–1430. PubMed PMID: 3201231

Wallace DC, Zheng X, Lott MT, Shoffner JM, Hodge JA, Kelley RI, Epstein CM, Hopkins LC (1988b) Familial mitochondrial encephalomyopathy (MERRF): genetic, pathophysiological, and biochemical characterization of a mitochondrial DNA disease. Cell 55(4):601–610. PubMed PMID: 3180221

Wallace DC, Lott MT, Procaccio V (2007) Mitochondrial genes in degenerative diseases, cancer and aging. In: Rimoin DL, Connor JM, Pyeritz RE, Korf BR (eds) Emery and Rimoin's principles and practice of medical genetics, 5th edn. Churchill Livingstone Elsevier, Philadelphia, pp 194–298

Wallace DC, Fan W, Procaccio V (2010) Mitochondrial energetics and therapeutics. Annu Rev Path 5:297–348. doi:10.1146/annurev.pathol.4.110807.092314. PubMed PMID: 20078222

Wang G, Chen HW, Oktay Y, Zhang J, Allen EL, Smith GM, Fan KC, Hong JS, French SW, McCaffery JM, Lightowlers RN, Morse HC 3rd, Koehler CM, Teitell MA (2010) PNPASE regulates RNA import into mitochondria. Cell 142(3):456–467. doi:10.1016/j.cell.2010.06.035. Epub 2010/08/10. PubMed PMID: 20691904

Wang G, Shimada E, Koehler CM, Teitell MA (2012a) PNPASE and RNA trafficking into mitochondria. Biochim Biophys Acta 1819(9–10):998–1007. doi:10.1016/j.bbagrm.2011.10.001. Epub 2011/10/26. PubMed PMID: 22023881

Wang G, Shimada E, Zhang J, Hong JS, Smith GM, Teitell MA, Koehler CM (2012b) Correcting human mitochondrial mutations with targeted RNA import. Proc Natl Acad Sci U S A 109(13): 4850–4855. doi:10.1073/pnas.1116792109. Epub 2012/03/14. PubMed PMID: 22411789

Wenz T, Diaz F, Spiegelman BM, Moraes CT (2008) Activation of the PPAR/PGC-1alpha pathway prevents a bioenergetic deficit and effectively improves a mitochondrial myopathy phenotype. Cell Metab 8(3):249–256. PubMed PMID: 18762025

Wenz T, Diaz F, Spiegelman BM, Moraes CT (2016) Retraction notice to: activation of the PPAR/PGC-1alpha pathway prevents a bioenergetic deficit and effectively improves a mitochondrial myopathy phenotype. Cell Metab 24(6):889. doi:10.1016/j.cmet.2016.11.006. PubMed PMID: 27974182; PMCID: PMC5206892

Wong A, Cavelier L, Collins-Schramm HE, Seldin MF, McGrogan M, Savontaus ML, Cortopassi GA (2002) Differentiation-specific effects of LHON mutations introduced into neuronal NT2 cells. Hum Mol Genet 11(4):431–438. PubMed PMID: 11854175

Yamada M, Emmanuele V, Sanchez-Quintero MJ, Sun B, Lallos G, Paull D, Zimmer M, Pagett S, Prosser RW, Sauer MV, Hirano M, Egli D (2016) Genetic drift can compromise mitochondrial

replacement by nuclear transfer in human oocytes. Cell Stem Cell 18(6):749–754. doi:10.1016/j.stem.2016.04.001. PubMed PMID: 27212703

Yu H, Koilkonda RD, Chou TH, Porciatti V, Ozdemir SS, Chiodo V, Boye SL, Boye SE, Hauswirth WW, Lewin AS, Guy J (2012) Gene delivery to mitochondria by targeting modified adenoassociated virus suppresses Leber's hereditary optic neuropathy in a mouse model. Proc Natl Acad Sci U S A 109(20):E1238–E1247. doi:10.1073/pnas.1119577109. Epub 2012/04/24. PubMed PMID: 22523243

Yu H, Koilkonda RD, Chou TH, Porciatti V, Mehta A, Hentall ID, Chiodo VA, Boye SL, Hauswirth WW, Lewin AS, Guy J (2015) Consequences of zygote injection and germline transfer of mutant human mitochondrial DNA in mice. Proc Natl Acad Sci U S A 112(42):E5689–E5E98. doi:10.1073/pnas.1506129112. PubMed PMID: 26438859

Yu-Wai-Man P (2015) Therapeutic approaches to inherited optic neuropathies. Semin Neurol 35(5):578–586. doi:10.1055/s-0035-1563574. PubMed PMID: 26444403

Yu-Wai-Man P, Chinnery PF (1993) Leber hereditary optic neuropathy [updated 2016 Jun 23]. In: Pagon RA, Adam MP, Ardinger HH, Wallace SE, Amemiya A, Bean LJH, Bird TD, Fong CT, Mefford HC, Smith RJH, Stephens K (eds) GeneReviews®. Seattle, WA

Yu-Wai-Man P, Griffiths PG, Chinnery PF (2011) Mitochondrial optic neuropathies – disease mechanisms and therapeutic strategies. Prog Retin Eye Res 30(2):81–114. doi:10.1016/j.preteyeres.2010.11.002. Epub 2010/11/30. PubMed PMID: 21112411

Yu-Wai-Man P, Spyropoulos A, Duncan HJ, Guadagno JV, Chinnery PF (2016) A multiple sclerosis-like disorder in patients with OPA1 mutations. Ann Clin Transl Neurol 3(9):723–729. doi:10.1002/acn3.323. PubMed PMID: 27656661; PMCID: PMC5018584

Zhang M, Zhou X, Li C, Zhao F, Zhang J, Yuan M, Sun YH, Wang J, Tong Y, Liang M, Yang L, Cai W, Wang L, Qu J, Guan MX (2010) Mitochondrial haplogroup M9a specific variant ND1 T3394C may have a modifying role in the phenotypic expression of the LHON-associated ND4 G11778A mutation. Mol Genet Metab 101(2–3):192–199. doi:10.1016/j.ymgme.2010.07.014. Epub 2010/08/24. PubMed PMID: 20728388

Zullo SJ, Parks WT, Chloupkova M, Wei B, Weiner H, Fenton WA, Eisenstadt JM, Merril CR (2005) Stable transformation of CHO cells and human NARP cybrids confers oligomycin resistance (oli(r)) following transfer of a mitochondrial DNA-encoded oli(r) ATPase6 gene to the nuclear genome: a model system for mtDNA gene therapy. Rejuvenation Res 8(1):18–28. doi:10.1089/rej.2005.8.18. Epub 2005/03/31. PubMed PMID: 15798371

Equipping Physiologists with an Informatics Tool Chest: Toward an Integerated Mitochondrial Phenome

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Contents

Abstract

Understanding the complex involvement of mitochondrial biology in disease development often requires the acquisition, analysis, and integration of large-scale molecular and phenotypic data. An increasing number of bioinformatics tools are currently employed to aid in mitochondrial investigations, most notably in predicting or corroborating the spatial and temporal dynamics of mitochondrial molecules, in retrieving structural data of mitochondrial components, and

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H. Singh, S.-S. Sheu (eds.), *Pharmacology of Mitochondria*,
Handbook of Experimental Pharmacology 240, DOI 10.1007/164_2016_93

in aggregating as well as transforming mitochondrial centric biomedical knowledge. With the increasing prevalence of complex Big Data from omics experiments and clinical cohorts, informatics tools have become indispensable in our quest to understand mitochondrial physiology and pathology. Here we present an overview of the various informatics resources that are helping researchers explore this vital organelle and gain insights into its form, function, and dynamics.

Keywords
Computation • Data science • FAIR data • Metadata • Omics • Open access • Visualization

1 Introduction

Mitochondria play a major role in a range of diseases and are promising targets for therapeutic approaches to neurodegenerative and metabolic disorders, ischemia-reperfusion injury and cardiomyopathies, and various forms of cancer (Lesnefsky et al. 2001; Vlasblom et al. 2014). In spite of this, very few mitochondrial drugs have completed clinical trials (Walters et al. 2012). Initially described as "bioblasts" in 1890 by Altmann, mitochondria are of vital importance in eukaryotic systems (Altmann 1894). In the 1940s, Claude developed methods for cell fractionation and differential centrifugation that allowed the separation of mitochondria from the cytosol and a closer study of these fascinating organelles in isolation (Bensley and Hoerr 1934; Claude and Fullam 1945; Claude 1946a, b). He established the localization of respiratory enzymes to the mitochondrion; Kennedy and Lehninger further identified this as the site of the citric acid cycle, oxidative phosphorylation, and fatty acid oxidation (Kennedy and Lehninger 1949). The distinctive dual-membrane characteristic of the mitochondrion and the organization of cristae within its matrix were uncovered in 1952 with the first high-resolution electron micrographs of isolated mitochondria by Palade (1952, 1953) (Daems and Wisse 1966). Imaging approaches also provided the means for the discovery of mitochondrial DNA (Nass 1963; Nass and Nass 1963; Schatz et al. 1964) which ultimately became the first component of the human genome to be completely sequenced (Anderson et al. 1981) more than 20 years before the massive Human Genome Project completed their goal of sequencing the entire human genome (Lander et al. 2001).

Technological advances and physiological discoveries have fomented renaissance periods in mitochondrial research. A notable example is the work of Peter Mitchell leading to the chemiosmotic theory (Mitchell 1961, 1966, 1968). Eventually accepted 20 years after its introduction, Mitchell's proposal broadened the scope from basic molecular biology and biochemistry to a more physiological approach, including explorations of the intricacies of the potassium cycle and matrix volume regulation through the study of channel and carrier membrane biology. These approaches have become progressively more advanced, leading to a

greater understanding of mitochondrial physiology and its role in health and disease. The scientific community is now progressing from the classical, organelle-based study of mitochondria to the digital era of the informatics-based mitochondrial phenome. Informatics combines computer science, engineering, and statistics to develop tools and methods that empower researchers to navigate biological data, put it into context, and extract knowledge. Under the increasing influx of data, prowess in data science methods and awareness of available informatics resources are becoming essential components of the modern researcher's tool chest.

The most common high-throughput data come in the form of omics datasets, which assess the global conditions within the cell through one dimension of data, be it whole-genome sequencing for genomics, RNA-sequencing (RNA-seq) for transcriptomics, tandem mass spectrometry (MS/MS) for proteomics, or NMR spectroscopy for metabolomics studies (Field et al. 2009). Integrating levels of expression across many dimensions can elucidate physiological mechanisms underlying healthy and diseased phenotypes not revealed or biasedly portrayed by a single dimension. Developments in these technologies have manifested a dramatic increase in the sheer volume of publicly available scientific data; genomics data has been growing at a rate that exceeds Moore's law by a factor of 4 since 2008 (Gomez-Cabrero et al. 2014; O'Driscoll et al. 2013). This deluge of data has presented unique and unforeseen challenges. Omics investigations rely heavily on effective database management and annotation to contextualize molecular data and infer biological significance through statistical enrichment and class discovery techniques. The completeness and precision of existing annotations are therefore instrumental to harness omics techniques for disease phenotyping and mechanistic investigations. In light of these challenges, funding agencies, research organizations, and publishers around the world are adopting FAIR data principles, which maintain that data should be findable, accessible, interoperable, and reusable (FORCE11 2014). Building on these principles, omics datasets and analysis platforms must also be citable and scalable to allow the attribution of the work to the appropriate research groups and expansion to new and improved techniques with larger capacity. In this changing landscape, the concept of open-access data is gaining traction, asserting that data should be freely available for researchers to use, reuse, and disseminate (Molloy 2011).

This chapter provides an overview of the informatics tools and resources available to the modern researcher and how they may be used to inform a greater understanding of mitochondrial physiology. We focus on integrated omics resources, including mitochondria-specific resources, mitochondrial components of general resources, available mitochondrial datasets, as well as analytical tools and computational methods for an informatics approach to mitochondrial physiology.

2 Mitochondria-Specific Resources

The mitochondrial research community has undergone several paradigm shifts in conceptual focus and experimental design, progressing from hypothesis to data-driven approaches. The discovery of mitochondrial DNA (mtDNA) fostered a period of renewed interest in mitochondrial physiology and the development of new tools and techniques. Mitochondrial DNA was first discovered in 1963 by Nass et al., who described them as "intramitochondrial fibers with DNA characteristics" (Nass 1963; Nass and Nass 1963). The entirety of the human mitochondrial genome was sequenced in 1981 by Anderson et al. (1981), and mutations were first discovered less than a decade later, identifying the genetic basis of LHON, Kearns-Sayre, MELAS, and MERRF (Wallace et al. 1988a, b). mtDNA is 3,000 kb long and encodes only 13 proteins in the mitochondrial proteome, compared to over 1,500 from nuclear DNA. Aberrations in the mitochondrial genetic code result in diseases that are most often fatal, demonstrating their integral role. Maternal inheritance of mtDNA has provided a method for genotyping and tracing of genetic lineages. Computational approaches have been employed to identify genetic pressures for phylogenetic retention of mitochondrial genes as well as the debated mtDNA bottleneck mechanism, whereby cell–cell variability is utilized to avoid aggregation of deleterious mutations and loss of function of the uniparental mtDNA (Johnston and Williams 2016; Johnston et al. 2015).

Characterizing the mitochondrial genome and corresponding proteome requires a tailored approach due to its unique quality of having genetic contribution from both nuclear and mitochondrial DNA. The databases that contain these datasets must delineate the genetic sources, and the protein localization information can vary between datasets and detection methods. As such, the mitochondrial community has created a variety of tools to decipher the principles of mitochondrial physiology. In this section, we highlight mitochondria-specific resources geared exclusively toward the storage, maintenance, and manipulation of mitochondrial datasets, from "omics" repositories to community-curated resources of mitochondrial knowledge.

In genomics, analyses of an individual's genome is compared to a reference genome to determine individual genetic variations and aberrations; accordingly, the Cambridge Reference Sequence (CRS) was created in 1981 (Anderson et al. 1981), and updated in 1999 (Andrews et al. 1999). This reference sequence is stored within GenBank [NCBI Reference Sequence: NC_012920.1] and within MITOMAP, which provides information relating specifically to the human mitochondrial genome; this includes polymorphisms, mutations, and control regions, and allows users to upload and analyze sequences through the MITOMASTER web interface (Lott et al. 2013). Similarly, MitoCarta 2.0 provides a curated inventory of 1,158 human and mouse genes, as well as the proteins that localize to the mitochondrion. The inventory is generated using mass spectra of mitochondria isolated from 14 tissues and protein localization is determined via GFP tagging, microscopy, and machine learning. MS and microscopy results are integrated with six other genome-scale datasets of mitochondrial localization, lending greater accuracy to the determination of protein location (Pagliarini et al. 2008). Importantly, MitoCarta has

integrated information from archived mitochondrial databases, such as MitoP2, in order to ensure that the knowledge contained within said databases remains accessible (Calvo et al. 2016). Datasets can be downloaded in Excel, MySQL, BED, and FASTA file formats and are publicly available. Datasets within MitoCarta have led to important insights such as the identification of targets for whole-exome sequencing disease analysis (Falk et al. 2012).

Characterizing the mitochondrial genome is particularly important in the context of human disease, where maternally inherited mutations can lead to deadly diseases such as MERAS and MERFF (Wallace et al. 1988a, b). Computational tools have been essential in characterizing these mutations (see Table 1). MitImpact is a repository of pathogenicity predictions as related to mitochondrial DNA mutations. Predictions are generated by assembling estimations as well as structural and evolutionary annotations for each missense mutation. The resource is comprehensive, and provides assessments of susceptibilities for previously characterized and unknown mutations resulting in amino acid sequence alteration (Castellana et al. 2015). Mutations currently characterized across populations are stored in the Human Mitochondrial DataBase (HmtDB), which focuses on mitochondrial diseases in population genetics (Rubino et al. 2012). The genome sequences within HmtDB are annotated based on population variability factors, using SiteVar software. Users can query and browse the database, analyze sequences for classification, and download the results with reference genomes. As of August 2016, HmtDB contains 28,196 complete normal genomes spanning multiple continents, and 3,539 complete patient genomes. Globally, the database contains data for close to 10,000 variant sites (Attimonelli et al. 2005). For more deleterious mutations, MitoBreak contains mitochondrial genome rearrangements comprising circular deletion, circular duplication, and linear breakpoints. Spanning seven species including human, each case lists the positions of the breakpoints, junction sequences, and clinical relevance found in publications. The resulting resource is crucial for studying structural alterations of mtDNA (Damas et al. 2014). MitoSeek is a software tool for obtaining various mitochondrial genome information from exome, whole genome, and RNA-seq data (Guo et al. 2013). The tool can be utilized for mitochondrial sequence extraction, assembly quality evaluation, relative copy number estimation, detection of mitochondrial heteroplasmy, somatic mutations, and structural mtDNA alterations (Jayaprakash et al. 2015). These mitochondria-specific tools have enabled greater efficiency and standardization in the analysis of genomics datasets.

Just a few short years after Marc Wilkins coined the term “proteome” in 1995 (Wasinger et al. 1995; Godovac-Zimmermann 2008), Rabilloud attempted the first characterization of all mitochondrial proteins using 2-D electrophoresis (Rabilloud et al. 1998). In the ensuing decades, tremendous progress has been made in defining the mitochondrial proteome and its subproteomes (Lotz et al. 2014), owing primarily to the remarkable developments in mass spectrometry technology (Yates 2013). To house the massive amount of data generated in these studies, MitoMiner is used as a data aggregator to store and analyze mitochondrial proteomics data obtained from MS and fluorescent protein tagging studies (Smith et al. 2012). It integrates with many other informatics resources, namely UniProt, Gene Homology, Online

Table 1 Mitochondrial resources and websites

Tool	URL	Description	Last updated
HmtDB	http://www.hmtdb.uniba.it:8080/hmdb/	Open resource hosting human mitochondrial genome sequences annotated with population and variability data, the latter being estimated through the application of SiteVar	09/2015
MitImpact	http://mitimpact.css-mendel.it/	Repository of pathogenicity predictions. These predictions are created through the assembly of precomputed and computed sets of estimations for all missense mutations; these mutations are then structurally and evolutionarily annotated	07/2016
MitoBreak	http://mitobreak.portugene.com	Database containing mitochondrial genome rearrangements through a list of circular deletion, circular duplication, and linear breakpoints	05/2014
MitoCarta 2.0	http://www.broadinstitute.org/node/7098/index.html	Provides a curated inventory of 1,158 human and mouse genes encoding proteins with strong scientific support of localization to the mitochondrion	06/2015
MitoFish/ MitoAnnotator	http://mitofish.aori.u-tokyo.ac.jp/	Contains the mitochondrial genomes of many model systems, including zebra fish. The database also contains phylogenetic information, as lineage can often be determined via mitochondrial DNA. MitoAnnotator automates the annotations of new sequences uploaded to the database, and has also reannotated the previously uploaded mitogenomes to gain new insights	08/2016
MITOMAP	http://www.mitomap.org/MITOMAP	Provides information relating specifically to the human mitochondrial genome, including polymorphisms, mutations, and control regions, and allows users to upload and analyze sequences through the MITOMASTER web interface	06/2016
MitoMiner	http://mitominer.mrc-mbu.cam.ac.uk	Data aggregator for the storage and analysis of mitochondrial proteomics data obtained from MS and fluorescent protein tagging studies	04/2016
MitoPedia	http://www.bioblast.at/index.php/MitoPedia	Encyclopedic resource and discussion platform specifically focused on mitochondrial knowledge relating to experimental design, methods, and terminology	05/2016

(continued)

Table 1 (continued)

Tool	URL	Description	Last updated
MitoSeek	https://github.com/riverlee/MitoSeek	Software tool for obtaining various types of mitochondrial genome information from exome, whole genome, and RNA-seq sequencing data	05/2015

Mendelian Inheritance in Man, HomoloGene, Integrated Mitochondrial Protein Index, KEGG, and PubMed. As such, MitoMiner provides an all-in-one platform for mitochondrial researchers interested in probing the mitochondrial proteome. MitoMiner currently encompasses 11 different species and integrates 46 large-scale proteomics studies in its database, providing output data in XML, JSON, GFF3, UCSC-BED, FASTA, and HTML formats, and programmatic access through REST APIs and platform-specific clients (Perl, Python, Ruby, and Java). Most importantly, MitoMiner is actively maintained and updated to accommodate changes to the integrated resources.

Other databases have been created for specific animal models, such as MitoFish. MitoFish contains the mitochondrial genomes of many fish species, including the common model system, zebra fish. The database also contains phylogenetic information, as lineage can often be determined via mitochondrial DNA. MitoAnnotator automates the annotations of new sequences uploaded to the database, and has also reannotated the previously uploaded mitogenomes to gain new insights (Iwasaki et al. 2013). MitoFish is particularly useful to mitochondrial researchers due to expert curation and automated annotation. Data contained within MitoFish has spurred efforts to determine the genetic basis for various adaptations in fish (Wang et al. 2016) as well as advancements in phylogeographic studies (Hirase et al. 2016). This includes the development of suffix tree-based marker detection methods for detecting short genetic sequences, resulting in improved approaches to annotating mitochondrial genomes or to detecting and correcting erroneous annotations (Moritz et al. 2014).

Collaboration among domain-specific communities is integral to creating studies for emerging physiological questions. Created in 2010 by Bioblast, MitoPedia was created as an encyclopedic resource and discussion platform specifically focused on mitochondrial knowledge relating to experimental design, methods, and terminology. Content is generated by contributions from domain scientists and mitochondrial physiologists with experience in cellular and mitochondrial isolation and experimentation. Experts in the field write, discuss, and contribute to articles relating to respirometry, fluorometry, spectrophotometry, mitochondrial swelling, membrane potential ($\Delta\psi$), and ion flux experiments. Members of the Mitochondrial Physiology Society (MiPs) comprise the primary user base of the MitoPedia platform, which has been accessed over 40,000 times, with approximately 100–200 page views per month (Oroboros 2015). Many of the articles presented deal with respirometry experiments and the MiPs group actively endorses a move to

standardized experimental protocols, drug concentrations, and terminology so as to have the most effective discussions among mitochondrial physiologists across the world.

Use Case for Investigating Mitochondrial DNA Mutations

Biomedical question: An investigator would like to study the role of mtDNA mutations in Leber's hereditary optic neuropathy (LHON), whether polymorphisms exist predominantly for a particular demographic, as well as information on current treatment efficacy and clinical trials.

Data science approach: Use HmtDB to find sequences, population genetics, and polymorphisms in human mitochondrial genomes. Searching for LHON returns 190 records in healthy and diseased patients. The same search on MITOMAP yields 61 selected references, and MitoMiner was recently used to identify mitochondrial proteins that are downregulated in LHON patients due to an mtDNA mutation (Tun et al. 2014). MitoPedia contains 13 entries of references and abstracts relating to LHON and mitochondrial function. Finally, clinicaltrials.gov (discussed in Sect. 3) lists a current Phase 2 randomized clinical trial on a small cohort (12 patients) "Investigating the Safety, Tolerability, and Efficacy of Elamipretide (MTP-131) Topical Ophthalmic Solution for the Treatment of Leber's Hereditary Optic Neuropathy" (https://clinicaltrials.gov/ct2/show/NCT02693119/). Using the patient records from HmtDB, one could determine differential prevalence across demographics, or predict other mutations that would result in a similar phenotype using the MitImpact tool.

3 General Bioinformatics Resources and Their Mitochondrial Components

Approaches to the study of mitochondrial physiology have undergone tremendous change with the advent of omics approaches and cloud computing, among other technologies and advancements. The resulting influx of information has the potential to generate vast amounts of knowledge, but only with the proper infrastructure in place to handle the load. Bioinformatics and cloud computing approaches allow more efficient and effective management of the wide variety of data sources that contribute to our generation of physiological knowledge and a greater understanding of mitochondria. Here we review the mitochondrial components of well-established, curated omics resources (see Table 2).

Many resources span multiple dimensions, providing information on two or more omics data types. Xfam is a collection of databases including Rfam for RNA families (Nawrocki et al. 2015), Pfam for protein families (Finn et al. 2014a), and iPfam for protein family interactions (Finn et al. 2014b). Each database provides annotations that are crowdsourced through Wikipedia and links to other databases for more information

Table 2 Mitochondrial entries in existing big resources

Tool	URL	Data type(s)	Mitochondrial relevant entries
Flybase	http://flybase.org/	Fly genes, mutations, and stocks	377 genes, 11,057 stocks
HMDB	http://www.hmdb.ca/	Metabolites	17,682 metabolites
IMSR	http://www.findmice.org/	Mouse strains	4,011 strains
KEGG	http://www.genome.jp/kegg/	Pathway maps	55 relevant pathway maps
PDB	http://www.rcsb.org/pdb/	Protein structures	2,107 protein structures
Reactome	http://www.reactome.org/	Reactions and pathways	153 human pathways
UniProt	http://www.uniprot.org/	Proteins	4,889 reviewed proteins
Xfam	http://xfam.org/		
Rfam	http://rfam.xfam.org/	RNA families	RNA families
Pfam	http://pfam.xfam.org/	Protein families	1,460 proteins
iPfam	http://ipfam.org/	Protein family interactions	325 protein families, 44 ligands

on the protein sequence, protein structure, or RNA sequence of interest. Rfam contains information about noncoding RNAs (ncRNAs), structured cis-regulatory elements, and self-splicing RNAs. Each entry is represented by multiple sequence alignments, consensus secondary structures, and covariance models (CMs), which allow simultaneous modeling of RNA structure and sequence (Nawrocki et al. 2015). Currently, there are 861 mitochondrial RNA families within this database. Pfam utilizes hidden Markov models (HMMs) to generate multiple protein sequence alignments, allowing users to search sequence databases for homologous proteins with a specialized computational package. Sequence information is organized into higher level groupings of related families called clans, based on collections of Pfam-A entries related by sequence similarity, structure, or profile HMM (Finn et al. 2014a). Pfam has over 16,000 manually curated entries to date, 1,460 of which are annotated as mitochondrial. iPfam provides protein interaction information, based on structural information from all known structures contained in the Protein Data Bank (PDB) (Berman et al. 2000). Protein crystal structure is analyzed to identify protein domains, bonds, and small chemical ligands in each structure and bond length is estimated based on geometric and chemical properties of the sites (Finn et al. 2014b). There are 325 protein families and 44 ligands within iPfam that are mitochondrially related.

The Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) is the premier resource for protein structure data. The PDB contains over 120,000 crystallographic structures of proteins, nucleic acids, and protein/

nucleic acid complexes, along with complementary analysis and visualization tools (Berman et al. 2000). The PDB also accepts data depositions from the community and provides tools for data preparation and validation. Most data provided by the PDB is available as PDBML or PDB files, and is accessible over two REST web services: the SEARCH service for querying the database and the FETCH service for retrieving the structure data. Over 2,000 of the structures within PDB are mitochondrially associated. Structural information about these proteins yields valuable insight into their conformational arrangements and sites of posttranslational modification, and can help identify active sites for drug development.

The PDB assists in these developments by providing a stable, organized resource for accessing and sharing structural data. To supplement the structural data within PDB, users rely on UniProt, a publicly accessible, comprehensive resource of protein sequence, annotation, and localization data created and maintained by the European Bioinformatics Institute. UniProt contains both highly curated and machine-generated protein annotations, and has become the de facto source for protein information. As of September 2016, this resource contains 550,000 manually reviewed proteins for human (SwissProt), of which 4,889 are related to the mitochondria.

Perturbation in protein expression is a crucial component of omics research; the manifestations of such changes can be studied through metabolomics, which assess the entire pool of small molecules within the cell or organelle. As proteins act on metabolites, their fluctuations provide another, highly dynamic dimension of phenotypic data. One database, the Human Metabolome Database (HMDB), contains or links chemical data, clinical data, and molecular biology/biochemistry data (Wishart et al. 2007). The database has detailed, curated information for 41,993 water- and lipid-soluble metabolites. Within each entry, there are 110 data fields, with a large emphasis on chemical and clinical data; significantly, quantification information is available on each MetaboCard entry, and there exists protein sequence information for 5,701 entries. The HMDB supports extensive text, sequence, chemical structure, and relational query searches (Wishart et al. 2009). The database interfaces with many others, including some of those aforementioned, as well as PubChem, MetaCyc, ChEBI, and GenBank (Wishart et al. 2013). Of the over 40,000 metabolites within the database, mitochondrially related metabolites comprise over 17,000 MetaboCard entries.

Metabolite fluctuations are best understood through visualization of their respective pathways, as it is becoming increasingly apparent that their complex interplay is a burgeoning area of investigation. Two databases provide pathway visualization tools: KEGG PATHWAY and Reactome. The Kyoto Encyclopedia of Genes and Genomes (KEGG) currently contains 17 databases, each with a different focus in the domains of systems biology, genomics, chemistry, and medicine. KEGG is publicly accessible and supports tab-separated, plain text or KEGG database entry data formats, while the KEGG Application Programming Interface (API) allows customization of KEGG analyses (Kanehisa et al. 2014). These tools provide an in-depth look at signaling pathways and interactions among proteins, allowing a more complete view of pathways of interest, providing insight into related processes and species that might deserve study. Specifically, within KEGG

PATHWAY, there are 55 mitochondrial relevant pathway maps, many of which are associated with the progression of common human diseases. Another resource, Reactome, is an open-source platform for biological pathway visualization that is manually curated and peer reviewed by experts, with a focus on human reaction pathways (Matthews et al. 2009). The database contains deeply annotated pathway information from 19 distinct species and includes 1,786 pathways for human as of September 2016. Of these, there are over 150 mitochondrially associated pathways, reactions, and complexes identified and annotated within the platform. Reactome bundles many pathway-related visualization, interpretation, and analysis tools in one resource (Stein 2004). The pathway data can be viewed and analyzed directly from the Pathway Browser, accessed programmatically through a REST API, or downloaded in BioPAX, PSI-MITAB, SBML, and SBGN formats (Jupe et al. 2015). Reactome is widely used for different analyses, including the identification of biomarkers in a neurological model of PTSD (Jia et al. 2012; Bai et al. 2007).

Use Case for Investigating Mitochondrial Localization or Involvement

Biomedical question: 12-Lipoxygenase has been implicated in ischemic preconditioning pathways, but has it been identified as having mitochondrial localization? What resource can an investigator use to answer this question? Are lipoxygenases mitochondrially targeted? What metabolic changes result from perturbations in lipoxygenase expression or function? What interacting partners exist or is there any relevance as a drug target?

Data science approach: UniProt reveals a GO annotation of positive regulation of mitochondrial depolarization. Querying HMDB for 12-lipoxygenase returns five metabolites that are associated with the enzyme. These results also connect the user to a multitude of resources, including primary research papers that have been used for annotation and other sites or resources with relevant information. KEGG and Reactome can also be used to investigate reaction pathways related to the lipoxygenases; a KEGG query for 12-lipoxygenase returns eight pathways and Reactome lists seven reactions and their corresponding pathways.

4 Public Mitochondrial Datasets and Data Sharing

The digitization of scientific literature through resources like PubMed has made scientific publications easy to find for a much wider audience. However, due to the incremental nature of science, where new studies are based on the conclusions reached by past endeavors, knowledge remains distributed across many publications. This creates a gap in our ability to reuse or repurpose existing knowledge and renders high-throughput computational analyses of the literature immensely difficult. The growing demand for access to the data behind scientific publications assigns data repositories an increasingly important role as the backbone of modern

scientific research. Sponsored by the National Institutes of Health, the National Center for Biotechnology Information (NCBI) created the Database of Genotypes and Phenotypes, or dbGaP, which contains hierarchical data of structured types. The inputted data are organized into investigations that explore the interface of genotype and phenotype and are linked to an accession number. Within the studies are phenotypic datasets, whereby certain variables are measured. Investigators can upload the raw datasets, provide important information, and give metadata to encourage reusability. If investigators performed analyses, those may also be uploaded. Upon study completion, investigators may upload supporting documents, which may contain further information such as study instructions, protocols/forms for data procurement, and other information necessary to use these datasets (Tryka et al. 2014). Conducting a query for "mitochondria" returns 21 mitochondrial related studies spanning 216 variables. Within this, there are ten supporting documents and 37 raw datasets. These are also documented in clinicaltrials.gov. Currently, the database is openly accessible to institutions, and there is an avenue by which individuals can gain controlled access. These resources are aggregated in Table 3.

As the creation of new data exponentially grew, the scientific community realized the need for consolidated, open omics repositories. One unique challenge was to take raw, unprocessed datasets and provide information and resources to enable

Table 3 Publicly accessible mitochondrial datasets

Dataset	URL	Description	Mitochondrial datasets and components
dbGaP	http://www.ncbi.nlm.nih.gov/gap	The database of genotypes and phenotypes contains hierarchical data of structured types	21 studies: 216 variables 1 analysis 10 documents 37 datasets
ProteomeXchange	http://www.proteomexchange.org/	Publicly accessible, centralized repository for proteomics datasets	32 datasets
OmicsDI	http://www.omicsdi.org/	Provides searchable metadata such as accession, description, sample/data protocols, and biological corroborations	2,819 total datasets: 326 proteomics 24 metabolomics 63 transcriptomics 122 genomics 208 multi-omics

access and collaborative analysis by other users. Founded by the creators of the prominent primary proteomics resource PRIDE (PRoteomics IDEntifications), the ProteomeXchange Consortium sought to create a repository for proteomics mass spectrometry datasets. The consortium consists of investigators who created many primary and secondary proteomics resources, and now has a publicly accessible, centralized repository (Vizcaino et al. 2014). Users can proceed through a full or partial submission workflow, using PRIDE as the initial resource. The PX Submission Tool facilitates the upload of datasets, as well as supporting documentation and metadata (Ternent et al. 2014). Of the over 2,500 datasets compiled on the ProteomeXchange resource, there are 32 proteomics datasets that are related to mitochondria. While many repositories exist that are specific to one omics domain, they are fragmented, operate independently, and highlight the need for an integrated repository, whereby omics datasets can be organized and disseminated in a systematic fashion. Within the Big Data to Knowledge (BD2K) Initiative, there exists OmicsDI, a repository for multiple datasets. One common challenge within omics datasets is that data is generated through differential protocols; as such, this hinders the analyses that can be performed. OmicsDI combats this by providing searchable metadata such as accession, description, sample/data protocols, and biological corroborations (Perez-Riverol et al. 2016). Querying OmicsDI for mitochondrial terms yields 2,800 datasets: of those, 122 are genomic, 63 are transcriptomic, 326 are proteomic, 24 are metabolomic, and 208 are multi-omic datasets.

The development of informatics resources for storage, maintenance, and analysis of scientific data addresses each component of the FAIR data doctrine. Standardization of experimental protocols in the "omics" sciences is necessary for more reliable data, while consistency in data formats and annotation facilitates more efficient data sharing. Curated resources and data repositories organize the information, rendering "omics" data findable and accessible, and lending added value to the data that might otherwise be undiscovered in an obscure format. Furthermore, analytic tools and platforms have been developed by the scientific community to facilitate the goals of interoperability and reusability. Analytic platforms make data mining and the generation of metadata much easier and more widely accessible, furthering data discovery and allowing others to try a novel approach on any of the available datasets. Scientists benefit from making their datasets publicly available and in a common format because their work becomes more visible, useable, and, ultimately, citable.

In addition to the technical challenges of data sharing, there exist legal and cultural considerations, especially with respect to clinical studies. One readily identifiable challenge is sharing clinical datasets in accordance with the Health Insurance Portability and Accountability Act (HIPAA), which mandates the protection of privacy with regard to medical information. Research efforts must establish protocols that de-identify information contained within electronic health/medical records (EHR/EMR), and package them into usable, queryable data for research purposes (Russo et al. 2016). More important, however, is the need to secure explicit consent from study participants such that their personal data can be shared across groups with different research foci. Currently, protocols are being developed to provide

standardized consent forms for larger scale purposes with wide applicability, rather than for individual studies. New studies benefit from incorporating these stipulations into consent documents during study design, whereas researchers wishing to disseminate older datasets must review their consent documents to determine if sharing is appropriate or legal. Alternatively, a follow-up may be necessary to gain further consent from study participants (Kaye and Hawkins 2014). Already, research groups and agencies have created repositories with these issues in mind.

Because these data repositories are general and allow the storage of datasets originating from a broad range of biological fields, researchers may question the utility of those that were not originally constructed with a mitochondrial focus. Previously, datasets were considered disposable material, only useful for supporting the conclusions of the particular study. In the data science landscape, new methods and protocols have been developed for managing and cataloging data, providing researchers the ability to recycle and reuse datasets. There lies untapped potential in these datasets for mitochondrial researchers to explore and gain new insights, all while avoiding expensive and laborious data generation until it is necessary. This is especially applicable to large-scale omics datasets, which provide the opportunity for data-driven, untargeted approaches to streamline experimental design and generate targeted approaches for further hypothesis-driven, physiological studies.

5 Pipelines and Tools for Data Processing and Analysis

While access to datasets is crucial, it is only one step in being able to truly harness the knowledge contained within them. The first and often most laborious aspect is to process the data and wrangle it into a format that will be usable by intended tools and pipelines. Investigative teams often perform their own processing protocols, and do not leave adequate metadata and/or annotation for others to be able to recreate the study. To mitigate this, some omics investigators have sought to create best practice pipelines. The effects of a unified system and set of tools can be most concretely seen in the area of genomics, specifically with the Genome Analysis ToolKit (GATK), which focuses on sequence analysis to discover relevant variants (McKenna et al. 2010). Having established best practices for genomics analyses, GATK has also been expanded to have copy number and structural variation analysis capacities (Tennessen et al. 2012). Currently, GATK hosts a wide variety of tools to assist investigators along all steps of analysis, including sequence data processing tools, such as sequence aligners and readers; variant discovery, evaluation, and manipulation tools, such as a Bayesian genotyper and a variant filter; and annotation modules. The toolkit was originally intended for human exomes and genomes sequenced from Illumina technologies, but has been expanded to other experimental protocols and model systems, regardless of ploidy. GATK also hosts third-party tools that can be integrated with the previously established pipelines (DePristo et al. 2011; Van der Auwera et al. 2013). GATK and other analysis tools and pipelines are highlighted in Table 4.

Table 4 Analysis pipelines and platforms

Tool	URL	Description
COPaKB	https://amino.heartproteome.org/	Centralized platform featuring high-quality cardiac proteomics data and relevant cardiovascular phenotype information. The organellar modules constitute the mass spectral library and are utilized by COPaKB's unique high-performance search engine to identify and annotate proteins in the mass spectra files that are submitted by the user in mzML or DTA formats
Cytoscape	http://www.cytoscape.org/	Open-source software platform for the visualization of complex biological systems, such as molecular interaction networks and biological pathways. The platform enables enhancement of the network data through integration of various formats of metadata into the network structure
Galaxy	https://usegalaxy.org/	Informatics workflow management system and data integration platform that aims to make computational biology accessible to researchers with limited experience in computer programming. Provides a graphical user interface, customizable plug-ins, access to public datasets, and other users' workflows
GATK	https://software.broadinstitute.org/gatk/	Toolkit that focuses on sequence analysis to discover relevant variants; originally intended for human exomes and genomes sequenced from Illumina technologies, but has been expanded to other experimental protocols and model systems, regardless of ploidy
MetaboAnalyst	http://www.metaboanalyst.ca/	Analysis pipeline spanning a wide variety of data types; has the capacity for biomarker identification, as well as a host of other bioinformatics tools for the best standard metabolomics analyses using an extensive spectral library for enhanced metabolite identification
MetaCore	https://portal.genego.com/	Standalone program and Web application comprising multiple different analysis methods for varying types of high-throughput molecular data, including sequencing and gene expression, proteomic data, and metabolomic data. Also contains a manually curated database
MetazSecKB	http://bioinformatics.ysu.edu/secretomes/animal/index.php	Database presenting subcellular protein location based on manual curation of the scientific literature combined with UniProt sequence data and annotations. Employs an algorithm that utilizes multiple prediction tools, combining the predictions of publicly

(continued)

Table 4 (continued)

Tool	URL	Description
		available tools including TargetP, SignalP, Phobius, ScanProsite, WolfPSORT, FragAnchor, and TMHMM
OmicsPipe	http://sulab.org/tools/omics-pipe/	Integrates multiple best practice pipelines to provide a platform for processing raw data; aims to reduce the overhead for processing large datasets, and provides visualizations produced; currently, the platform has automated workflows for processing RNA-seq, whole-exome sequencing (WES), whole-genome sequencing (WGS), and ChIP-seq datasets
ProTurn	http://heartproteome.org/proturn	Scalable analysis platform that assesses the turnover rate of proteins. Uses a deuterium oxide (D_2O) labeling protocol and determines the kinetics by integrating MS peaks, determining isotope abundances, and using multivariate optimization. Available for use on a wide variety of datasets
TargetP	http://www.cbs.dtu.dk/services/TargetP/	Web-based tool for predicting the subcellular location of eukaryotic proteins and identity of secretory signal or transit peptides using N-terminal sequence information and a combination of machine learning methods. Commonly used by mitochondrial physiologists analyzing proteomics datasets

Many other emerging areas of omics are developing best practice guidelines; however, creating these guidelines requires significant effort for their implementation. To help investigators with this, OmicsPipe integrates multiple best practice pipelines to provide a platform for processing raw data. OmicsPipe aims to reduce the overhead for processing large datasets, and provides visualizations produced; currently, the platform has automated workflows for processing RNA-seq, whole-exome sequencing (WES), whole-genome sequencing (WGS), and ChIP-seq datasets (Fisch et al. 2015).

Upon processing the data, investigators are faced with myriad tools to perform analyses; without distinct computational knowledge, navigating these platforms can be daunting. Operating on a unified system can substantially streamline omics analyses; that being said, there are many stand-alone tools specific to one branch of omics that can elucidate valuable knowledge. Within the realm of proteomics, identifying the subcellular location of a protein is important for determination of function, genome annotation, drug development, and disease identification. Proteins are designed to play their role in specific locations, defining their function based on their environment. This becomes particularly important when discussing mitochondrial proteins. Generally speaking, there are two parallel approaches to

determine whether a protein is localized to the mitochondrion: the approach of the biologist or biochemist, and that of the informatician. The former will devise a plan for isolation, fractionation, assays, and copurification with other known mitochondrial markers to look for biochemical evidence of localization to one organelle or another. The latter would investigate features of the gene or protein sequence utilizing computational approaches that would identify it as localized to mitochondria.

Proteins bound for localization in mitochondrial membranes contain an amino acid sequence signal at the N-terminus or an internal targeting sequence to direct them to their appropriate position, both of which are managed by different species of the translocase inner- and outer-membrane (TIM and TOM) complexes. An amphipathic helix in a protein's presequence is cleaved upon delivery (von Heijne 1986; Schatz and Gottfried 1993), while proteins lacking a presequence region remain in the cytosol (Fox 2012).

TargetP is a Web-based tool for predicting the subcellular location of eukaryotic proteins and identity of secretory signal or transit peptides using N-terminal sequence information and a combination of machine learning methods. This tool is commonly used by mitochondrial physiologists analyzing proteomics datasets, as evidenced by over 2,000 citations of the tool's two papers in the scientific literature. The user submits either an amino acid sequence or a FASTA file as the input, and retrieves a plain text file outlining the predictions (Emanuelsson et al. 2007). The predictions were found to be 90% accurate for non-plant proteins and 85% accurate for plant proteins (Klee and Ellis 2005; Emanuelsson et al. 2000). The tool is publicly accessible as a Web service or downloadable for local computation. It is one of the prediction tools used by UniProt to annotate mitochondrial peptides, along with Predotar, TMHMM, and Phobius, all of which make predictions based on N-terminal targeting sequences (Small et al. 2004; Käll et al. 2004; Krogh et al. 2001). In addition to localization prediction, TMHMM predicts transmembrane helices in protein sequences with 97% accuracy (Krogh et al. 2001) while Phobius was shown to predict the secondary structure of proteins with fewer instances of false classification and identify signaling proteins with fewer false positives than TargetP and TMHMM (Käll et al. 2004).

MetazSecKB combines results from each of these tools to increase prediction accuracy for secretome and subcellular proteome localization. The database presents subcellular protein location based on manual curation of the scientific literature combined with UniProt sequence data and annotations. When this annotation is lacking, MetazSecKB employs an algorithm that utilizes multiple prediction tools, combining the predictions of publicly available tools including TargetP, SignalP, Phobius, ScanProsite, WolfPSORT, FragAnchor, and TMHMM (Meinken et al. 2015). The accuracy of localization predictions increased significantly when these tools were utilized in concert, rather than individually. Accordingly, the algorithm combs data from each of the tools simultaneously, and then applies statistical and data mining techniques to acquire the most accurate localization predictions for eukaryotic secreted proteins (Min 2010). Over 135,000 proteins in *Homo sapiens* are represented in the database, approximately 21,000 of which

localize to mitochondria; 3,737 of these are associated with the mitochondrial membrane and 17,623 are non-membrane proteins.

The Cardiac Organellar Protein Atlas Knowledgebase (COPaKB) is a centralized platform featuring high-quality cardiac proteomics data and relevant cardiovascular phenotype information (Zong et al. 2013). As of September 2016, COPaKB features 11 organellar modules, comprising 4,467 LC-MS/MS experiments from human, mouse, drosophila, and *Caenorhabditis elegans*. There are four mitochondrial specific modules for each species with over 1,000 proteins represented in each species. The organellar modules constitute the mass spectral library and are utilized by COPaKB's unique high-performance search engine to identify and annotate proteins in the mass spectra files that are submitted by the user in mzML or DTA formats. Data in COPaKB can be viewed within the browser, accessed via the REST API or downloaded in Excel XLS, XML, and JSON formats.

Protein expression data does not take into account the rate of synthesis and degradation of a certain protein, termed protein turnover. As such, measuring expression alone is not sufficient to understand the dynamics of protein levels within the mitochondria. Accordingly, tools have been developed that align with dynamics protocols. One such tool is ProTurn, which uses a deuterium oxide (D_2O) labeling protocol and determines the kinetics by integrating MS peaks, determining isotope abundances, and using multivariate optimization. Most importantly, this tool is scalable, which enables users to perform analysis on a wide range of datasets (Wang et al. 2014).

To elucidate the small-molecule perturbations that may occur in varying mitochondrial physiological states, two types of tools exist: one assesses the quantitative levels of metabolites, while the other synthesizes metabolite lists into known networks, so that these can be visualized by the investigator. Originally developed in 2009 (Xia et al. 2009), MetaboAnalyst has gone through many iterations, with updates in 2012 (Xia et al. 2012) and 2015 (Xia et al. 2015) bringing vital improvements. The current version accepts a wide variety of data types, including NMR spectra, MS spectra, and compound/concentration data. The user interface guides investigators through the analysis pipeline, beginning with dataset quality control by the user. Once quality control standards have been met, the platform will analyze the data, using an extensive spectral library for enhanced metabolite identification. The most current version, MetaboAnalyst 3.0, has the capacity for biomarker identification, as well as a host of other informatics tools for the best standard metabolomics analyses. The graphical output allows users to view the analysis results in a user-friendly format. This platform has been used extensively in mitochondrial studies to understand mitochondrial physiological function in the spinal cord in an ALS model, identify therapeutic targets of cardiomyopathy, and uncover the role of mitochondrial protein quality control in the context of physiological stress across many systems (Quintana et al. 2016; Cacabelos et al. 2016; Picard et al. 2015).

Cytoscape is an open-source software platform for the visualization of complex biological systems, such as molecular interaction networks and biological pathways. The platform enables enhancement of the network data through integration of various formats of metadata into the network structure. One powerful aspect of Cytoscape is its extendibility; third-party developers can access its API and develop applications on top of Cytoscape that readily implement the desired functionality. As of September 2016, the Cytoscape App Store contains 228 Apps, with 305,000 downloads in total (Ono 2015). Cytoscape is an excellent fit for visualization of networks, such as microRNA networks in the brain stem (DeCicco et al. 2015).

Stand-alone tools created by members of the omics community have proven integral to furthering omics research. However, these tools exist as fragments, which makes dissemination to the broader community a significant challenge. To combat this, Galaxy was created as an informatics workflow management system and data integration platform that aims to make computational biology accessible to researchers with limited experience in computer programming (Goecks et al. 2010). Galaxy provides a graphical user interface, customizable plug-ins, and access to public datasets and other users' workflows. This offers a robust peer-review mechanism in which the analyses conducted previously can be reproduced with little effort (Sandve et al. 2013). Because the workflows are hosted on the cloud and Galaxy servers perform the computational work, this greatly reduces the requirement for setting up expensive infrastructure to achieve research goals. One branch of Galaxy, Galaxy-P, contains workflows specifically designed to analyze proteomics datasets and integrate them with other forms of omics data, such as transcriptomics (Sheynkman et al. 2014). In addition to tools developed in the academic community, commercial tools have been developed with similar infrastructure. MetaCore™, developed by Thomson Reuters, exists as a stand-alone program as well as a Web application. The tool contains multiple different analysis methods for varying types of high-throughput molecular data, including sequencing and gene expression, proteomic data, and metabolomic data. In addition to an internal, manually curated database, MetaCore™ contains genomic analysis tools, a data mining toolkit, a pathway editor, and data parsers to adapt the wide range of omics data that can be uploaded (Cambiaghi et al. 2016).

Using these tools requires significant computational power; the previously established paradigm for in-house platforms is becoming extremely costly. The hardware usually becomes dated in about 3 years, and must be updated in order to maintain relevance. Even with extensive collaboration and shared infrastructure, the servers are used for only a fraction of the time they are available, which creates a highly inefficient cost per analysis. These problems have illustrated the need for a unified resource in which researchers can take advantage of ever-improving capacities and features, with significantly less up-front expense. Recent advancements point to cloud-based computing and storage systems. Operations on the cloud provide the same computational power but represent only a tiny fraction of the hardware and operational costs for an in-house server-based computational platform. The National Institutes of Health has proposed the cloud-based Commons environment, a cost-sharing model that will provide access to scalable storage and computational resources for the entire biomedical community. Commons Credits will serve as the currency for computational efforts and

require minimal effort to apply so as to reduce the administrative overhead of establishing computational infrastructure, be it in-house or on business cloud servers (https://www.commons-credit-portal.org).

6 Conclusion

The current deluge of data brings significant challenges to which researchers must respond by continually improving methods and technologies for data management and dissemination. In adhering to the FAIR doctrine, data shall be accessible in all respects, and their analyses will also be presented in an intuitive, structured manner. As such, the resulting dataset and knowledge will be open to reuse, repurpose, and reanalysis so as to investigate different targets of interest. In this chapter, we have outlined a collection of tools and resources that serve to aid a deeper understanding of mitochondrial physiology and its role in health and disease. These tools range from community-generated encyclopedic resources, expert-curated databases, and repositories for data management and access to tools for analysis and visualization of biological processes. They allow greater access and reuse of data through annotation, metadata, and analysis platforms. The development of these tools and resources, as well as the openness of scientific data, has had a dramatic impact on the breadth, depth, and structure of data, as well as the reproducibility of experiments and analysis pipelines.

The physiology discipline stands at a unique cross section, and bridges data and clinical applications. It is through these tools that investigators are able to unlock mechanistic insight and access the potential for clinical translation. The advancement of multi-omic approaches, bioinformatics analyses, and open-access data has improved our basic understanding of physiology and pathology while spurring the development of personalized medicine and discovery of biomarkers for disease (Almeida 2010; de Graaf 2013). EHR is becoming more detailed, accessible, and multidimensional, and natural language processing is making it easier to conduct meta-analyses of disease treatments from de-identified patient records. With the concept of precision medicine, the paradigm of health and disease classification is shifting from broad generalization to distinct and individualized medical profiling (Hayes et al. 2014). In this landscape, researchers can significantly benefit from using computational and informatics tools to enable better scientific investigations.

Informatics science is transforming the scope of biomedical research, providing ample tools and methods by which to address the requirements of Big Data, personalized medicine, and next-generation scientific questions. New and improved infrastructure in the research and health sectors have resulted in a burgeoning expansion of data that requires research scientists and clinicians alike to investigate novel approaches in data science and informatics. It is at the interface of domain knowledge and computational bandwidth that mitochondrial research can synergistically propel forward, at a velocity not seen in isolated studies. As this data is shifting from disposable to indispensable, integrated approaches are rapidly

demonstrating themselves as invaluable components of a biomedical researcher's tool chest.

Acknowledgements This work was supported by the NIH Big Data to Knowledge (BD2K) Center of Excellence Award (U54GM114883).

References

Almeida JS (2010) Computational ecosystems for data-driven medical genomics. Genome Med 2(9):67

Altmann R (1894) Die Elementarorganismen und ihre Beziehungen zu den Zellen. Verlag von Veit & Comp, Leipzig

Anderson S et al (1981) Sequence and organization of the human mitochondrial genome. Nature 290(5806):457–465

Andrews RM et al (1999) Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. Nat Genet 23(2):147

Attimonelli M et al (2005) HmtDB, a human mitochondrial genomic resource based on variability studies supporting population genetics and biomedical research. BMC Bioinformatics 6(Suppl 4): S4

Bai X et al (2007) Third-generation human mitochondria-focused cDNA microarray and its bioinformatic tools for analysis of gene expression. Biotechniques 42(3):365–375

Bensley RR, Hoerr NL (1934) Studies on cell structure by the freezing-drying method VI. The preparation and properties of mitochondria. Anat Rec 60(4):449–455

Berman HM et al (2000) The Protein Data Bank. Nucleic Acids Res 28:235–242 Oxford University Press

Cacabelos D et al (2016) Early and gender-specific differences in spinal cord mitochondrial function and oxidative stress markers in a mouse model of ALS. Acta Neuropathol Commun 4:3

Calvo SE, Clauser KR, Mootha VK (2016) MitoCarta2.0: an updated inventory of mammalian mitochondrial proteins. Nucleic Acids Res 44(D1):D1251–D1257

Cambiaghi A, Ferrario M, Masseroli M (2016) Analysis of metabolomic data: tools, current strategies and future challenges for omics data integration. Brief Bioinform. doi:10.1093/bib/bbw031 (Epub ahead of print)

Castellana S, Ronai J, Mazza T (2015) MitImpact: an exhaustive collection of pre-computed pathogenicity predictions of human mitochondrial non-synonymous variants. Hum Mutat 36(2): E2413–E2422

Claude A (1946a) Fractionation of mammalian liver cells by differential centrifugation: II. Experimental procedures and results. J Exp Med 84(1):61–89

Claude A (1946b) Fractionation of mammalian liver cells by differential centrifugation: I. Problems, methods, and preparation of extract. J Exp Med 84(1):51–59

Claude A, Fullam EF (1945) An electron microscope study of isolated mitochondria: method and preliminary results. J Exp Med 81(1):51–62

Daems WT, Wisse E (1966) Shape and attachment of the cristae mitochondriales in mouse hepatic cell mitochondria. J Ultrastruct Res 16(1):123–140

Damas J et al (2014) MitoBreak: the mitochondrial DNA breakpoints database. Nucleic Acids Res 42(Database issue):D1261–D1268

de Graaf D (2013) Multi-omic biomarkers unlock the potential of diagnostic testing. MLO Med Lab Obs 45(8):40, 42

DeCicco D et al (2015) MicroRNA network changes in the brain stem underlie the development of hypertension. Physiol Genomics 47(9):388–399

DePristo MA et al (2011) A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat Genet 43(5):491–498

Emanuelsson O et al (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J Mol Biol 300(4):1005–1016
Emanuelsson O et al (2007) Locating proteins in the cell using TargetP, SignalP and related tools. Nat Protoc 2(4):953–971
Falk MJ et al (2012) Mitochondrial disease genetic diagnostics: optimized whole-exome analysis for all MitoCarta nuclear genes and the mitochondrial genome. Discov Med 14(79):389–399
Field D et al (2009) Omics data sharing. Science 326(5950):234–236
Finn RD et al (2014a) Pfam: the protein families database. Nucleic Acids Res 42(Database issue): D222–D230
Finn RD et al (2014b) iPfam: a database of protein family and domain interactions found in the Protein Data Bank. Nucleic Acids Res 42(Database issue):D364–D373
Fisch KM et al (2015) Omics Pipe: a community-based framework for reproducible multi-omics data analysis. Bioinformatics 31(11):1724–1728
FORCE11 (2014) Guiding principles for findable, accessible, interoperable and re-usable data publishing version b1.0. https://www.force11.org/node/6062
Fox TD (2012) Mitochondrial protein synthesis, import, and assembly. Genetics 192(4):1203–1234
Godovac-Zimmermann J (2008) 8th Siena meeting. From genome to proteome: integration and proteome completion. Expert Rev Proteomics 5(6):769–773
Goecks J et al (2010) Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. Genome Biol 11(8):R86
Gomez-Cabrero D et al (2014) Data integration in the era of omics: current and future challenges. BMC Syst Biol 8(Suppl 2):I1
Guo Y et al (2013) MitoSeek: extracting mitochondria information and performing high-throughput mitochondria sequencing analysis. Bioinformatics 29(9):1210–1211
Hayes DF et al (2014) Personalized medicine: risk prediction, targeted therapies and mobile health technology. BMC Med 12:37
Hirase S et al (2016) Parallel mitogenome sequencing alleviates random rooting effect in phylogeography. Genome Biol Evol 8(4):1267–1278
Iwasaki W et al (2013) MitoFish and MitoAnnotator: a mitochondrial genome database of fish with an accurate and automatic annotation pipeline. Mol Biol Evol 30(11):2531–2540
Jayaprakash AD et al (2015) Mito-seek enables deep analysis of mitochondrial DNA, revealing ubiquitous, stable heteroplasmy maintained by intercellular exchange. Nucleic Acids Res 43(4):2177–2187
Jia M et al (2012) Biomarkers in an animal model for revealing neural, hematologic, and behavioral correlates of PTSD. J Vis Exp (68)
Johnston IG, Williams BP (2016) Evolutionary inference across eukaryotes identifies specific pressures favoring mitochondrial gene retention. Cell Syst 2(2):101–111
Johnston IG et al (2015) Stochastic modelling, Bayesian inference, and new in vivo measurements elucidate the debated mtDNA bottleneck mechanism. Elife 4:e07464
Jupe S, Fabregat A, Hermjakob H (2015) Expression data analysis with reactome. Curr Protoc Bioinformatics 49:8.20.1–8.20.9
Käll L, Krogh A, Sonnhammer ELL (2004) A combined transmembrane topology and signal peptide prediction method. J Mol Biol 338(5):1027–1036
Kanehisa M et al (2014) Data, information, knowledge and principle: back to metabolism in KEGG. Nucleic Acids Res 42(Database issue):D199–D205
Kaye J, Hawkins N (2014) Data sharing policy design for consortia: challenges for sustainability. Genome Med 6(1):4
Kennedy EP, Lehninger AL (1949) Oxidation of fatty acids and tricarboxylic acid cycle intermediates by isolated rat liver mitochondria. J Biol Chem 179(2):957–972
Klee EW, Ellis LB (2005) Evaluating eukaryotic secreted protein prediction. BMC Bioinformatics 6:256
Krogh A et al (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol 305(3):567–580

Lander ES et al (2001) Initial sequencing and analysis of the human genome. Nature 409 (6822):860–921
Lesnefsky EJ et al (2001) Mitochondrial dysfunction in cardiac disease: ischemia–reperfusion, aging, and heart failure. J Mol Cell Cardiol 33(6):1065–1089
Lott MT et al (2013) mtDNA variation and analysis using MITOMAP and MITOMASTER. Curr Protoc Bioinformatics 1(123):1.23.1–1.23.26
Lotz C et al (2014) Characterization, design, and function of the mitochondrial proteome: from organs to organisms. J Proteome Res 13(2):433–446
Matthews L et al (2009) Reactome knowledgebase of human biological pathways and processes. Nucleic Acids Res 37(Database issue):D619–D622
McKenna A et al (2010) The genome analysis toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 20(9):1297–1303
Meinken J et al (2015) MetazSecKB: the human and animal secretome and subcellular proteome knowledgebase. Database 2015
Min XJ (2010) Evaluation of computational methods for secreted protein prediction in different eukaryotes. J Proteomics Bioinform 3:143–147
Mitchell P (1961) Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. Nature 191:144–148
Mitchell P (1966) Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. Biol Rev Camb Philos Soc 41(3):445–502
Mitchell P (1968) Chemiosmotic coupling and energy transduction. Glynn Research, Bodmin, Cornwall
Molloy JC (2011) The Open Knowledge Foundation: open data means better science. PLoS Biol 9(12):e1001195
Moritz RL, Bernt M, Middendorf M (2014) Local similarity search to find gene indicators in mitochondrial genomes. Biology (Basel) 3(1):220–242
Nass MM, Nass S (1963a) Intramitochondrial fibers with DNA characteristics. I. Fixation and electron staining reactions. J Cell Biol 19:593–611
Nass S, Nass MM (1963b) Intramitochondrial fibers with DNA characteristics. II. Enzymatic and other hydrolytic treatments. J Cell Biol 19:613–629
Nawrocki EP et al (2015) Rfam 12.0: updates to the RNA families database. Nucleic Acids Res 43(Database issue):D130–D137
O'Driscoll A, Daugelaite J, Sleator RD (2013) 'Big data', Hadoop and cloud computing in genomics. J Biomed Inform 46(5):774–781
Ono, K (2015) Cytoscape: an open source platform for complex network analysis and visualization. http://www.cytoscape.org/
Oroboros (2015) MitoPedia – bioblast. http://www.bioblast.at/index.php/MitoPedia
Pagliarini DJ et al (2008) A mitochondrial protein compendium elucidates complex I disease biology. Cell 134(1):112–123
Palade GE (1952) The fine structure of mitochondria. Anat Rec 114(3):427–451
Palade GE (1953) An electron microscope study of the mitochondrial structure. J Histochem Cytochem 1(4):188–211
Perez-Riverol Y et al (2016) Omics discovery index – discovering and linking public omics datasets. bioRxiv:049205
Picard M et al (2015) Mitochondrial functions modulate neuroendocrine, metabolic, inflammatory, and transcriptional responses to acute psychological stress. Proc Natl Acad Sci U S A 112(48): E6614–E6623
Quintana MT et al (2016) Cardiomyocyte-specific human Bcl2-associated anthanogene 3 P209L expression induces mitochondrial fragmentation, Bcl2-associated anthanogene 3 haploin sufficiency, and activates p38 signaling. Am J Pathol 186(8):1989–2007

Rabilloud T et al (1998) Two-dimensional electrophoresis of human placental mitochondria and protein identification by mass spectrometry: toward a human mitochondrial proteome. Electrophoresis 19(6):1006–1014
Rubino F et al (2012) HmtDB, a genomic resource for mitochondrion-based human variability studies. Nucleic Acids Res 40(Database issue):D1150–D1159
Russo E et al (2016) Challenges in patient safety improvement research in the era of electronic health records. Healthcare (Amsterdam, Netherlands) pii: S2213-0764(15)30090-7. doi:10.1016/j.hjdsi.2016.06.005 (Epub ahead of print)
Sandve GK et al (2013) Ten simple rules for reproducible computational research. PLoS Comput Biol 9(10):e1003285
Schatz G, Gottfried S (1993) The protein import machinery of mitochondria. Protein Sci 2(2):141–146
Schatz G, Haslbrunner E, Tuppy H (1964) Deoxyribonucleic acid associated with yeast mitochondria. Biochem Biophys Res Commun 15(2):127–132
Sheynkman GM et al (2014) Using Galaxy-P to leverage RNA-Seq for the discovery of novel protein variations. BMC Genomics 15:703
Small I et al (2004) Predotar: a tool for rapidly screening proteomes for N-terminal targeting sequences. Proteomics 4(6):1581–1590
Smith AC, Blackshaw JA, Robinson AJ (2012) MitoMiner: a data warehouse for mitochondrial proteomics data. Nucleic Acids Res 40(Database issue):D1160–D1167
Stein LD (2004) Using the reactome database. Curr Protoc Bioinformatics Chapter 8:Unit8.7
Tennessen JA et al (2012) Evolution and functional impact of rare coding variation from deep sequencing of human exomes. Science 337(6090):64–69
Ternent T et al (2014) How to submit MS proteomics data to ProteomeXchange via the PRIDE database. Proteomics 14(20):2233–2241
Tryka KA et al (2014) NCBI's database of genotypes and phenotypes: dbGaP. Nucleic Acids Res 42(Database issue):D975–D979
Tun AW et al (2014) Profiling the mitochondrial proteome of Leber's Hereditary Optic Neuropathy (LHON) in Thailand: down-regulation of bioenergetics and mitochondrial protein quality control pathways in fibroblasts with the 11778G>A mutation. PLoS One 9(9):e106779
Van der Auwera GA et al (2013) From FastQ data to high confidence variant calls: the Genome Analysis Toolkit best practices pipeline. Curr Protoc Bioinformatics 43:11.10.1–11.1033
Vizcaino JA et al (2014) ProteomeXchange provides globally coordinated proteomics data submission and dissemination. Nat Biotechnol 32(3):223–226
Vlasblom J et al (2014) Exploring mitochondrial system properties of neurodegenerative diseases through interactome mapping. J Proteomics 100:8–24
von Heijne G (1986) Mitochondrial targeting sequences may form amphiphilic helices. EMBO J 5(6):1335–1342
Wallace DC et al (1988a) Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. Science 242(4884):1427–1430
Wallace DC et al (1988b) Familial mitochondrial encephalomyopathy (MERRF): genetic, pathophysiological, and biochemical characterization of a mitochondrial DNA disease. Cell 55(4):601–610
Walters AM, Porter GA Jr, Brookes PS (2012) Mitochondria as a drug target in ischemic heart disease and cardiomyopathy. Circ Res 111(9):1222–1236
Wang D et al (2014) Characterization of human plasma proteome dynamics using deuterium oxide. Proteomics Clin Appl 8(7–8):610–619
Wang Y et al (2016) Mitogenomic perspectives on the origin of Tibetan loaches and their adaptation to high altitude. Sci Rep 6:29690
Wasinger VC et al (1995) Progress with gene-product mapping of the Mollicutes: Mycoplasma genitalium. Electrophoresis 16(7):1090–1094

Wishart DS et al (2007) HMDB: the Human Metabolome Database. Nucleic Acids Res 35(Database issue):D521–D526
Wishart DS et al (2009) HMDB: a knowledgebase for the human metabolome. Nucleic Acids Res 37(Database issue):D603–D610
Wishart DS et al (2013) HMDB 3.0 – The Human Metabolome Database in 2013. Nucleic Acids Res 41(Database issue):D801–D807
Xia J et al (2009) MetaboAnalyst: a web server for metabolomic data analysis and interpretation. Nucleic Acids Res 37(Web Server issue):W652–W660
Xia J et al (2012) MetaboAnalyst 2.0 – a comprehensive server for metabolomic data analysis. Nucleic Acids Res 40(Web Server issue):W127–W133
Xia J et al (2015) MetaboAnalyst 3.0 – making metabolomics more meaningful. Nucleic Acids Res 43(W1):W251–W257
Yates JR 3rd (2013) The revolution and evolution of shotgun proteomics for large-scale proteome analysis. J Am Chem Soc 135(5):1629–1640
Zong NC et al (2013) Integration of cardiac proteome biology and medicine by a specialized knowledgebase. Circ Res 113(9):1043–1053

Mitochondrial Flashes: Elemental Signaling Events in Eukaryotic Cells

Gaomin Feng, Beibei Liu, Tingting Hou, Xianhua Wang, and Heping Cheng

Contents

Abstract

Mitochondrial flashes (mitoflashes) are recently discovered mitochondrial activity which reflects chemical and electrical excitation of the organelle. Emerging evidence indicates that mitoflashes represent highly regulated, elementary signaling events that play important roles in physiological and pathophysiological processes in eukaryotes. Furthermore, they are regulated by mitochondrial ROS, Ca^{2+}, and protons, and are intertwined with mitochondrial metabolic processes. As such, targeting mitoflash activity may provide a novel means for the control of mitochondrial metabolism and signaling in health and disease. In this brief review, we summarize salient features and mechanisms of biogenesis of mitoflashes, and synthesize data on mitoflash biology in the context of metabolism, cell differentiation, stress response, disease, and ageing.

Keywords

Dynamic activity • Mitochondrial permeability transition pore • Mitoflash • Reactive oxygen species (ROS) • Signaling

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H. Singh, S.-S. Sheu (eds.), *Pharmacology of Mitochondria*,
Handbook of Experimental Pharmacology 240, DOI 10.1007/164_2016_129

1 Introduction

Originated through endosymbiosis 1.5 billion years ago, the mitochondrion is a fascinating organelle that captivates the attention and imagination of generations of scientists. Ever since their visualization and morphological characterization in the late nineteenth century, mitochondria have been instrumental in the development of some of the most profound concepts in modern biology. The chemiosmotic theory established about half a century ago has put mitochondria in the center of cellular energy metabolism (Mitchell 1961). The past two and a half decades, however, have witnessed a new wave of vigorous research that unveils their roles as a signaling hub in vital processes ranging from programmed cell death to innate immunity, from calcium regulation to redox and reactive oxygen species (ROS) signaling (Wallace 2012; Shadel and Horvath 2015; Weinberg et al. 2015). As such, mitochondrial dysfunction and mal-signaling are deeply interlinked to the etiology of major diseases including metabolic disorders, cardiomyopathy, neurodegenerative diseases, and cancer as well as the biology of senescence.

As a transformative new understanding of this organelle, we now increasingly appreciate that mitochondria are dynamic, morphologically and functionally. Inside live cells, they move, divide, fuse, and undergo kissing and nanotunneling for inter-mitochondrial communication (Huang et al. 2013; Mishra and Chan 2014). At the single-organelle level, they are also pulsing, chemically and electrically. Oscillation of the mitochondrial membrane potential occurs incessantly over a broad spectrum of frequency (Aon et al. 2003). Through the mitochondrial Ca^{2+} uniporter, mitochondrial matrix Ca^{2+} transients arise in response to dynamic Ca^{2+} changes in the cytosol and the endoplasmic/sarcoplasmic reticulum (Baughman et al. 2011; De Stefani et al. 2011; Patron et al. 2013). Strikingly, we and others have recently unveiled a novel type of functional dynamics of the organelle, i.e., mitochondrial flash or mitoflash (Wang et al. 2008), which has been hotly pursued by many research groups (Azarias and Chatton 2011; Fang et al. 2011; Schwarzlander et al. 2011, 2012; Wei et al. 2011; Santo-Domingo et al. 2013; Wei-LaPierre et al. 2013). In this brief review, we intend to summarize distinctive features and mechanisms of this newly discovered mitochondrial phenomenon, with a focus on the emerging notion that mitoflashes are elemental signaling events in eukaryotes. We will synthesize data on mitoflash biology in the context of metabolism, cell differentiation, stress response, disease, and ageing. Extensive reviews on related topics can be found in references from different groups (Wang et al. 2012, 2016a; Wei and Dirksen 2012; Hou et al. 2014).

2 Mitoflashes: A New Type of Dynamic Activity of the Organelle

Mitoflashes are 10-s dynamic events occurring suddenly and spontaneously in individual mitochondria in milieu of intact cells under physiological conditions (Wang et al. 2008). First detected with the mitochondrial matrix-targeted biosensor

mt-cpYFP, which dually senses superoxide and pH, mitoflashes have since been visualized with a panel of biosensors and chemical probes, including mitoSOX for superoxide and 2, 7-dichlorodihydrofluorescein diacetate (DCF) for total ROS (Zhang et al. 2013), grx1-roGFP2 for redox potential (Breckwoldt et al. 2014; Wang et al. 2016b), mitoSypHer, pHTomato, and SNARF-1 for matrix pH (Santo-Domingo et al. 2013; Wei-LaPierre et al. 2013; Wang et al. 2016b). In addition, label-free imaging of NADH and FAD autofluorescence revealed also a transient oxidation of NADH and $FADH_2$ (Wei-LaPierre et al. 2013; Hou et al. 2014; Wang et al. 2016b). Simultaneous measurement using TMRM or TMRE showed a rapid, partial loss of mitochondrial membrane potential ($\Delta\psi_m$) followed by a gradual recovery, whose time course mirrors or sometimes outlasts the other indicator-reported signals (Wang et al. 2008; Wei et al. 2011). Overall, there is a positive correlation between the amplitudes of cpYFP-reported mitoflashes and the TMRM-reported $\Delta\psi_m$ depolarization (Wang et al. 2016b), indicating that these parameters are likely interlinked. Interestingly, only a subset of $\Delta\psi_m$ oscillations correspond to cpYFP-reported mitoflashes (Wang et al. 2008; Li et al. 2012b), indicating that the two types of mitochondrial dynamics have overlapping but different origins. Hence, mitoflashes represent a new mitochondrial dynamic phenomenon and each reflects chemical and electrical excitation at the single-organelle level. By integrating data from multiparametric measurements from different groups, we conclude that a mitoflash event comprises multifaceted and interlinked signals – bursting superoxide production, redox shift toward oxidation, matrix alkalinization, and action potential-like membrane depolarization (Hou et al. 2014; Wang et al. 2016b) (Fig. 1). It is conceivable that relative contributions of these sub-components may vary in a cell type-specific and context-sensitive manner.

The mitoflash activity is universally present in all types of cells, tissues, and organisms examined so far. These include mammalian cardiomyocytes, skeletal muscle fibers, neurons, glial cells, fibroblasts, chondrocytes, epithelial cells, and many types of cancer cells (Wang et al. 2008; Pouvreau 2010; Fang et al. 2011; Ma et al. 2011; Wei et al. 2011; Hou et al. 2012, 2013; Cao et al. 2013; Wei-LaPierre et al. 2013). It is well-conserved in species from plants to animals and, for animals, from *C. elegans* to zebrafish to rodents and to humans. Even for single-cell eukaryotic species like yeast, robust mitoflash activity can be detected upon metabolic challenge (Ouyang and Cheng, unpublished data). The experimental systems used for the demonstration of mitoflashes range from isolated single mitochondria (Wei-LaPierre et al. 2013; Zhang et al. 2013) to permeabilized cells (Jian et al. 2014; Gong et al. 2015), to explanted beating hearts (Wang et al. 2008), and even live worms (Shen et al. 2014), zebrafish (Zhang et al. 2015), and mice (Fang et al. 2011) (Fig. 2). The universality of mitoflashes, together with the fact that mitoflashes occur in cell-free respiring mitochondria, attests an evolutionally ancient origin of this unique biophysical and physiological activity. Indeed, this speculation is somewhat bolstered by the finding that mitoflash properties in terms of amplitude and duration are remarkably similar across the vast breadth of species and cell types, in sharp contrast to the diversity in mitochondrial shape, density, motility, metabolic rate, and functionality.

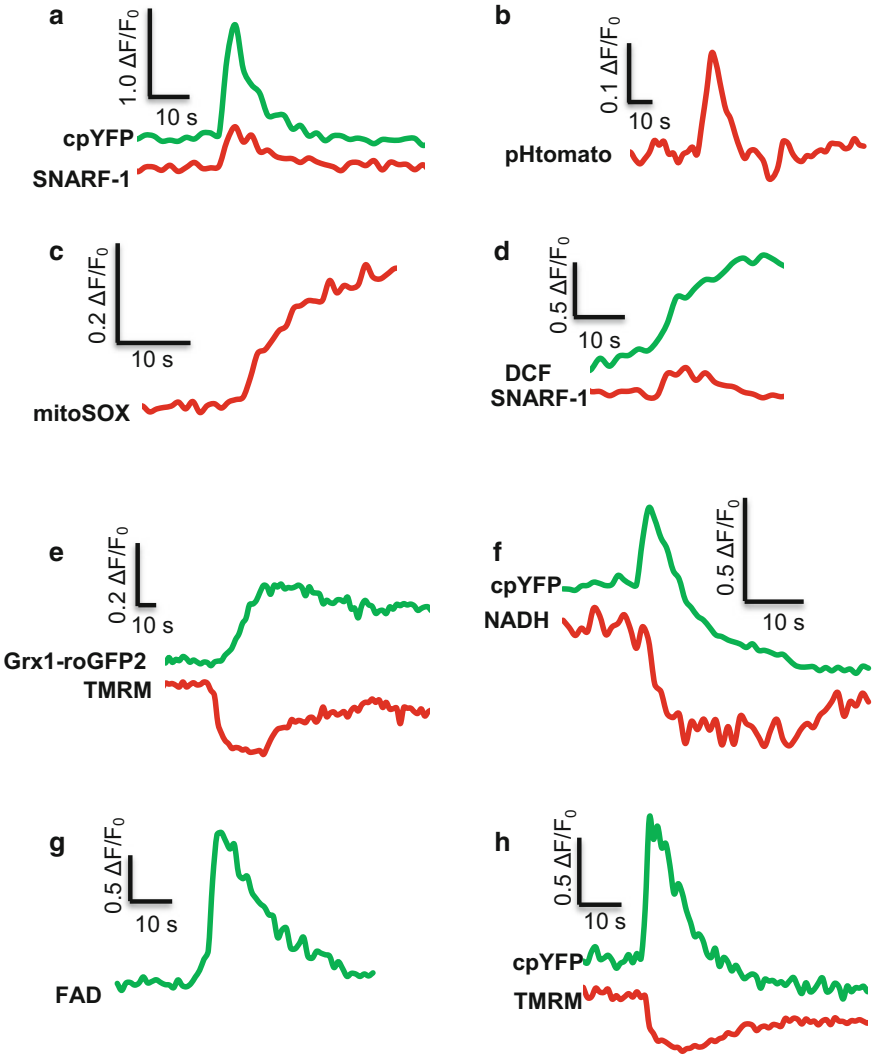

Fig. 1 Multifaceted signals in a mitoflash. Transient matrix alkalinization is detected by cpYFP (**a**, **f**, **h**), pHTomato (**b**), and SNARF-1 (**d**); MitoSox (**c**) is used to detect superoxide and DCF (**d**) for the total ROS; Membrane depolarization is measured using TMRM (**e**, **h**); Transient redox shift toward oxidation was visualized with Grx1-roGFP2 (**e**) in conjunction with label-free imaging of NADH (**f**) and FAD (**g**) autofluorescence. From Wang et al. (2016b)

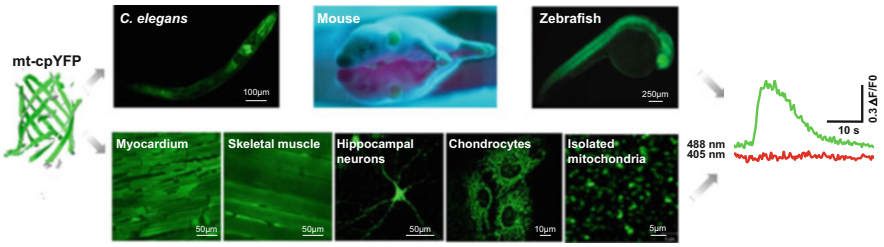

Fig. 2 Mitoflashes: universal and elemental mitochondrial events. Mitoflashes of highly conserved properties are found in different tissues, cells, and isolated mitochondria across eukaryotic species

3 Biogenesis of Mitoflashes

Since mitoflash is an excitable phenomenon, the first and foremost prerequisite for mitoflash biogenesis is that the mitochondria must be energized at an excitable state. Mitoflash activity is abrogated upon dissipation of $\Delta\psi_m$ and pH gradients across the inner mitochondrial membrane (IMM), by nanomolar FCCP, a charge-uncompensated proton ionophore, or micromolar nigericin, an electroneutral H^+/K^+ antiporter, or micromolar monensin, a H^+/Na^+ antiporter (Wang et al. 2008, 2016b; Wei-LaPierre et al. 2013). Mitoflash is completely absent in ρ^0 143B human osteosarcoma cells devoid of mitochondrial DNA and electron transfer chain (ETC) activity (Wang et al. 2008). ETC inhibitors, including rotenone (Complex I), myxothiazol (Complex II), antimycin A (Complex III), sodium cyanide (Complex IV), are all effective blockers of mitoflashes (Wang et al. 2008; Santo-Domingo et al. 2013). Moreover, limiting electron entry at complex I by cardiac-specifically knocking out Ndufs4, an assembling subunit of complex I, suppresses mitochondrial flash activity in cardiac myocytes (Gong et al. 2015).

In normal mitochondrial respiration, oxygen is required to act as the four-electron acceptor and form H_2O at complex IV, pulling the electron flow forward through the ETC, whose activity is coupled with proton pumping at complexes I, III, and IV to establish the proton gradients across the IMM, and contributes to $\Delta\psi_m$. Oxygen can also act as the one-electron acceptor for the formation of superoxide anions at intermediary ETC sites. Thus, no mitoflashes exist in the conditions of stringent anoxia (Huang et al. 2011). Further, to permit mitoflash occurrence, the pool of electron donors, NADH to donate electrons at Complex I and $FADH_2$ at Complex II, should not be in exhausted states.

A stochastic, flickering opening of the still-elusive mitochondrial permeability transition pore (mPTP) is thought to ignite a mitoflash in such an excited system. Most, though not all, mitoflashes accompany sudden and irreversible loss of Rhod-2 (MW = 752 Da) (Wang et al. 2008) or its Ca^{2+}-insensitive analog (MW = 980 Da) (Wang et al. 2012), suggesting that small molecules leak out of the matrix through mPTP at the onset of a mitoflash. Electrophysiological characterization has

revealed that mPTP exhibits a range of conductance, and likewise, its putative pore is variably permeable to small molecules of MW up to 1.5 KDa (Bernardi et al. 2015). This might explain the fact that not all mitoflashes are associated with discernible loss of matrix-preloaded Rhod-2 or its analog.

Several additional lines of evidence support the role of mPTP opening in mitoflash biogenesis. Mitoflashes are activated by atractyloside (ATR, an mPTP activator) and inhibited by bongkrekic acid (BA, an mPTP inhibitor) (Wang et al. 2008; Hou et al. 2013). Mitoflash frequency is decreased by cyclosporine A (CsA) which targets cyclophilin D (CyPD), an important regulator of mPTP (Wang et al. 2008). Overexpression of CyPD increases and knockout of CyPD halves cardiac mitoflash frequency (Shang et al. 2016). However, it should be noted that neither deletion of CyPD nor its inhibition by CsA alters mitoflash frequency in skeletal muscles (Wei et al. 2011; Shang et al. 2016). This apparent tissue-to-tissue disparity can be explained for two reasons. First, it has been recently shown that classic mPTP can operate in the absence of any CypD and in a CsA-insensitive manner (Baines et al. 2005; Basso et al. 2005; Li et al. 2012a), indicating that CypD is not obligatory to flickering mPTP gating. Second, the expression of CyPD in skeletal muscles is only 1/4 of that in cardiac muscles (Shang et al. 2016), suggesting that mPTP gating in skeletal muscles might be largely CypD-independent. Hence, neither CypD-independence nor CsA-insensitivity, as observed in skeletal muscles, should be taken as evidence excluding the involvement of mPTP in mitoflash biogenesis. Nevertheless, the exact mechanism whereby mPTP opening leads to a cascade of changes that manifest as a mitoflash is not fully understood.

Upon mPTP opening, electrochemically downhill movement of protons from the inter-membrane space to the matrix would acidify the matrix. Unexpectedly, we and others have found no evidence of matrix acidosis at the onset of a mitoflash. This apparent paradox might be reconciled for three different reasons. The opening of mPTP, as judged from leakage of small molecules, is brief (<2 s); the matrix pH buffering power is high, reaching 500,000 at pH 8.0 (Poburko et al. 2011). Furthermore, partial loss of $\Delta\psi_m$ accelerated proton pumping by the ETC complexes and this would counteract the mPTP-mediated proton influx.

Recently, Santo-Domingo and colleagues have proposed a model of mitoflash ignition, in which the formation of the IMM fusion pore serves the trigger (Santo-Domingo et al. 2013). This model was based on their observation that knockout of Opa1, which is an IMM fusion protein, completely abolishes mitoflashes in mouse embryonic fibroblasts (MEFs) (Santo-Domingo et al. 2013). We revisited this issue and found that, albeit markedly inhibited, basal mitoflash frequency is not completely vanished in Opa1$^{-/-}$ MEFs (Li et al. 2016). Strikingly, in response to hyperosmotic stress, which stimulates mitoflash production via Ca^{2+}- and ROS-dependent mechanisms (Hou et al. 2013), the rate of mitoflash occurrence is similarly elevated regardless of Opa1, excluding IMM fusion as a prerequisite of mitoflash biogenesis. Meanwhile, we and others have found that mitochondrial respiration is markedly inhibited in the absence of Opa1, due presumably to the fact that Opa1 is required for proper formation of cristae and ETC supercomplexes (Mishra et al. 2014). We

conclude that Opa1 deficiency secondarily impairs the biogenesis of spontaneous mitoflashes via its direct suppression of respiratory activity.

Within a heart cell containing thousands of mitochondria, mitoflashes seen in a confocal plane distribute randomly amongst the mitochondrial population, and the interval between two consecutive events in a given cell obeys a decaying exponential function, consistent with the idea that mitoflash ignition is governed by a Poisson process (Li et al. 2012b). Most recently, we have proposed a stochastic trigger model for the ignition of mitoflashes (Li et al. 2016). Specifically, the propensity of mitoflash ignition in a mitochondrion (in the form of granule, thread, or reticular network) is determined by nP_o, where n refers to the number of discrete trigger sites (e.g., number of mPTPs) and P_o the probability of transient opening. This model is supported by the observation that at the whole-cell level mitoflash frequency is largely invariant when mitochondria network fragmentizes in Mfn1/Mfn2 knockout cells or hyperfuses in Drp1- or Mff-deficient cells. As such, at the single-mitochondrion level, fragmented or hyperfused mitochondria display fewer or more frequent events per organelle. This is in contrast to an oscillatory trigger model in which mitoflashes arise from intrinsic oscillation of the mitochondria (Romashko et al. 1998; Aon et al. 2003). The oscillatory trigger model predicts a constant oscillation frequency independent of mitochondrial fission and fusion (Fig. 3). An exemplary oscillator system can be found in cardiac pacemaking – the rate of action potential firing in the whole sinoatrial node, which consists of hundreds of pacemaker cells that are electrically coupled to form a functional syncytium, is similar to that of individual cells acting in isolation (Lakatta et al. 2010).

Once activated, a mitoflash appears to evolve in an autonomous fashion. In a working model recently refined (Wang et al. 2016b) (Fig. 4), we hypothesized that mPTP opening results in water and ionic fluxes to electrically depolarize and mechanically strain the IMM. The mechanical strain is evidenced by the conspicuous mitochondrial swelling that sometimes even masquerade as "contraction" during a mitoflash. Recent super-resolution microscopy studies further revealed that the contents in the inter-membrane space can be squeezed by matrix expansion while the whole mitochondria volume is kept constant (Booth et al. 2016), indicating that mechanical strain may occur even in the absence of mitochondrial swelling. In either case, mechanical strain may cause dislocation of ETC molecular assembly (e.g., supercomplexes) and dis-insulation of the normal electron path particularly at the intermolecular junctures (Mechanism 1), and thus greatly increases intermediary electron leakage to oxygen, giving rise to a burst of superoxide formation. Meanwhile, depolarization accelerates electron transfer from the donor pool to ETC acceptors (e.g., from NADH to complex I, and from $FADH_2$ to complex II) and shifts the redox potential toward oxidation (Mechanism 2). The flux of superoxide production can be put in an equation [modified from a previous review (Wang et al. 2012)]:

$$J_{superoxide} = \beta\,[O_2][e^-]$$

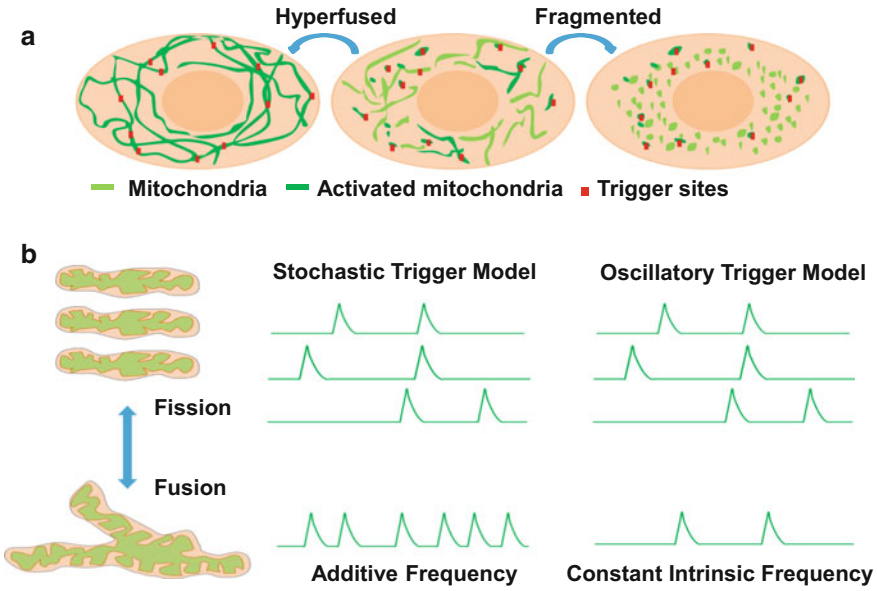

Fig. 3 Stochastic trigger model for mitoflash biogenesis. (**a**) Putative mitoflash trigger sites, marked with *red blocks*, remain constant when the mitochondrial network hyperfuses or fragmentizes. (**b**) In the stochastic trigger model, hyperfused mitochondrial reticulum exhibits additive rate of mitoflash production. In contrast, oscillatory trigger model predicts a near constant oscillation frequency, independent of mitochondrial fission or fusion. Modified from Li et al. (2016)

where $[e^-]$ refers to the electron occupancy at transferable sites, $[O_2]$ the local oxygen concentration, and β the transfer efficiency. In this scenario, Mechanism 1 is related to an increase in β whereas Mechanism 2 is related to change in $[e^-]$.

Depolarization and bursting superoxide formation stimulate ETC activity and accelerate coupled proton pumping across the IMM, resulting in matrix alkalinization. In this way, different facets of the mitoflashes are mechanistically interlinked. Termination of a mitoflash ensues following stochastic closure of the mPTP, restoration of matrix ionic homeostasis, re-insulation of the ETC path as the membrane stain decreases, and recovery of normal ETC activity as $\Delta\psi_m$ repolarizes. It is noteworthy that a subset of cpYFP-reported mitoflashes exhibit a prolonged tail of undershoot. We interpret this tail component to reflect a sustained matrix acidosis, likely arising from decreased ETC proton pumping (as the electron-donor pool diminishes) and/or enhanced proton entry due, for instance, to incomplete closure of mPTP. In this regard, multiparametric measurement in neurons shows that mito-SypHer reports a brief matrix alkalinization followed by a prolonged acidosis, while mito-Grx1-roGFP2 reports a persistent oxidative redox shift during the tail component (Breckwoldt et al. 2014).

As to properties of individual mitoflashes, quantitative analysis has been aided with an automated mitoflash detection algorithm, the Flashsniper (Li et al. 2012b),

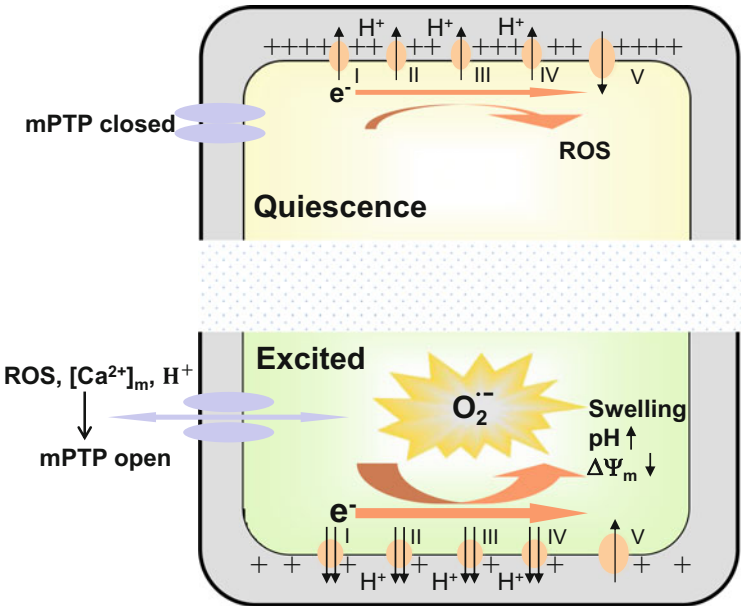

Fig. 4 Schematic model for mitoflash biogenesis. Acting solo or in concert, matrix Ca^{2+}, ROS, H^+, or other factors trigger a transient opening of mPTP. A stochastic, flickering opening of the mPTP results in water and ionic fluxes to strain and depolarize the inner mitochondrial membrane (IMM). The membrane depolarization may trigger additional mPTP opening throughout the mitochondrion or interconnected mitochondrial network. The mechanical strain may cause dislocation of ETC molecules and disruption of the normal electron-flow path, and thus greatly increases electron leakage to oxygen at intermediary ETC sites, giving rise to a burst production of superoxide and its ROS derivatives. Meanwhile, depolarization accelerates electron transfer from the donor pool to ETC acceptors (e.g., from NADH to complex I, and from $FADH_2$ to complex II), and shifts redox potential toward oxidation. Accelerated ETC activity, driven dually by depolarization and oxygen deprivation of ETC electrons, stimulates coupled proton pumping by ETC complexes across the IMM, giving rise to matrix alkalinization. Modified from Wang et al. (2008)

and the latest data obtained from hyperfused mitochondria with improved detection ability and minimized out-of-focus detection bias (Li et al. 2016). We found that the mitoflash amplitude follows a decaying exponential distribution (Li et al. 2016). This indicates that the chemical and electrical excitation of the mitochondria, in the form of mitoflashes, is polymorphic rather than stereotypical. This variability in mitoflash magnitude is presumably under the control of trigger strength (e.g., mPTP open duration and conductance). Thus, albeit discrete and digital, mitoflash biogenesis is not an all-or-none phenomenon as we initially thought.

Notably, we found that, over reticular mitochondrial network that extend up to 50 μm in skeletal muscles or the entire cell in HeLa cells, mitoflash ignition can be synchronous at the millisecond temporal resolution. This observation implicates that electrical signals, instead of diffusive messengers, are most likely responsible for the spatial synchrony. This hypothesis is in general agreement with the results

that membrane depolarization activates mPTP (Bernardi 1992), even though not all membrane depolarization events are associated with mitoflashes (Wang et al. 2008). Functionally, we can define mitoflash signal mass as amplitude × duration × area (Li et al. 2016), and the aforementioned finding implicates that mitochondrial morphological remodeling can regulate mitoflash signaling.

4 Mitoflashes: Elemental Signaling Events

At the level of a single mitochondrion, however, a mitoflash engulfs the entire organelle, all its genomic, protein, and lipid contents. The bursting formation of superoxide and its derivative ROS would put the mitochondrion in jeopardy. Moreover, it occurs at the expense of a significant sum of energy, evidenced by the dissipation of $\Delta\psi_m$ and the depletion of electron donor pool. The question arisen is why on the earth such mitoflash activity is universally present and highly conserved across all the eukaryotic cells and organisms examined? Is it an unavoidable, hazardous byproduct of the organelle at work? Or is it a necessary evil invented and harnessed for some beneficiary deeds, e.g., cellular and organelle signaling?

Much work has been unveiling a multi-layered link of mitoflash with energy metabolism. As discussed above, mitochondrial energization and respiration are prerequisites to the occurrence of mitoflashes in intact cells, and mitoflash frequency positively correlates with energy metabolism in many (but not all, see below) situations. Superfluous substrate supply (glucose or fatty acid in intact cells, or pyruvate in permeabilized cells) stimulates mitochondrial respiration and increases mitoflash frequency (Pouvreau 2010; Gong et al. 2015). By generating mt-cpYFP transgenic mice and developing in vivo mitoflash imaging technique, we have demonstrated that mitoflash frequency of skeletal muscles surges in response to systemic glucose challenge or insulin stimulation (Fang et al. 2011). Likewise, glucose supplementation in the culture plates enhances mitoflash activity in *C. elegans* pharyngeal muscles, whereas removal of food for 3–6 h diminishes it (Shen et al. 2014). Mitoflash frequency is also linked to energy expenditure and muscle activities, apparently in a more complex way: it significantly increases as an aftereffect of pacing of cardiac myocytes (2 min at 2 Hz) (Gong and Wang 2013; Gong et al. 2014); or, in skeletal muscle fibers, a brief tetanic stimulation (5 tetani) increases, but prolonged tetanic stimulation (40 tetani) markedly decreases mitoflash frequency observed right after stimulation (Wei et al. 2011). Taking these as a whole, mitoflash frequency is thought to be a biomarker of mitochondrial metabolic state. However, a caveat is that mitoflash frequency is not a unique function of the rate of metabolism under different conditions (see below).

We have recently shown that protons at nanodomains of IMM are powerful trigger of mitoflashes (Wang et al. 2016b). Matrix proton uncaging elicits immediate and robust mitoflash response over a broad dynamic range in cardiac myocytes and HeLa cells. Similar effects can be induced by electroneutral proton–cation antiport mediated by low concentrations of nigericin and monensin. Because the

estimated lifetime and diffusion distance of free matrix proton are merely 1.42 ns and 2.06 nm, the mitoflash-triggering effect is likely mediated by nanodomain, short-lived free protons rather than changes of bulk matrix pH (Wang et al. 2016b). For steady pH changes, mitoflash incidence in cardiac cells shows little change over the physiological pH ranges or even decreases at extreme pH values (Wang et al. 2016b). Since concepts such as proton gradients, vectorial proton movements, and proton motive force occupy the center stage of bioenergetics, that protons trigger mitoflashes indicates that mitoflash biogenesis is mechanistically coupled with energy metabolism at the deepest level. Future investigations are warranted to determine whether and how mitoflash activity reciprocally regulates mitochondrial energy metabolism, fulfilling a signaling role.

However, this mitoflash-metabolism link is only one facet of the whole story. Mitochondria are multifunctional, so situations are there when mitoflash activity is regulated independently of energy metabolism. Indeed, mitoflash biogenesis involves molecular players and mechanisms that overlap but are distinctly different from those of energy metabolism (see above). For instance, mPTP, which is obligatory to mitoflash ignition, does not directly participate in oxidative phosphorylation, but serves a hub to integrate convergent signals including Ca^{2+}, ROS, $\Delta\psi_m$, pH, P_i (Bernardi et al. 2015). Indeed, mitoflash frequency is actively regulated alongside yet relatively independent of the regulation of mitochondrial respiration. In particular, three major mitoflash trigger signals have been identified, i.e., physiological levels of matrix Ca^{2+} elevation, basal ROS, and recently, protons at nanodomains of IMM (Hou et al. 2013; Jian et al. 2014; Wang et al. 2016b). In HeLa cells, a prolonged sub-micromolar matrix Ca^{2+} elevation increases mitoflash frequency, with EFHD1 functioning as a novel mitochondrial Ca^{2+} sensor (Hou et al. 2016). H_2O_2 and oxidants including selenite, menadione, and hypochlorite also dose-dependently enhance mitoflash activity (Ma et al. 2011; Hou et al. 2013; Zhang et al. 2014). Importantly, the co-presence of ROS can allow an otherwise sub-threshold Ca^{2+} signal to significantly augment mitoflash frequency, indicating a synergistic effect between Ca^{2+} and ROS in mitoflash regulation (Hou et al. 2013). In a nutshell, regulation of mitoflash ignition is qualitatively very similar to, but quantitatively distinctive from, the regulation of persistent mPTP opening – the mitoflash ignition involves much weaker, physiological changes of the trigger signals (Hou et al. 2014). Collectively, these results indicate that mitoflashes are not merely a byproduct or epiphenomenon of mitochondrial respiration. In addition, mitoflash is a complex event regulated by multiple factors, so different regulators may be dominant in different experimental conditions.

An emerging concept is that the ubiquitous, yet potentially hazardous mitoflashes are harnessed to act as elemental signaling events. In this regard, we have proposed that the ROS components or superoxide flashes are well-poised to serve as elemental, dynamic signaling units (Wang et al. 2012). It has been increasingly appreciated that ROS are important messengers participating in many signaling pathways including cell migration, cell proliferation, autophagy, apoptosis, and senescence (Shadel and Horvath 2015; Zhang et al. 2016). During cell migration, Rho, a critical mediator of actin polymerization and the formation of focal adhesions, can be activated by ROS

through direct oxidation of a redox-sensitive motif (Aghajanian et al. 2009). As an inhibitor of cell migration, the low molecular weight protein tyrosine phosphatase is inactivated by ROS through direct oxidation on Cys12 and Cys17 to form an inactivating disulfide bond (Chiarugi et al. 2001). Moreover, matrix pH change in a mitoflash or pH flash can also act as a signal. For example, nutrient stimulation increases mitochondrial matrix pH in pancreatic β cell and the matrix alkalinization promotes ATP synthase and metabolism-coupled insulin secretion (Wiederkehr et al. 2009; Akhmedov et al. 2010). Therefore, both the ROS and the pH components endow mitoflashes the potential to act as signaling events.

Mitoflash signaling occurs mainly in the frequency-modulated (FM) mode, because the amplitude and duration of mitoflashes are stochastic variables whose mean values are relatively constant in different conditions. Many groups have independently demonstrated that mitoflash frequency can be actively regulated over two orders of dynamic range. This implicates a high signal-to-background contrast, high signal-to-noise ratio, and high information-encoding capacity. Mitoflashes are "digital," and unique advantages of a digital versus analog signal are as the follows. (1) *Linearity*. Its signaling strength is linearly determined by the event rate, while an analog system may subject to non-linear transformation and undergo saturation; (2) *Coordination among multiple effectors*. If two or more effectors are targeted, a digital mechanism causes the same fold-change in each and every effector affected, whereas an analog signal differentially activates its effectors in accordance with their respective affinities (e.g., K_d) (Cai et al. 2008); (3) *Efficiency*. For any hyperlinear processes, spatially and temporally focused signals would be more effective than those if the same total signal mass spread evenly over space and time. (4) *Specificity*. In the digital paradigm, compartmentalization of signals provides a way to achieve signaling specificity by strategic localization of decoder and effector proteins; (5) *Diversity*. With specificity and compartmentalization, diverse signals can be activated in different contexts. Furthermore, as individual mitoflashes constitute elemental signaling events, higher orders of spatial and temporal pattern of mitoflash activation can greatly expand the information-encoding capacity in a combinatorial fashion. (6) *Safety*. Take ROS for an example. Whereas sustained elevation of global ROS is undoubtedly detrimental, brief and compartmentalized ROS pulses may reliably activate high-threshold ROS effectors and pathways locally, limiting cellular and mitochondrial damage.

These recent advances have also brought forth new insights into the "digital-analog" duality of cellular ROS signaling. Specifically, mitochondrial basal ROS production provides the primary source of analog, "hemostatic ROS" for setting basal ROS level and redox potential. Amidst this background, mitoflashes form a distinctive, discrete system of digital, "signaling ROS" (Hou et al. 2014). As discussed above, key features of the digital subsystem include confinement of mitoflashes to single mitochondria, autonomous mitoflash evolvement (with stochastic variability), and FM encoding and decoding of biological information. Such a digital subsystem does not operate independently of to the analog ROS subsystem. Basal ROS directly triggers mitoflash events, whilst the contribution of digital signaling ROS to homeostatic ROS appears to be marginal (Ma et al. 2011; Hou

et al. 2013). In analogy to "Ca^{2+} sparks" for Ca^{2+} signaling (Cheng and Lederer 2008), mitoflashes as the elemental ROS units may support hierarchical multi-scaled intracellular ROS dynamics, including ROS waves supported by ROS-induced ROS release (Romashko et al. 1998; Yan et al. 2008; Zorov et al. 2014) and cell-wide ROS oscillations under stressed conditions (Aon et al. 2003). Through spatial and temporal coordination, complemented with the multiplicity of mitoflash signal components, mitoflash signaling may achieve efficiency, diversity, and cell type- and context-dependent specificity, all at once.

In this scenario, we speculate that the mitochondrion in its symbiotic life might have been harnessed by its host not only as a powerhouse, but also for a handy, digital ROS (and pH) signal generator. That is, it provides a digital machinery to generate and terminate discrete signaling ROS and other signals. Evidently, this compartmentalized ROS signaling is intimately interlinked with other core functions and signals of the organelle, including bioenergetics, basal ROS production for redox homeostasis, and mitochondrial stress responses. Investigation of mitoflash signaling should shed new light on our understanding of mitochondrial functions and their integration to cellular and organismal biology.

5 Mitoflash Signaling in Biology

While the consensus on the origin and nature of mitoflashes emerges, more research groups are beginning to explore possible roles of mitoflashes in diverse physiological and pathophysiological contexts. This is an enormous and important undertake, because we anticipate that, despite the universality of mitoflashes, mitoflash signaling might vary in a tissue- and cell type-specific and context-dependent manner. In this section, we present a few sets of examples to give a glimpse of the significance and complexity of mitoflash signaling in biology (Fig. 5). An intimate relationship between mitoflashes and mitochondrial respiration and metabolism has been discussed above and will not be repeated.

In the first set of examples, mitoflashes serve as an important senor and responder to oxidative stresses. For instance, in cardiac myocytes exiting from hypoxia treatment, a flurry of mitoflash activity surges within 5–10 windows of reoxygenation (Wang et al. 2008). Chemical preconditioning with adenosine, which alleviates reoxygenation-associated oxidative stress, effectively suppresses this transient overshoot of mitoflash activity (Wang et al. 2008). Hyperosmotic stress elicits a reversible 20-fold increase of mitoflash frequency in an ROS- and Ca^{2+}-dependent manner (Hou et al. 2013). This elevated mitoflash activity is implicated in the activation of JNK and p38 involved in cellular adaptive responses for survival (Hou et al. 2013). In HeLa cells undergoing oxidative stress-related apoptosis, an increase in mitoflash activity is an early stress responder, occurring prior to global ROS increase, mitochondrial fragmentation, Bax translocation, and cytochrome C release (Ma et al. 2011). Such mitoflashes drive the progression toward the commitment of the cell to death, and are sensitive to CypD- and Bcl-2-mediated pro- and anti-apoptotic regulation (Ma et al. 2011). In *C. elegans* with transgenic expression of cpYFP, Shen et al. have shown that

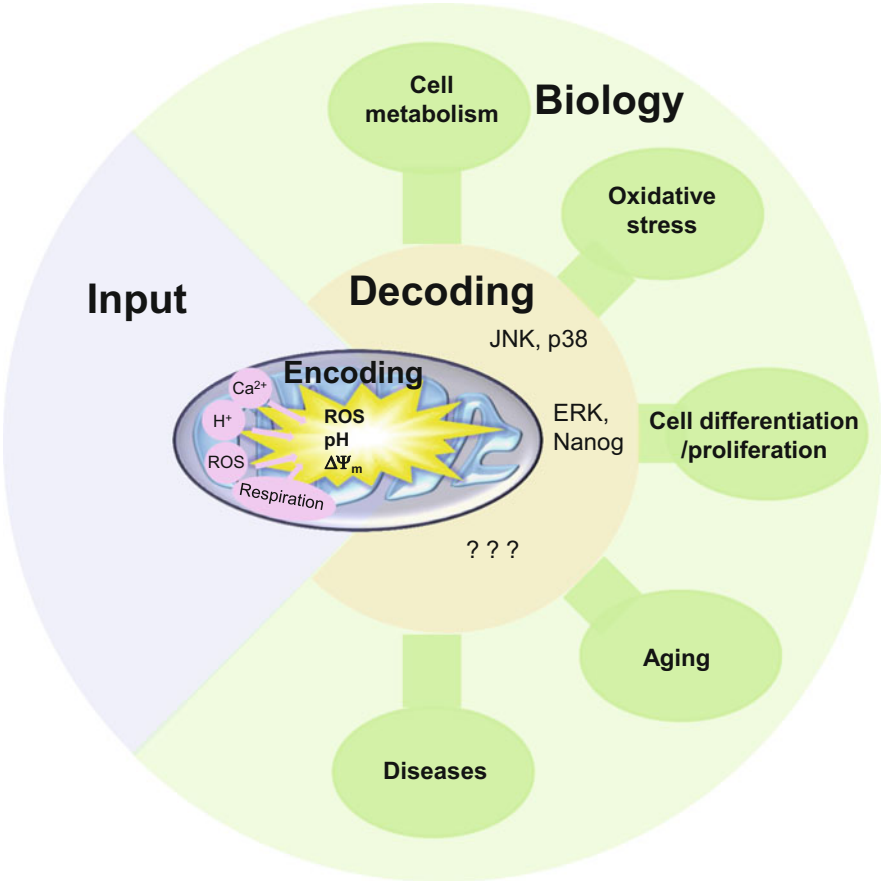

Fig. 5 Mitoflash signaling in biology. Input signals, such as matrix Ca^{2+}, ROS, H^+, and other factors, converge on triggering discrete mitoflash events. Mitoflash encodes the input signals in the form of ROS burst, redox shift toward oxidation, matrix alkalinization, and/or IMM depolarization. While intra-mitochondrial decoding processes are largely unknown at present, cytosolic decoding of mitoflash signals appears to invoke effectors such as ERK, JNK, p38, and Nanog. As such, mitoflashes play a signaling role in vital biological processes ranging from metabolism to cell differentiation, to stress response, diseases, and aging

oxidative stressors, H_2O_2, or paraquat or photochemically induced ROS from the fluorescent protein KillerRed, almost immediately increase mitoflash frequency, by 15-fold in the pharyngeal muscles (Shen et al. 2014). These results provide compelling evidence that mitoflash frequency is a sensitive gauge of oxidative stress, and mitoflashes constitute an early component of oxidative stress response.

Mitoflashes also participate in the regulation of cell differentiation, cell proliferation, and stemness maintenance. The mitoflash frequency negatively regulates the self-renewal of embryonic mouse cerebral cortical neural progenitor cells (NPCs) (Hou et al. 2012). SOD2 deletion and mPTP opener increase mitoflash frequency

and decrease NPC proliferation, whereas ROS scavengers or mPTP inhibitors decrease mitoflash frequency and increase NPS proliferation, with little change in the global ROS level (Hou et al. 2012). At mid-gestation, fewer proliferative NPCs and differentiated neurons are found in the embryonic cerebral cortex of SOD2 knockout mice compared with wild-type littermates (Hou et al. 2012). This mitoflash signaling is in part mediated by ERK1/2 activation, and is in contrast to the role of global ROS which is generally thought to promote cell differentiation (Hou et al. 2012). More recently, Ying et al. reported that mitoflash plays a role at the early stages of somatic stem cell reprogramming through increasing the expression of Nanog at both mRNA and protein level (Ying et al. 2016). These findings are in general agreement with that mitochondrial ROS play signaling roles in a variety of pathways in differentiation and organogenesis (Owusu-Ansah and Banerjee 2009).

Shen et al. reported that mitoflash frequency in early adulthood is a predictor of *C. elegans* lifespan (Shen et al. 2014). The day-3 mitoflash frequency in pharyngeal muscles negatively correlates with the lifespan of individual animals. This negative correlation holds when lifespan is altered genetically, or by environmental factors, or from stochastic variability, suggesting that the tempo of mitoflashes reflects the tick of the mitochondria which, in turn, are deeply rooted in the putative ageing program.

It has also been reported that increased mitoflash activity is associated with an over-all doubled level of mtDNA mutation in both fibroblasts from patients with Huntington's disease (HD) and the HD mouse model (Wang et al. 2013). Xu and Chisholm have shown that physical damage of the worm skin induces a wave of dramatically increased mitoflash activity in the wake of a tissue-level propagating Ca^{2+} wave initiated from the injury site. Functionally, this mitochondrial ROS burst promotes actin-based wound closure (Xu and Chisholm 2014). Moreover, in the skeletal muscle fibers from RYR1 Y522S/WT mice, a model of malignant hyperthermia, a significant temperature-dependent increase of mitoflash frequency has also been documented (Wei et al. 2011). The decreased mitoflash frequency in Ndufs4 cKO mice is accompanied by accelerated heart failure (Karamanlidis et al. 2013; Gong et al. 2015). In mt-cpYFP-expressing db/db mouse model with the obesity and insulin-resistance, we uncovered a progressive increase of mitoflash frequency along with its mitochondrial morphological changes. Pioglitazone treatment normalized mitoflash frequency and morphology while restoring mitochondrial respiratory function and insulin sensitivity (Ding et al. 2015). This set of findings illustrates that mitoflashes provide a powerful investigative tool in pursuing the etiology and therapeutic intervention of major mitochondrion-related diseases.

6 Perspectives

According to Shannon's information theory, a signaling pathway can be regarded as a communication system of information transmission, and involves encoding, communicating, and decoding components (Shannon 1997; Uda and Kuroda

2016). When a signaling pathway operates in cells and organisms, there are also biological input signals feeding to the pathway, and downstream effectors that respond to the output signals. In this perspective, we are only at the beginning of appraising the mitoflash signaling pathways. With Ca^{2+}, basal ROS, protons as the major input signals, how does a mitoflash encode them into new signals inside the mitochondria and beyond? How are the mitoflash signals decoded, and which signaling molecules and pathways are invoked? What are the ultimate biological effectors of mitoflashes? These are important questions that beg for answers.

We have by now identified several biological inputs – matrix Ca^{2+}, H^+, and basal ROS as the triggers. Candidate encoding signals include ROS burst, pH transient, redox shift, and action potential-like $\Delta\psi_m$ oscillation. These signals blanket over the entire mitochondrion or interconnected mitochondrial network. At present, almost nothing is known about how a mitoflash affects mitochondrion-residing signaling and functioning molecules – the protein machineries for tricarboxyl acid cycle and lipid metabolism, a plethora of enzymes for phosphorylation and acetylation, and other post-translation modifications, and metal proteins for redox–sensing and regulation, as well as transcription factors and protein synthesis machinery serving the mitochondrial genome. Likewise, little is understood about how the mitoflash activates downstream events beyond a mitochondrion. In principle, it might communicate with neighbor mitochondria and other organelles (e.g., endoplasmic/sarcoplasmic reticulum) directly via stable and diffusive ROS species derived from superoxide anions. In contrast, single-mitochondrion pH change is unlikely to propagate over a long distance, because free proton lifetime and diffusion distance are on nanosecond and nanometer scales in milieu of heavily buffered cellular environments. It might also be possible that signaling molecules are activated intra-mitochondrially and then traffic beyond single mitochondrion to relay mitoflash signaling. In the cytosol, only a few decoding signaling molecules have been identified to link mitoflashes to cell survival and stress responses. Nonetheless, biological studies have depicted an increasingly interesting picture where mitoflashes play salient and versatile roles in metabolism, biogenetics, cell fate regulation, stress responses, diseases, and aging.

With superoxide flash as one facet of a mitoflash, recent advances in mitoflash research shed new light on ROS signaling. Mitoflash ROS bursts are digital, local (single-mitochondrion), and stochastic. By contrast, ROS oscillations in the form of ROS-induced ROS release and ROS waves are oscillatory phenomena involving long-range and even cell-wide inter-mitochondrial communication. Future investigation is warranted to integrate such a broad spectrum of ROS dynamics into a unifying framework. Finally, as mitoflashes emerge as elemental events underlying ROS signaling and beyond, targeting mitoflash activity may provide a novel means for the control of mitochondrial metabolism and signaling in health and disease. The field is widely open to new researchers for new discoveries.

References

Aghajanian A, Wittchen ES, Campbell SL, Burridge K (2009) Direct activation of RhoA by reactive oxygen species requires a redox-sensitive motif. PLoS One 4:e8045

Akhmedov D, Braun M, Mataki C, Park KS, Pozzan T, Schoonjans K, Rorsman P, Wollheim CB, Wiederkehr A (2010) Mitochondrial matrix pH controls oxidative phosphorylation and metabolism-secretion coupling in INS-1E clonal beta cells. FASEB J 24:4613–4626

Aon MA, Cortassa S, Marban E, O'Rourke B (2003) Synchronized whole cell oscillations in mitochondrial metabolism triggered by a local release of reactive oxygen species in cardiac myocytes. J Biol Chem 278:44735–44744

Azarias G, Chatton JY (2011) Selective ion changes during spontaneous mitochondrial transients in intact astrocytes. PLoS One 6:e28505

Baines CP, Kaiser RA, Purcell NH, Blair NS, Osinska H, Hambleton MA, Brunskill EW, Sayen MR, Gottlieb RA, Dorn GW et al (2005) Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. Nature 434:658–662

Basso E, Fante L, Fowlkes J, Petronilli V, Forte MA, Bernardi P (2005) Properties of the permeability transition pore in mitochondria devoid of Cyclophilin D. J Biol Chem 280:18558–18561

Baughman JM, Perocchi F, Girgis HS, Plovanich M, Belcher-Timme CA, Sancak Y, Bao XR, Strittmatter L, Goldberger O, Bogorad RL et al (2011) Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter. Nature 476:341–345

Bernardi P (1992) Modulation of the mitochondrial cyclosporin A-sensitive permeability transition pore by the proton electrochemical gradient. Evidence that the pore can be opened by membrane depolarization. J Biol Chem 267:8834–8839

Bernardi P, Rasola A, Forte M, Lippe G (2015) The mitochondrial permeability transition pore: channel formation by F-ATP synthase, integration in signal transduction, and role in pathophysiology. Physiol Rev 95:1111–1155

Booth DM, Enyedi B, Geiszt M, Varnai P, Hajnoczky G (2016) Redox nanodomains are induced by and control calcium signaling at the ER-mitochondrial interface. Mol Cell 63:240–248

Breckwoldt MO, Pfister FM, Bradley PM, Marinkovic P, Williams PR, Brill MS, Plomer B, Schmalz A, St Clair DK, Naumann R et al (2014) Multiparametric optical analysis of mitochondrial redox signals during neuronal physiology and pathology in vivo. Nat Med 20:555–560

Cai L, Dalal CK, Elowitz MB (2008) Frequency-modulated nuclear localization bursts coordinate gene regulation. Nature 455:485–490

Cao Y, Zhang X, Shang W, Xu J, Wang X, Hu X, Ao Y, Cheng H (2013) Proinflammatory cytokines stimulate mitochondrial superoxide flashes in articular chondrocytes in vitro and in situ. PLoS One 8:e66444

Cheng H, Lederer WJ (2008) Calcium sparks. Physiol Rev 88:1491–1545

Chiarugi P, Fiaschi T, Taddei ML, Talini D, Giannoni E, Raugei G, Ramponi G (2001) Two vicinal cysteines confer a peculiar redox regulation to low molecular weight protein tyrosine phosphatase in response to platelet-derived growth factor receptor stimulation. J Biol Chem 276:33478–33487

De Stefani D, Raffaello A, Teardo E, Szabo I, Rizzuto R (2011) A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter. Nature 476:336–340

Ding Y, Fang H, Shang W, Xiao Y, Sun T, Hou N, Pan L, Sun X, Ma Q, Zhou J et al (2015) Mitoflash altered by metabolic stress in insulin-resistant skeletal muscle. J Mol Med 93:1119–1130

Fang H, Chen M, Ding Y, Shang W, Xu J, Zhang X, Zhang W, Li K, Xiao Y, Gao F et al (2011) Imaging superoxide flash and metabolism-coupled mitochondrial permeability transition in living animals. Cell Res 21:1295–1304

Gong G, Liu X, Wang W (2014) Regulation of metabolism in individual mitochondria during excitation-contraction coupling. J Mol Cell Cardiol 76:235–246

Gong G, Liu X, Zhang H, Sheu SS, Wang W (2015) Mitochondrial flash as a novel biomarker of mitochondrial respiration in the heart. Am J Physiol Heart Circ Physiol 309:H1166–H1177

Gong G, Wang W (2013) Confocal imaging of single mitochondrial superoxide flashes in intact heart or in vivo. J Vis Exp. e50818

Hou T, Jian C, Xu J, Huang AY, Xi J, Hu K, Wei L, Cheng H, Wang X (2016) Identification of EFHD1 as a novel Ca(2+) sensor for mitoflash activation. Cell Calcium 59:262–270

Hou T, Wang X, Ma Q, Cheng H (2014) Mitochondrial flashes: new insights into mitochondrial ROS signalling and beyond. J Physiol 592:3703–3713

Hou T, Zhang X, Xu J, Jian C, Huang Z, Ye T, Hu K, Zheng M, Gao F, Wang X et al (2013) Synergistic triggering of superoxide flashes by mitochondrial Ca2+ uniport and basal reactive oxygen species elevation. J Biol Chem 288:4602–4612

Hou Y, Ouyang X, Wan R, Cheng H, Mattson MP, Cheng A (2012) Mitochondrial superoxide production negatively regulates neural progenitor proliferation and cerebral cortical development. Stem Cells 30:2535–2547

Huang X, Sun L, Ji S, Zhao T, Zhang W, Xu J, Zhang J, Wang Y, Wang X, Franzini-Armstrong C et al (2013) Kissing and nanotunneling mediate intermitochondrial communication in the heart. Proc Natl Acad Sci U S A 110:2846–2851

Huang Z, Zhang W, Fang H, Zheng M, Wang X, Xu J, Cheng H, Gong G, Wang W, Dirksen RT et al (2011) Response to "A critical evaluation of cpYFP as a probe for superoxide". Free Radic Biol Med 51:1937–1940

Jian C, Hou T, Yin R, Cheng H, Wang X (2014) Regulation of superoxide flashes by transient and steady mitochondrial calcium elevations. Sci China Life Sci 57:495–501

Karamanlidis G, Lee CF, Garcia-Menendez L, Kolwicz SC Jr, Suthammarak W, Gong G, Sedensky MM, Morgan PG, Wang W, Tian R (2013) Mitochondrial complex I deficiency increases protein acetylation and accelerates heart failure. Cell Metab 18:239–250

Lakatta EG, Maltsev VA, Vinogradova TM (2010) A coupled SYSTEM of intracellular Ca^{2+} clocks and surface membrane voltage clocks controls the timekeeping mechanism of the heart's pacemaker. Circ Res 106:659–673

Li B, Chauvin C, De Paulis D, De Oliveira F, Gharib A, Vial G, Lablanche S, Leverve X, Bernardi P, Ovize M et al (2012a) Inhibition of complex I regulates the mitochondrial permeability transition through a phosphate-sensitive inhibitory site masked by cyclophilin D. Biochim Biophys Acta 1817:1628–1634

Li K, Zhang W, Fang H, Xie W, Liu J, Zheng M, Wang X, Wang W, Tan W, Cheng H (2012b) Superoxide flashes reveal novel properties of mitochondrial reactive oxygen species excitability in cardiomyocytes. Biophys J 102:1011–1021

Li W, Sun T, Liu B, Wu D, Qi W, Wang X, Ma Q, Cheng H (2016) Regulation of mitoflash biogenesis and signaling by mitochondrial dynamics. Sci Rep 6:32933

Ma Q, Fang H, Shang W, Liu L, Xu Z, Ye T, Wang X, Zheng M, Chen Q, Cheng H (2011) Superoxide flashes: early mitochondrial signals for oxidative stress-induced apoptosis. J Biol Chem 286:27573–27581

Mishra P, Carelli V, Manfredi G, Chan DC (2014) Proteolytic cleavage of Opa1 stimulates mitochondrial inner membrane fusion and couples fusion to oxidative phosphorylation. Cell Metab 19:630–641

Mishra P, Chan DC (2014) Mitochondrial dynamics and inheritance during cell division, development and disease. Nat Rev Mol Cell Biol 15:634–646

Mitchell P (1961) Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism. Nature 191:144–148

Owusu-Ansah E, Banerjee U (2009) Reactive oxygen species prime Drosophila haematopoietic progenitors for differentiation. Nature 461:537–541

Patron M, Raffaello A, Granatiero V, Tosatto A, Merli G, De Stefani D, Wright L, Pallafacchina G, Terrin A, Mammucari C et al (2013) The mitochondrial calcium uniporter (MCU): molecular identity and physiological roles. J Biol Chem 288:10750–10758

Poburko D, Santo-Domingo J, Demaurex N (2011) Dynamic regulation of the mitochondrial proton gradient during cytosolic calcium elevations. J Biol Chem 286:11672–11684

Pouvreau S (2010) Superoxide flashes in mouse skeletal muscle are produced by discrete arrays of active mitochondria operating coherently. PLoS One 5:e13035

Romashko DN, Marban E, O'Rourke B (1998) Subcellular metabolic transients and mitochondrial redox waves in heart cells. Proc Natl Acad Sci U S A 95:1618–1623

Santo-Domingo J, Giacomello M, Poburko D, Scorrano L, Demaurex N (2013) OPA1 promotes pH flashes that spread between contiguous mitochondria without matrix protein exchange. EMBO J 32:1927–1940

Schwarzlander M, Logan DC, Fricker MD, Sweetlove LJ (2011) The circularly permuted yellow fluorescent protein cpYFP that has been used as a superoxide probe is highly responsive to pH but not superoxide in mitochondria: implications for the existence of superoxide 'flashes'. Biochem J 437:381–387

Schwarzlander M, Logan DC, Johnston IG, Jones NS, Meyer AJ, Fricker MD, Sweetlove LJ (2012) Pulsing of membrane potential in individual mitochondria: a stress-induced mechanism to regulate respiratory bioenergetics in Arabidopsis. Plant Cell 24:1188–1201

Shadel GS, Horvath TL (2015) Mitochondrial ROS signaling in organismal homeostasis. Cell 163:560–569

Shang W, Gao H, Lu F, Ma Q, Fang H, Sun T, Xu J, Ding Y, Lin Y, Wang Y et al (2016) Cyclophilin D regulates mitochondrial flashes and metabolism in cardiac myocytes. J Mol Cell Cardiol 91:63–71

Shannon CE (1997) The mathematical theory of communication. 1963. MD Comput 14:306–317

Shen EZ, Song CQ, Lin Y, Zhang WH, Su PF, Liu WY, Zhang P, Xu J, Lin N, Zhan C et al (2014) Mitoflash frequency in early adulthood predicts lifespan in *Caenorhabditis elegans*. Nature 508:128–132

Uda S, Kuroda S (2016) Analysis of cellular signal transduction from an information theoretic approach. Semin Cell Dev Biol 51:24–31

Wallace DC (2012) Mitochondria and cancer. Nat Rev Cancer 12:685–698

Wang JQ, Chen Q, Wang X, Wang QC, Wang Y, Cheng HP, Guo C, Sun Q, Chen Q, Tang TS (2013) Dysregulation of mitochondrial calcium signaling and superoxide flashes cause mitochondrial genomic DNA damage in Huntington disease. J Biol Chem 288:3070–3084

Wang W, Fang H, Groom L, Cheng A, Zhang W, Liu J, Wang X, Li K, Han P, Zheng M et al (2008) Superoxide flashes in single mitochondria. Cell 134:279–290

Wang W, Gong G, Wang X, Wei-LaPierre L, Cheng H, Dirksen R, Sheu SS (2016a) Mitochondrial flash: integrative reactive oxygen species and pH signals in cell and organelle biology. Antioxid Redox Signal 25:534–549

Wang X, Jian C, Zhang X, Huang Z, Xu J, Hou T, Shang W, Ding Y, Zhang W, Ouyang M et al (2012) Superoxide flashes: elemental events of mitochondrial ROS signaling in the heart. J Mol Cell Cardiol 52:940–948

Wang X, Zhang X, Huang Z, Wu D, Liu B, Zhang R, Yin R, Hou T, Jian C, Xu J et al (2016b) Protons trigger mitochondrial flashes. Biophys J 111:386–394

Wei-LaPierre L, Gong G, Gerstner BJ, Ducreux S, Yule DI, Pouvreau S, Wang X, Sheu SS, Cheng H, Dirksen RT et al (2013) Respective contribution of mitochondrial superoxide and pH to mitochondria-targeted circularly permuted yellow fluorescent protein (mt-cpYFP) flash activity. J Biol Chem 288:10567–10577

Wei L, Dirksen RT (2012) Perspectives on: SGP Symposium on Mitochondrial Physiology and Medicine. Mitochondrial superoxide flashes: from discovery to new controversies. J Gen Physiol 139:425–434

Wei L, Salahura G, Boncompagni S, Kasischke KA, Protasi F, Sheu SS, Dirksen RT (2011) Mitochondrial superoxide flashes: metabolic biomarkers of skeletal muscle activity and disease. FASEB J 25:3068–3078

Weinberg SE, Sena LA, Chandel NS (2015) Mitochondria in the regulation of innate and adaptive immunity. Immunity 42:406–417

Wiederkehr A, Park KS, Dupont O, Demaurex N, Pozzan T, Cline GW, Wollheim CB (2009) Matrix alkalinization: a novel mitochondrial signal for sustained pancreatic beta-cell activation. EMBO J 28:417–428

Xu S, Chisholm AD (2014) Methods for skin wounding and assays for wound responses in *C. elegans*. J Vis Exp (94):51959

Yan Y, Liu J, Wei C, Li K, Xie W, Wang Y, Cheng H (2008) Bidirectional regulation of Ca2+ sparks by mitochondria-derived reactive oxygen species in cardiac myocytes. Cardiovasc Res 77:432–441

Ying Z, Chen K, Zheng L, Wu Y, Li L, Wang R, Long Q, Yang L, Guo J, Yao D et al (2016) Transient activation of mitoflashes modulates Nanog at the early phase of somatic cell reprogramming. Cell Metab 23:220–226

Zhang J, Wang X, Vikash V, Ye Q, Wu D, Liu Y, Dong W (2016) ROS and ROS-mediated cellular signaling. Oxid Med Cell Longev 2016:4350965

Zhang M, Sun T, Jian C, Lei L, Han P, Lv Q, Yang R, Zhou X, Xu J, Hu Y et al (2015) Remodeling of mitochondrial flashes in muscular development and dystrophy in zebrafish. PLoS One 10: e0132567

Zhang W, Li K, Zhu X, Wu D, Shang W, Yuan X, Huang Z, Zheng M, Wang X, Yang D et al (2014) Subsarcolemmal mitochondrial flashes induced by hypochlorite stimulation in cardiac myocytes. Free Radic Res 48:1085–1094

Zhang X, Huang Z, Hou T, Xu J, Wang Y, Shang W, Ye T, Cheng H, Gao F, Wang X (2013) Superoxide constitutes a major signal of mitochondrial superoxide flash. Life Sci 93:178–186

Zorov DB, Juhaszova M, Sollott SJ (2014) Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. Physiol Rev 94:909–950

Mitochondria-Targeted Agents: Mitochondriotropics, Mitochondriotoxics, and Mitocans

Diana Guzman-Villanueva and Volkmar Weissig

Contents

Abstract

Mitochondria, the powerhouse of the cell, have been known for many years for their central role in the energy metabolism; however, extensive progress has been made and to date substantial evidence demonstrates that mitochondria play a critical role not only in the cell bioenergetics but also in the entire cell metabolome. Mitochondria are also involved in the intracellular redox poise, the regulation of calcium homeostasis, and the generation of reactive oxygen species (ROS), which are crucial for the control of a variety of signaling pathways. Additionally, they are essential for the mitochondrial-mediated apoptosis process. Thus, it is not surprising that disruptions of mitochondrial functions can lead or be associated with human pathologies. Because of diseases like diabetes, Alzheimer, Parkinson's, cancer, and ischemic disease are being increasingly linked to mitochondrial dysfunctions, the interest in mitochondria as a prime pharmacological

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H. Singh, S.-S. Sheu (eds.), *Pharmacology of Mitochondria*,
Handbook of Experimental Pharmacology 240, DOI 10.1007/164_2016_37

target has dramatically risen over the last decades and as a consequence a large number of agents, which could potentially impact or modulate mitochondrial functions, are currently under investigation. Based on their site of action, these agents can be classified as mitochondria-targeted and non-mitochondria-targeted agents. As a result of the continuous search for new agents and the design of potential therapeutic agents to treat mitochondrial diseases, terms like mitochondriotropics, mitochondriotoxics, mitocancerotropics, and mitocans have emerged to describe those agents with high affinity to mitochondria that exert a therapeutic or deleterious effect on these organelles. In this chapter, mitochondria-targeted agents and some strategies to deliver agents to and/or into mitochondria will be reviewed.

Keywords
Lipophilic cations • Mitocans • Mitochondria • Mitochondriotropics • Mitochondriotoxics • Triphenylphosphonium

1 Introduction

Mitochondria are sub-cellular organelles commonly recognized as the powerhouse of the cell; however, in addition to their role in the energy metabolism it has increasingly become evident that mitochondria play important roles in a large variety of process including apoptosis, cell differentiation, oxygen sensing, and calcium metabolism (Smith et al. 2012).

The growing interest of different research groups to investigate mitochondria as a pharmaceutical target has been triggered by the realization that a large variety of neuromuscular and neurodegenerative disorders, as well as diseases like cancer, ischemia-reperfusion injury, and diabetes, are associated with mitochondrial dysfunctions.

An increasing number of bioactive molecules potentially able to act on mitochondrial molecular targets, as well as strategies to deliver such molecules to and/or into mammalian mitochondria in living cells have been described (D'Souza et al. 2011). According to their site of action, agents able to interfere with mitochondrial functions can be classified into two main categories: as mitochondria-targeted and non-mitochondria-targeted agents (Smith et al. 2012). Mitochondria-targeted agents are those molecules that have mitochondria as their primary target and alter mitochondria functionality by directly acting on them. On the other hand, non-mitochondrial-targeted agents are those molecules that have a secondary target in a different sub-cellular location other than mitochondria but their interaction results in modifications of mitochondria functionality (Szewczyk and Wojtczak 2002). Figure 1 illustrates multiple targets of mitochondria including the respiratory chain complexes and the mitochondrial permeability transition pore complex (PTPC), as well as the whole cell representing additional sub-cellular organelles as secondary targets of mitochondria.

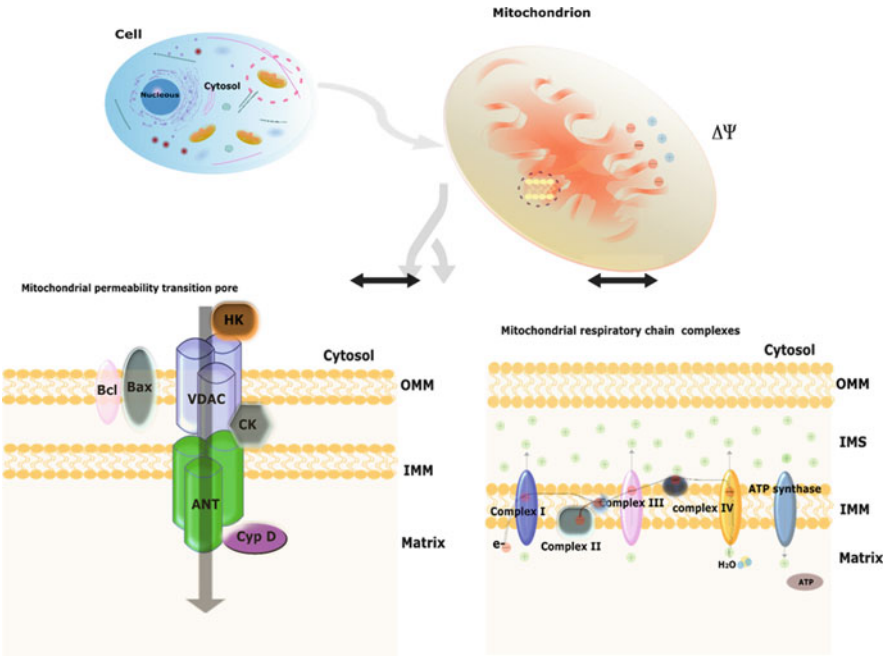

Fig. 1 Schematic representation of potential pharmacological targets in the cell. Mitochondria-targeted agents directly act on molecular targets such as the mitochondrial respiratory chain complexes and the mitochondria permeability transition pore in the mitochondrion. Some other agents accumulate within mitochondria driven by the large mitochondrial membrane potential ($\Delta\Psi$). Non-mitochondria-targeted agents, on the other hand, act on different sub-cellular organelles in the cell but their interaction also triggers modifications in mitochondria functionality

The search for mitochondria-targeted drugs and the development of new strategies to deliver low-molecular weight compounds to mitochondria have created a variety of terms including "mitochondriotropics," "mitochondriocancerotropics," "mitochondriotoxics," and "mitocans" to describe their high affinity for mitochondria as well as their ability to interfere with the multiple metabolic pathways that take place in these organelles. Among these agents are antioxidants, anticancer, nonsteroidal anti-inflammatory, and immunosuppressive drugs (Horobin et al. 2007).

2 Mitochondriotropic Agents

The term "mitochondriotropics" generally refers to low-molecular weight compounds, which based on their physicochemical properties and reactivity show high intrinsic affinity towards mitochondria. Once inside mammalian cells, mitochondriotropics accumulate either at or inside mitochondria without requiring the assistance of any mitochondrial-targeted delivery system (Weissig and

Torchilin 2001a; Weissig and Torchillin 2001b). Skulachev's group (Liberman et al. 1969) described the first of such compounds, the methyl-triphenylphosphonium cation in 1969. Skulachev's group found that this compound selectively accumulated inside mammalian mitochondria in response to the large mitochondrial membrane potential ($\Delta\Psi_m$).

Horobin, Trapp, and Weissig (Horobin et al. 2007) analyzed over 100 agents known to accumulate at or inside mitochondria. Using physicochemical classification, QSAR, and Fick–Nerst–Plank models these authors concluded that the agents with potential mitochondriotropic properties included those with the following features: lipophilic molecules: $8 > \log P > 5$, amphiphilic molecules: $8 > AL > 5$, weak acids with lipophilic less-ionized species: $Z \leq 0$; $\log P_{\text{less ionized}} > 0$, $pKa = 7 \pm 3$, lipophilic to strong lipophilic cations: $Z > 0$; $5 \log P > 0$, and moderately hydrophilic to moderately lipophilic cations: $Z > 0$; $-2 > \log P > 2$. In addition, they also concluded that the mechanisms behind the mitochondrial accumulation of those molecules were mainly the electric potential, ion trapping, or complex formation with cardiolipin (Horobin et al. 2007).

A long with the physicochemical properties, molecular weight seems to be another significant parameter involved in the specific accumulation of agents within mitochondria, as it directly impacts their diffusion and membrane permeability (Zheng et al. 2011). In a meta-analysis performed to determine the accumulation of approximately 900 small molecules in specific locations within cell, Zheng reported that compounds with molecular weights <500 Daltons were highly localized in mitochondria (Zheng et al. 2011). A few examples of the chemical compounds analyzed in this meta-analysis that showed accumulation within mitochondria included valproic acid, amytal, lonidamine, zidovudine, diclofenac, and haloperidol.

The recognition of specific physicochemical properties found in low-molecular weight compounds with high affinity for mitochondria now makes possible to predict the mitochondriotropic behavior of certain drugs or molecules, which in turn has tremendously increased the number of pharmacological agents under investigation for clinical applications. Equally important is the identification of molecular targets, pathways, or mechanisms of action responsible for the pharmacological effects of mitochondriotropic agents. In this regard, multiple potential mitochondrial molecular targets either at the inner (IMM) or the outer (OMM) mitochondria membranes, matrix or intermembrane space (IMS) have been described (Weissig et al. 2004). Among them are mitochondria respiratory chain complexes, mitochondrial DNA (mtDNA), mitochondrial PTPC, and proapoptotic and antiapoptotic proteins (see Fig. 1), (Milane et al. 2015). Table 1 provides a detailed list of the main primary targets of mitochondria, while Table 2 describes a vast number of mitochondria-targeted agents and their pharmacologic effect.

Table 1 Molecular targets of mitochondria divided according to their sub-localization (Milane et al. 2015; Horobin et al. 2007; Weissig et al. 2004)

Localization	Target
OMM	Bcl-2 Voltage-dependent anion channels, VDACs Hexokinase Mitochondrial benzodiazepine receptor, BzPR p53 Carnitine acyltransferase I, CPT1 P-glycoprotein, Pgp Translocase of the outer membrane (TOM) Bax
IMM	Adenine nucleotide translocase, ANT Complex I Complex II Complex III Complex IV Complex V Cytochrome *c* Ca^{2+} uniport ATP-sensitive channel, mitoK_{ATP} channel Mitochondrial inner membrane K+ channel, mitoK_{Ca} Uncoupling proteins, UCP ATPase
Matrix	Mitochondrial cyclophilin, CyP-D Mitochondrial estrogen receptor, ER Tricarboxylic acid cycle, TCA Mitochondrial DNA, mDNA Sirtuin-silent information regulator proteins, SIRT
IMS	Glycerol phosphate dehydrogenase, GPD

2.1 Delocalized Lipophilic Cations

Low-molecular weight compounds lacking of mitochondriotropism but that could potentially act on a molecular target at or inside mitochondria often need to be directed towards these organelles. One of the most commonly used approaches to deliver these low-molecular weight compounds to mitochondria is their conjugation to mitochondriotropic lipophilic cations such as triphenylphosphonium (TPP, see Fig. 2). Following their first description by Skulachev's group in 1969, delocalized lipophilic cations have become very well known for being rapidly taken up by mitochondria without requiring a specific type of transport. Due to their delocalized positive charge, lipophilic cations can permeate mitochondrial membranes and accumulate in or at mitochondria in response to their larger mitochondrial membrane potential (negative inside) (Modica-Napolitano and Aprille 2001; Horobin et al. 2007; Murphy 2008). The utilization of delocalized lipophilic cations, especially TPP cations for targeting a variety of agents to mitochondria, has been pioneered and extensively studied by Mike Murphy's group. They have successfully linked TPP to a significant number of molecules to

Table 2 Pharmacologic effect of mitochondrial-targeted agents (Milane et al. 2015; Horobin et al. 2007; Zheng et al. 2011)

Agent	Effect
Valproic acid Glibenclamide Tolbutamide	These agents are known to inhibit or interfere with mitochondrial β-oxidation
Menadione Rotenone Paraquat Memantine Imipramine Cinnarizine Flunarizine Haloperidol Myxothiazol Mahanine Cyanide Amytal Vacor	These agents are able to directly inhibit complexes of the mitochondrial respiratory chain, and subsequently trigger cell death
Ubiquinol Flupirtine Resveratrol Ranolazine MitoQ MitoE MitoPBN MitoPeroxidase SS31	The overproduction of reactive oxygen species (ROS) appears to be a common underlying mechanism of many pathologies, as it damages cellular components, including proteins, lipids, and DNA. These agents have been used to counteract these detrimental effects, based on their ability to act as antioxidants and radical scavengers
Cromakalim Nicorandil Diazoxide	These potassium channel openers are able to depolarize the mitochondrial membrane potential and cause reversible oxidation of flavoproteins of the respiratory chain and increase mitochondrial ROS production
Cyclosporin A	Permeability transition pore opening is major step towards apoptosis. Cyclosporin A is a well-known mitochondrial permeability transition pore opening blocker
Zidovudine Zalcitabine Stavudine Didanosine Ditercalinium 5-Aminolevuliniv acid	Cellular exposure to these compounds generates oxidative damage to mitochondrial DNA (mtDNA), including single- and double-strand breaks and base damage
Aspirin Ibuprofen Diclofenac Mefenamic acid Tolfenamic acid Piroxicam Salicylic acid Acetylsalicylic acid	These agents act as uncouplers of the oxidative phosphorylation by reducing the ATP production

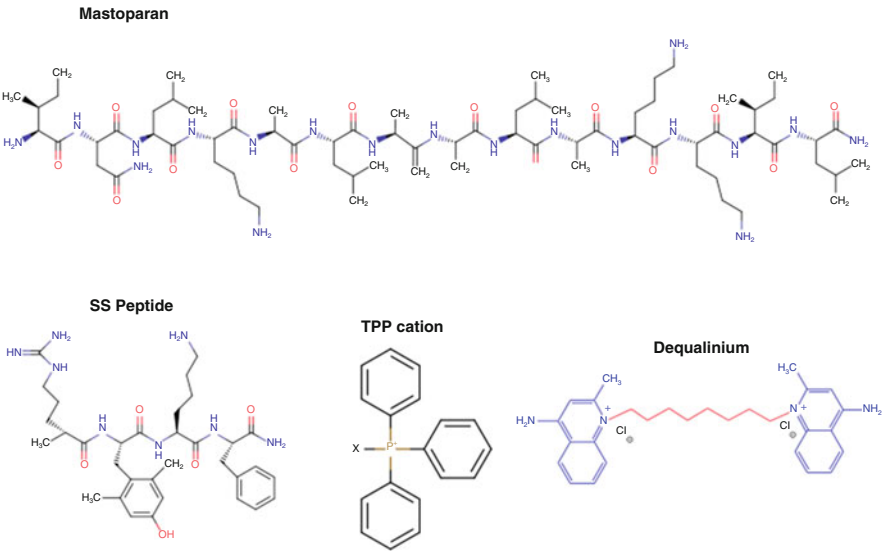

Fig. 2 Schematic representation of the most commonly used mitochondriotropic and mitochondriotoxic systems

selectively target mitochondria, particularly, antioxidant molecules to protect the cell from oxidative stress damage. (Murphy 2008). A few examples of these TPP-conjugates include MitoE, MitoPeroxidase, MitoTempo, MitoPC, and at last MitoQ, the most studied antioxidant-targeted molecule (Armstrong 2007; Starenki and Park 2013; Szeto 2008; Smith and Murphy 2011).

Another strategy to deliver molecules with pharmacological effect to mitochondria is their incorporation into pharmaceutical nanocarriers such as liposomes. This strategy is particularly useful for those molecules that exhibit low affinity to mitochondria, low solubility, or are easily degraded. Boddapati (Boddapati et al. 2008), for example, conjugated the lipophilic cation TPP to a stearyl residue to obtain the STPP conjugate, which was subsequently incorporated into liposomes to form the so-called mitochondriotropic liposomes. These mitochondriotropic liposomes were loaded with ceramide and when added into the cancer cells they efficiently induced apoptosis. In the same way, Malhi (Malhi et al. 2012) prepared what he called "mitocancerotropic liposomes," which consisted in folic acid-functionalized STPP liposomes loaded with doxorubicin.

Since delocalized lipophilic cations are toxic at high concentrations. Biswas (Biswas et al. 2012) conjugated polyethylene glycol-phosphatidylethanolamine (PEG-PE) to the TPP cation in order to reduce its toxicity. The resulting TPP-PEG-PE conjugate was incorporated into liposomes loaded with paclitaxel, which reduced TPP toxicity and at the same time improved paclitaxel anticancer properties. Benien (Benien et al. 2015) and Guzman-Villanueva (Guzman-Villanueva et al. 2015) also achieved reduction of the TPP cation toxicity by

conjugating TPP to other commercially available phospholipids including phosphatidylethanolamine (PE). Possible reasons for the reduced cytotoxicity of the TPP cation conjugated to two-tailed phospholipids compared to the TPP conjugated to a single fatty acid have been discussed by Guzman-Villanueva et al. STPP (TPP conjugated to a stearyl residue), for example, possesses an alkyl-lysolipid-like structure. Lysolipids (or single-tailed lipids) are not bilayer forming lipids, which generate high positive curvatures and membrane re-arrangements when inserted into phospholipid bilayer membranes. One can easily imagine STPP contributing to the permeabilization of the mitochondria membrane, thereby creating instability and modifications of the properties of the lipid bilayer (Lucken-Ardjomande and Martinou 2005; Fuller and Rand 2001). It has indeed been reported that excessive accumulation of lysolipids in membranes induces cell lysis, which is consistent with the concentration-dependent toxicity of STPP, as observed by Guzman-Villanueva. Along with these membrane structural modifications, Guzman-Villanueva also suggested that STPP may induce cell death by disturbing the lipid metabolism and lipid signaling in cell membranes, as described by van del Luit (van der Luit et al. 2002) and Marco (Marco et al. 2014).

In summary, as of today a substantial number of TPP conjugates for antioxidant and anticancer therapy have been described and the covalent attachment of TPP to different drugs has become a well-establish approach to prepare mitochondriotropic agents. However, the use of mitochondriotropics, in particular, delocalized lipophilic cations as single agents has also been studied for anticancer therapy. Because cancer cells exhibit a considerably higher mitochondrial membrane potential than non-transformed cells due to altered bioenergetics, higher (10-fold greater) accumulation of lipophilic actions in mitochondria has been documented (Modica-Napolitano and Aprille 2001). Examples of these lipophilic cations include Rhodamine 123 and MKT-077. MKT-077 has shown to selectively accumulate in tumor cell mitochondria including colon, pancreatic, bladder and breast carcinoma and now is being investigated in clinical trials (Koya et al. 1996; Propper et al. 1999).

2.2 Dequalinium Vesicles

Dequalinium, 1,1'-Decamethylene bis (4-aminoquinaldinium chloride), a cationic bolaamphiphile composed of two quinaldinium rings linked by ten methylene groups (Fig. 2), is another agent that displays mitochondriotropic properties (Lasch et al. 1999; Weissig et al. 1998). Weissig (Weissig et al. 1998) named these vesicles DeQAlinium-based lipoSOMES, DQAsomes, after having accidently discovered dequalinium's capacity to self-assemble into vesicles-like liposomes. Recent reports have shown that DQAsomes have been used to deliver drugs like paclitaxel, curcumin, and even DNA constructs to mammalian mitochondria (Zupančič et al. 2014; Lasch et al. 1999; Vaidya and Vyas 2012; Lyrawathi et al. 2011; D'Souza et al. 2008; D'Souza et al. 2005).

2.3 Mitochondriotropic Peptides

Many reports in the literature have described the ability of certain peptides to readily penetrate plasma membrane (Lindgren et al. 2000; Deshayes et al. 2005). However, it was recently reported that some of these peptides have the ability not only to permeate the plasma membrane but also to selectively accumulate within mitochondria (Szeto 2006). Examples of such peptides are the so-called Szeto-Schiller (SS peptides). These small, water-soluble peptides are tetrapeptides composed of Tyr-Tyrosine or Dmt-dimethyltyrosine, Arg-Arginine, Phe-Phenylalanine, and Lys-Lysine residues, which selectively accumulate in the inner mitochondrial membrane (IMM), scavenge ROS, and inhibit the mitochondrial permeability transition pore opening (Szeto 2008). The sequence of these SS peptides is characterized by the alternation of the aromatic motifs (Tyr, Dmt, and Phe) with the basic amino acids (Arg, Lys), which contributes to their net positive charge (Szeto 2006), as seen in Fig. 2. This amino acid sequence also allows them to freely penetrate membranes in an energy-independent and non-saturable manner. Among these peptide antioxidants SS-01 (Tyr-D-Arg-Phe-Lys-NH_2), SS-02 (Dmt-D-Arg-Phe-Lys-NH_2), and SS-31 (D-Arg-Dmt-Lys-Phe-NH_2) have been described (Szeto 2008).

The aromatic amino acids Dmt and Tyr appear to be accountable for the antioxidant activity of the SS peptides, as they efficiently scavenge reactive oxygen species (ROS) to form unreactive tyrosyl or dityrosine radicals, which can also react with superoxide radicals to form tyrosine hydroperoxide (Zhao et al. 2004). Moreover, seems like SS-31 inhibits cardiolipin peroxidation and the onset of mitochondrial transition pore (Birk et al. 2013).

3 Mitochondriotoxic Peptides

Viral protein R (Vpr), a protein encoded in the HIV-1 genome, is a small soluble protein consisting of 96 amino acids and 3 alpha-helical domains. Vpr is present in most IHV-1 infected patients and it has been reported to induce cell death via the intrinsic pathway of apoptosis, which involves mitochondrial membrane permeabilization (Brenner and Kroemer 2003). Upon incubation with isolated mitochondria, Vpr has been demonstrated to cross the outer membrane via the voltage-dependent anion channel (VDAC) and to subsequently interact with the adenine nucleotide translocator (ANT) forming a channel that dissipates the mitochondrial membrane potential triggering apoptosis. In ANT-deficient cells, Vpr failed to induce apoptosis, thus demonstrating that the ANT is critical for Vpr to induce apoptosis (Brenner and Kroemer 2003). The mitochondriotoxic effect of Vpr has been associated with a dodecapeptide (amino acid 72–83) located in the C-terminal α-helix. This dodecapeptide contains three arginine residues (positively charged) distributed in one side of the C-terminal α-helix, which physically interacts with the first loop (amino acids 104–116) of the transmembrane domain

ANT exposed to the mitochondrial intermembrane space (Brenner and Kroemer 2003).

Mastoparan is another peptide able to permeate plasma and mitochondria membranes. Mastoparan consists of a sequence of 14 amphipathic and cationic amino acids (Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH_2), isolated from the venom of *Vespula lewisii* (de Azevedo et al. 2015). When added to isolated mitochondria, mastoparan induces mitochondrial membrane permeabilization. Incubation of the whole cells with mastoparan induces mitochondria-mediated apoptosis, which is characterized by morphological changes in mitochondria, translocation of phosphatidylserine, DNA fragmentation, nuclear condensation, loss of mitochondrial membrane potential, and activation of caspases.

Previous work has reported that mastoparan shows potent antitumor activity against melanoma including A2058, MCF7, Jurkat, SiHa, SK-BR3, and other cancer cell lines. Interestingly, mastoparan appears to be less toxic in non-transformed cells (de Azevedo et al. 2015).

Mastoparan possesses an α-helical conformation with a positive charge distributed on one side of the helix. This "mitochondriotoxic" motif has also been found in the proapoptotic viral protein R (Vpr) from human immunodeficiency virus-1 (described above), and other derivatives, which suggests that this structural motif is essential to initiate this mitochondriotoxic effect (Yamamoto et al. 2014).

Another example of mitochondriotoxic molecules is the $p13^{II}$ protein, an 87 amino acid protein coded by the X-II open reading frame of the human T-cell leukemia/lymphotropic virus type I (HTLV-1). This $p13^{II}$ protein selectively targets mitochondria producing alterations in the mitochondrial morphology and disruptions in the mitochondrial membrane potential (Ciminale et al. 1999). According to Ciminale (Ciminale et al. 1999), the mitochondria-targeting effect of the $p13^{II}$ protein is the result of a 10 amino acid sequence in the amino-proximal region that contains positively charged residues (also Arg residues) that are distributed in the α-helix. Mitochondriotoxic agents like mastoparan, viral protein R, and $p13^{II}$ protein appear to share unique structural and conformational features: sequence motifs located at the amino-proximal or amino-terminal region of the protein, a positive charge, and an amphipathic α-helical conformation. Together, these properties have demonstrated to be recognized as mitochondrial targeting signals (Ciminale et al. 1999) that provide mitochondrial selectivity.

4 Mitocans

"Mitocans," a term coined by Ralph (Ralph et al. 2006), has recently been used to classify all the agents that show anticancer activity by specifically acting on mitochondria. A few of these mitocans are described below and their schematic representation is shown in Fig. 3.

Lonidamine, 1-[(2,4-dichlorophenyl)methyl]-1H-indazole-3-carboxylic acid or LND is a drug with antitumoral properties, which in combination with other

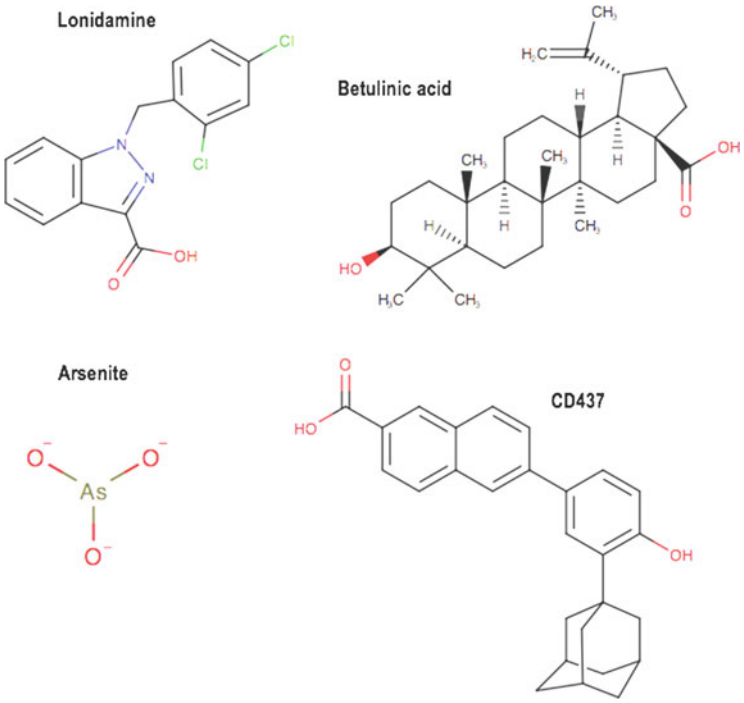

Fig. 3 Chemical structures of mitocans, agents that exhibit with high affinity to mitochondria as well as anticancer properties

chemotherapeutic agents, seems to enhance the cellular apoptotic response (Floridi et al. 1987). For example, the combination of lonidamine with cisplatin potentiates cisplatin toxicity in ovarian carcinoma cell lines. This adjuvant property of lonidamine with other standard antineoplastic agents like doxorubicin, cyclophosphamide, and radiotherapy has also been investigated for the treatment of solid tumors and metastatic cancers (Di Cosimo et al 2003). Although mechanisms of action like intracellular lactate accumulation, increase in cytosolic Ca^{2+}, interference of the function of mitochondrial hexokinase, and respiratory chain have been proposed, it has been now demonstrated that lonidamine induces apoptosis by directly affecting the mitochondrial PTPC. It specifically inhibits the adenine nucleotide translocase (ANT). When added to the cells, lonidamine causes PT pore opening, decreases mitochondrial membrane potential, generates uncoupling of the respiratory chain, and produces swelling of the mitochondrial matrix. All these events trigger the release of apoptogenic factors, subsequently leading to nuclear apoptosis (Ravagnan et al. 1999). Lonidamine is currently undergoing phase III/IV clinical trials, although it has shown to be toxic in non-tumor tissue (Neuzil et al. 2013).

Arsenite is another agent that shows anticancer activity against acute promyelocytic leukemia and myeloma cells. Similar to other mitocans, the

mechanism of action of arsenite was initially unclear; however, caspase activation, generation of ROS, and downregulation of Bcl-2 have been described as events involved in the cell death (Larochette et al. 1999). Later, it was confirmed that mitochondria are the intracellular target of arsenite, as nuclear apoptosis failed when arsenite was added to isolated nuclei but it was activated in the presence of mitochondria. In addition, apoptotic-inducing factor (AIF) was found in supernatants of mitochondria treated with arsenite. PT pore opening inhibitors like CsA and the overexpression of Bcl-2 prevented the release of AIF. This effect immediately suggested that the proapoptotic effect of arsenite was mediated by acting on the mitochondrial permeability transition pore (MPTP) (Larochette et al. 1999; Costantini et al. 2000). Finally, it has been reported that arsenite induces permeabilization of ANT and requires mitochondria to induce nuclear apoptosis, as shown by cell-free systems (Neuzil et al. 2013).

Betulinic acid, a natural pentacyclic triterpene, has also been demonstrated to possess potent antitumoral activity against a number of cancer cells, including melanoma, head and neck, colon, breast, lung, prostate, and ovarian carcinoma. Betulinic acid also shows activity in drug-resistant cancer cells and has reported a low cytotoxicity in normal cells and tissue (Fulda 2008). The anticancer properties of betulinic acid have been attributed to its ability to induce the mitochondrial pathway of apoptosis through the loss of mitochondrial membrane potential, which in turn triggers mitochondrial outer membrane permeabilization (MOMP), as well as modulates the activity of Bcl-2 proteins (Neuzil et al. 2013).

CD437 (6[3-adamantyl-4-hydroxyphenyl]2-naphthalene carboxylic acid) is a well-known anticancer agent. The anticancer properties of this mitocan are also linked to mitochondrial membrane permeabilization by inhibiting the ANT protein. CD437 has shown proapoptotic activity against breast, prostate, lung, and ovarian cells (Costantini et al. 2000; Belzacq et al. 2001). Other chemotherapy agents directly acting on mitochondria include diamide, PK11195, MT-21, verteporfin (Belzacq et al. 2001).

Mitochondria membrane permeabilization has shown to constitute a critical event in the apoptotic process induced by chemotherapy agents, as it promotes the release of AIF and cytochrome *c*, which activate caspases and nucleases. Additionally, it reduces ATP production and causes loss of redox balance. Hence, it is evident that targeting the MPTP represents a promising strategy for anticancer therapy, as the mitochondrial PT pore opening precedes a series of signaling events that lead to apoptotic cell death.

Shifting the balance between antiapoptotic and proapoptotic Bcl-2 family proteins represents another strategy to induce apoptosis in cancer cells. For example, BH3 mimetics have shown to interfere with the interaction between antiapoptotic proteins (Bcl-2, Blc-xL, or Mcl) and the prop-apoptotic proteins Bax or Bak, which leads to the oligomerization of BAx or Bak to form channels and to activate MOMP-dependent apoptosis (Oliver et al. 2004). ABT-737, ABT-263, and AT-101 (gossypol) are the best-characterized BH3 mimetics and all of them are now under investigation in clinical trials alone or in combination with other standard chemotherapeutic agents (Neuzil et al. 2013).

Disruptors of the HK II and VDAC interaction offer an additional alternative for the development of new mitocans, as HK II is frequently overexpressed in cancer and its binding with VDAC is usually tighter in cancer cells than in nonmalignant cells. Methyl jasmonate is a small molecule known to disrupt the HK II-VDAC interaction, which favors the MOMP in cancer cell lines (Goldin et al 2008).

Honokiol is another small molecule, isolated from Magnolia, which has recently shown antitumor activity. Induction of CyP-D and MPTP opening has been suggested as its major mechanism of action (Neuzil et al. 2013).

Mitochondria respiratory chain complexes are also target of mitocans. Tamoxifen, a nonsteroidal selective estrogen receptor modulator commonly used in estrogen-positive breast cancer chemotherapy, for example, induced apoptosis by interfering with the FAD binding site (Moreira et al 2006).

Mitocans targeting mitochondrial DNA (mtDNA) have also been reported. The toxin 1-methyl-4-phenylpyridinium reduces the copy number of mtDNA by destabilizing the structure of the mitochondrial D-loop (Neuzil et al. 2013).

In summary, it has been well established that agents acting directly on mitochondrial molecular targets can potentially be powerful anticancer agents. For this reason, it is important to discriminate between those chemotherapeutic agents that directly act on mitochondria to induce apoptosis from those classical chemotherapeutic agents that compromise mitochondrial function by activating extramitochondrial proapoptotic signal pathways like etoposide, cisplatin, or doxorubicin. This discrimination is particularly important because mitocans offer two major advantages over the classical chemotherapeutic agents: selectivity and their ability to bypass drug resistance (Kroemer and de Thé 1999).

5 Final Remarks

Mitochondria have emerged over the last two decades as a prime pharmacological target. Subsequently, the amount of agents able to act on mitochondrial molecular targets has significantly increased. Terms like mitochondriotropics, mitochondriotoxics, mitocancerotropics, and mitocans have emerged to describe those agents with high affinity to mitochondria. Investigations aimed to better understand mitochondrial-targeted agents and their interaction with their corresponding molecular targets will eventually translate into an increasing number of pharmacological alternatives for the treatment of mitochondria-associated disorders including cancer.

References

Armstrong JS (2007) Mitochondrial medicine: pharmacological targeting of mitochondria in disease. Br J Pharmacol 151(8):1154–1165

Belzacq AS, El Hamel C, Vieira HL, Cohen I, Haouzi D, Métivier D, Marchetti P, Brenner C, Kroemer G (2001) Adenine nucleotide translocator mediates the mitochondrial membrane permeabilization induced by lonidamine, arsenite and CD437. Oncogene 20(52):7579–7587

Benien P, Solomon MA, Nguyen P, Sheehan EM, Mehanna AS, D'Souza GG (2015) Hydrophobized triphenyl phosphonium derivatives for the preparation of mitochondriotropic liposomes: choice of hydrophobic anchor influences cytotoxicity but not mitochondriotropic effect. J Liposome Res 26(1):21–27

Birk AV, Liu S, Soong Y, Mills W, Singh P, Warren JD, Seshan SV, Pardee JD, Szeto HH (2013) The mitochondrial-targeted compound SS-31 re-energizes ischemic mitochondria by interacting with cardiolipin. J Am Soc Nephrol 24(8):1250–1261

Biswas S, Dodwadkar NS, Deshpande PP, Torchilin VP (2012) liposomes loaded with paclitaxel and modified with novel triphenylphosphonium-peg-pe conjugate possess low toxicity, target mitochondria and demonstrate enhanced antitumor effects *in vitro* and *in vivo*. J Control Release 159(3):393–402

Boddapati SV, D'Souza GG, Erdogan S, Torchilin VP, Volkmar W (2008) Organelle-targeted nanocarriers: specific delivery of liposomal ceramide to mitochondria enhances its cytotoxicity *in vitro* and *in vivo*. Nano Lett 8(8):2559–2563

Brenner C, Kroemer G (2003) The mitochondriotoxic domain of Vpr determines HIV-1 virulence. J Clin Invest 111(10):1455–1457

Ciminale V, Zotti L, D'Agostino DM, Ferro T, Casareto L, Franchini G, Bernardi P, Chieco-Bianchi L (1999) Mitochondrial targeting of the p13II protein coded by the X-II ORF of human T-Cell leukemia/lymphotropic virus type I (HTLV-I). Oncogene 18(31):4505–4514

Costantini P, Jacotot E, Decaudin D, Kroemer G (2000) Mitochondrion as a novel target of anticancer chemotherapy. J Natl Cancer Inst 92(13):1042–1053

D'Souza GG, Boddapati SV, Weissig V (2005) Mitochondrial leader sequence-plasmid DNA conjugates delivered into mammalian cells by DQAsomes co-localize with mitochondria. Mitochondrion 5(5):352–358

D'Souza GG, Cheng SM, Boddapati SV, Horobin RW, Weissig V (2008) Nanocarrier-assisted sub-cellular targeting to the site of mitochondria improves the pro-apoptotic activity of paclitaxel. J Drug Target 16(7):578–585

D'Souza GGM, Mayura AW, Vaibhav S, Shah A (2011) Approaches for targeting mitochondria in cancer therapy. Biochim Biophys Acta 1807(6):689–696

de Azevedo RA, Figueiredo CR, Ferreira AK, Matsuo AL, Massaoka MH, Girola N, Auada AV et al (2015) Mastoparan induces apoptosis in B16F10-Nex2 melanoma cells via the intrinsic mitochondrial pathway and displays antitumor activity *in vivo*. Peptides 68(June):113–119

Deshayes S, Morris MC, Divita G, Heitz F (2005) Cell-penetrating peptides: tools for intracellular delivery of therapeutics. Cell Mol Life Sci 62(16):1839–1849

Di Cosimo S, Ferretti G, Papaldo P, Carlini P, Fabi A, Cognetti F (2003) Lonidamine: efficacy and safety in clinical trials for the treatment of solid tumors. Drugs Today 39(3):157–174

Floridi A, Bianchi C, Bagnato A, Gambacurta A, Paggi MG, Silvestrini B, Caputo A (1987) Lonidamine-induced outer membrane permeability and susceptibility of mitochondria to inhibition by adriamycin. Anticancer Res 7(6):1149–1152

Fulda S (2008) Betulinic acid for cancer treatment and prevention. Int J Mol Sci 9(6):1096–1107

Fuller N, Rand RP (2001) The influence of lysolipids on the spontaneous curvature and bending elasticity of phospholipid membranes. Biophys J 81(1):243–254

Goldin N, Arzoine L, Heyfets A, Israelson A, Zaslavsky Z, Bravman T, Bronner V, Notcovich A, Shoshan-Barmatz V, Flescher E (2008) Methyl jasmonate binds to and detaches mitochondria-bound hexokinase. Oncogene 27(34):4636–4643

Guzman-Villanueva D, Mendiola MR, Nguyen XN, Weissig V (2015) Influence of triphenylphosphonium (TPP) cation hydrophobization with phospholipids on cellular toxicity and mitochondrial selectivity. SOJ Pharm Pharm Sci 2(1):1–9

Horobin RW, Trapp S, Weissig V (2007) Mitochondriotropics: a review of their mode of action, and their applications for drug and DNA delivery to mammalian mitochondria. J Control Release 121(3):125–136

Koya K, Li Y, Wang H, Ukai T, Tatsuta N, Kawakami M, Shishido CLB (1996) MKT-077, a novel rhodacyanine dye in clinical trials, exhibits anticarcinoma activity in preclinical studies based on selective mitochondrial accumulation. Cancer Res 56(3):538–543

Kroemer G, de Thé H (1999) Arsenic trioxide, a novel mitochondriotoxic anticancer agent?". J Natl Cancer Inst 91(9):743–745

Larochette N, Decaudin D, Jacotot E, Brenner C, Marzo I, Susin SA, Zamzami N, Xie Z, Reed J, Kroemer G (1999) Arsenite induces apoptosis via a direct effect on the mitochondrial permeability transition pore. Exp Cell Res 249(2):413–421

Lasch J, Meye A, Taubert H, Koelsch R, Mansa-ard J, Weissig V (1999) Dequalinium vesicles form stable complexes with plasmid DNA which are protected from DNase attack. Biol Chem 380(6):647–652

Liberman EA, Topaly VP, Tsofina LM, Jasaitis AA, Skulachev VP (1969) Mechanism of coupling of oxidative phosphorylation and the membrane potential of mitochondria. Nature 222 (5198):1076–1078

Lindgren M, Hällbrink M, Prochiantz A, Langel Ü (2000) Cell-Penetrating Peptides. Trends Pharmacol Sci 21(3):99–103

Lucken-Ardjomande S, Martinou JC (2005) Newcomers in the process of mitochondrial permeabilization. J Cell Sci 118(Pt 3):473–483

Lyrawati D, Trounson A, Cram D (2011) Expression of GFP in the mitochondrial compartment using DQAsomes-mediated delivery of an artificial mini-mitochondrial genome. Pharm Res 28 (11):2848–2862

Malhi SS, Budhiraja A, Arora S, Chaudhari KR, Nepali K, Kumar R, Sohi H, Murthy RS (2012) Intracellular delivery of redox cycler-doxorubicin to the mitochondria of cancer cell by folate receptor targeted mitocancerotropic liposomes. Int J Pharm 432(1–2):63–74

Marco C, Ríos-Marco P, Jiménez-López JM, Segovia JL, Carrasco MP (2014) Antitumoral alkylphospholipids alter cell lipid metabolism. Anticancer Agents Med Chem 14(4):545–558

Milane L, Trivedi M, Singh A, Talekar M, Amiji M (2015) Mitochondrial biology, targets, and drug delivery. J Control Release 207(June):40–58

Modica-Napolitano JS, Aprille JR (2001) Delocalized lipophilic cations selectively target the mitochondria of carcinoma cells. Adv Drug Deliv Rev 49(1-2):63–70

Moreira PI, Custódio JMA, Oliveira CR, Santos MS (2006) Tamoxifen and estradiol interact with the flavin mononucleotide site of complex i leading to mitochondrial failure. J Biol Chem 281 (15):10143–10152

Murphy MP (2008) Targeting lipophilic cations to mitochondria. Biochim Biophys Acta 1777 (7–8):1028–1031

Neuzil J, Dong LF, Rohlena J, Truksa J, Ralph SJ (2013) Classification of mitocans, anti-cancer drugs acting on mitochondria. Mitochondrion 13(3):199–208

Oliver CL, Bauer JA, Wolter KG, Ubell ML, Narayan A, O'Connell KM, Fisher SG et al (2004) *In vitro* effects of the bh3 mimetic, (-)-gossypol, on head and neck squamous cell carcinoma cells. Clin Cancer Res 10(22):7757–7763

Propper DJ, Braybrooke JP, Taylor DJ, Lodi R, Styles P, Cramer JA, Collins WC et al (1999) Phase I trial of the selective mitochondrial toxin MKT077 in chemo-resistant solid tumours. Ann Oncol 10(8):923–927

Ralph S, Low P, Dong L, Lawen A, Neuzil J (2006) Mitocans: mitochondrial targeted anti-cancer drugs as improved therapies and related patent documents. Recent Pat Anticancer Drug Discov 1(3):327–346

Ravagnan L, Marzo I, Costantini P, Susin SA, Zamzami N, Petit PX, Hirsch F et al (1999) Lonidamine triggers apoptosis via a direct, Bcl-2-inhibited effect on the mitochondrial permeability transition pore. Oncogene 18(16):2537–2546

Smith RA, Murphy MJ (2011) Mitochondria-targeted antioxidants as therapies. Discov Med 11 (57):106–114

Smith RA, Hartley RC, Cochemé HM, Murphy MP (2012) Mitochondrial pharmacology. Trends Pharmacol Sci 33(6):341–352

Starenki D, Park J (2013) Mitochondria-targeted nitroxide, mito-cp, suppresses medullary thyroid carcinoma cell survival *in vitro* and *in vivo*. J Clin Endocrinol Metab 98(4):1529–1540

Szeto HH (2006) Cell-permeable, mitochondrial-targeted, peptide antioxidants. AAPS J 8(2): E277–E283

Szeto HH (2008) Mitochondria-targeted cytoprotective peptides for ischemia-reperfusion injury. Antioxid Redox Signal 10(3):601–619

Szewczyk A, Wojtczak L (2002) Mitochondria as a pharmacological target. Pharmacol Rev 54 (1):101–127

Vaidya B, Vyas SP (2012) Transferrin coupled vesicular system for intracellular drug delivery for the treatment of cancer: development and characterization. J Drug Target 20(4):372–380

van der Luit AH, Budde M, Ruurs P, Verheij M, van Blitterswijk WJ (2002) Alkyl-lysophospholipid accumulates in lipid rafts and induces apoptosis via raft-dependent endocytosis and inhibition of phosphatidylcholine synthesis. J Biol Chem 277(42):39541–39547

Weissig V, Torchilin VP (2001) Towards mitochondrial gene therapy: dqasomes as a strategy. J Drug Target 9(1):1–13

Weissig V, Torchillin VP (2001) Cationic bolasomes with delocalized charge centers as mitochondria-specific DNA delivery systems. Adv Drug Deliv Rev 49(1–2):127–149

Weissig V, Lasch J, Erdos G, Meyer HW, Rowe TC, Hughes J (1998) DQAsomes: a novel potential drug and gene delivery system made from dequalinium. Pharm Res 15(2):334–337

Weissig V, Boddapati SV, D'Souza GG, Cheng SM (2004) Targeting of low-molecular weight drugs to mammalian mitochondria. Drug Des Rev 1(1):15–28

Yamamoto T, Ito M, Kageyama K, Kuwahara K, Yamashita K, Takiguchi Y, Kitamura S, Terada T, Shinohara Y (2014) Mastoparan peptide causes mitochondrial permeability transition not by interacting with specific membrane proteins but by interacting with the phospholipid phase. FEBS J 281(17):3933–3944

Zhao K, Zhao GM, Wu D, Soong Y, Birk AV, Schiller PW, Szeto HH (2004) Cell-permeable peptide antioxidants targeted to inner mitochondrial membrane inhibit mitochondrial swelling, oxidative cell death, and reperfusion injury. J Biol Chem 279(33):34682–34690

Zheng N, Tsai HN, Zhang X, Shedden K, Rosania GR (2011) The subcellular distribution of small molecules: a meta-analysis. Mol Pharm 8(5):1611–1618

Zupančič Š, Kocbek P, Zariwala MG, Renshaw D, Gul MO, Elsaid Z, Taylor KE, Somavarapu S (2014) Design and development of novel mitochondrial targeted nanocarriers, DQAsomes for curcumin inhalation. Mol Pharm 11(7):2334–2345

Role of Mitochondrial Reactive Oxygen Species in the Activation of Cellular Signals, Molecules, and Function

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Handbook of Experimental Pharmacology 240, DOI 10.1007/164_2016_117

Abstract

Mitochondria are a major source of intracellular energy and reactive oxygen species in cells, but are also increasingly being recognized as a controller of cell death. Here, we review evidence of signal transduction control by mitochondrial superoxide generation via the nuclear factor-κB (NF-κB) and GATA signaling pathways. We have also reviewed the effects of ROS on the activation of MMP and HIF. There is significant evidence to support the hypothesis that mitochondrial superoxide can initiate signaling pathways following transport into the cytosol. In this study, we provide evidence of TATA signal transductions by mitochondrial superoxide. Oxidative phosphorylation via the electron transfer chain, glycolysis, and generation of superoxide from mitochondria could be important factors in regulating signal transduction, cellular homeostasis, and cell death.

Keywords

Activation • GATA • HIF • Mitochondria • Mitochondrial ROS • MMP • MnSOD • NF-κB • Reactive oxygen species • Signal transduction • Superoxide

1 Introduction

Our solar system and planet were formed ~4.6 billion years ago, and evidence has been presented for the development of life on earth 1 billion years later. The evolution of algae is believed to have given rise to the presence of oxygen (O_2) in the planetary atmosphere, with this now containing ~20.5% O_2. Before this, atmospheric O_2 levels were extremely low (Arnold et al. 2004; Kerr 2005). This rise in atmospheric O_2

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resulted in major evolutionary survival pressures due to the increased incidence and severity of oxidative damage and intracellular peroxidation, especially in mitochondria (Halliwell and Gutteridge 2007a). Organisms have therefore had to evolve prevention and protection systems against oxidative damage imposed by higher O_2 levels, which can be toxic to non-adapted cells. These protective systems include enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxiredoxins, and glutathione peroxidase (GPX), and non-enzymatic substances including glutathione (GSH); vitamins A, C, and E; urate; carotenoids; and polyphenols (Halliwell and Gutteridge 2007b). Current data suggest that about 1 billion years ago, infection or uptake of a kind of a *Rickettsia prowazekii*, a free-living bacteria, into cells resulted in the formation of a symbiotic relationship with the host cell (Gray et al. 1999; Andersson et al. 1998). This symbiosis, which is supported by genetic analysis, appears to have given rise to the development of the mitochondria, and underlies the development of most eukaryotes. The incorporated bacterium appears to have evolved to share cellular functions with the host, and particularly the production of large quantities of cellular "energy" in the form of phosphorylated compounds by oxidative phosphorylation (OXPHOS) via electron transfer from cellular substrates to molecular O_2. Other organisms on earth (anaerobes) have adapted to live without O_2, with most of these, using glucose to produce energy via glycolysis. Mammalian cells retain the capacity to use glycolysis to produce energy, in addition to OXPHOS, but this pathway only produces 2 molecules of adenosine triphosphate (ATP; the major "energy fuel" in cells) compared to the 31 molecules of ATP produced in mitochondria via OXPHOS (Salway 2004), indicating the considerable advantages of the symbiosis with *Rickettsia prowazekii*, and the development of mitochondria.

The evidence that mitochondria are of bacterial origin, such as from *Rickettsia*, is considerable (Wang and Wu 2015). Thus, mitochondria have the double-membrane structure typical of prokaryotes compared to the single membranes of eukaryotes, together with their own deoxyribonucleic acid (DNA; mtDNA), and protein production system. The mtDNA is constituted of 16,569 base pairs (bp) (compared to $\sim 3 \times 10^9$ bp in human nuclear DNA), and has 13 genes that code for protein production, i.e., transcription and translation, all of which are for subunits of the OXPHOS system. In contrast, protein formation from nuclear DNA occurs within the cytosol (DiMauro and Schon 2006). Mitochondria are comprised of almost 1,000 proteins, with nearly all of the genes that code for these, except the 13 mitochondrial genes, present in the nuclear genome indicating a dramatic symbiosis (Indo et al. 2007, 2015; Majima et al. 2016).

Evolutionary development of complex organisms from simple multicellular systems appears to have required an efficient intracellular energy source, so that without the acquisition of mitochondria and OXPHOS, it is less likely that the complexity of organisms that are currently present on earth could have developed. Similar efficient intracellular energy production systems are likely to be required for all complex species, whether they have evolved on earth, or elsewhere. The tremendous production of intracellular energy afforded by the presence of mitochondria has allowed the development both of complex organisms and their considerable life spans. Why should this be the case? The links between mitochondria, life span, and

aging are of considerable interest and there is now a large body of evidence that links the electron transport chains of mitochondria, with oxidative damage and life span. Oxidative stress resulting from the generation of oxidants from electron leakage from the OXPHOS process has been strongly implicated in both aging and neurodegenerative disorders (Indo et al. 2015; Majima et al. 2016). However, reactive oxygen species produced by OXPHOS might have other signaling functions in normal and tumor cells.

2 Reactive Oxygen Species (ROS) Activate Transduction Signals: Nrf2 and NF-κB

There is increasing evidence that reactive oxygen species (ROS), peroxides and other reactive species formed on several proteins, lipids, and DNAs, can activate transductional signals. One of the major signaling pathways activated in this manner is the Kelch-like ECH-associated protein 1 (Keap1)–nuclear factor E2-related factor 2 (Nrf2) system (Kobayashi and Yamamoto 2006). Multiple reactive oxidants can modify amino acid residues of these proteins (Stadtman and Berlett 1991), and it has been established that damage to Keap1 results in dissociation of the Nrf2–Keap1 complex, and activation of Nrf2 (Itoh et al. 2004). There is also evidence that simultaneous targeting of both the Nrf2 and NF-κB systems can have therapeutic effects against neuro-inflammation and oxidative stress via alteration of nerve damage, impaired blood supply, pain hypersensitivity, and neuronal apoptosis (Ganesh Yerra et al. 2013).

In cells, signal transduction networks act to maintain homeostasis and prevent major changes in intracellular status including alterations to redox potentials. Among the multiple pathways involved, signal transduction via NF-κB appears to play a key role during inflammation, immunity, development, cell growth, and survival. NF-κB regulates over 100 genes, including those with both antioxidant and pro-oxidant functions (Morgan and Liu 2011). Tumor necrosis factor-α (TNF-α) is a well-established inducer of NF-κB, with this occurring in a ROS-dependent manner (Jamaluddin et al. 2007). Although antioxidants have been reported to induce NF-κB activation (Liu et al. 2008), there is overwhelming evidence for a key role of numerous oxidants in this process, which is postulated to be important in disease (Schieven et al. 1993; Peng et al. 1995; Li and Karin 1999; Bowie and O'Neill 2000; Kamata et al. 2002; Schoonbroodt and Piette 2000; Takada et al. 2003; Gloire et al. 2006; Gloire and Piette 2009). The protein, NF-κB essential modulator (NEMO, also known as inhibitor of NF-κB kinase subunit gamma, IKK-γ), is a subunit of the IκB kinase complex that activates NF-κB when present in a dimer (disulfide-bond) form. The formation of these disulfide bonds requires Cys54 and Cys347, and it has been shown that treatment of cells with H_2O_2 enhances the formation of NEMO dimers, suggesting that oxidants can activate the NF-κB-related system via this pathway (Herscovitch et al. 2008).

Several studies have shown that antioxidants such as N-acetyl-L-cysteine (NAC) can inhibit NF-κB activation by preventing nuclear translocation of active NF-κB (Dirsch et al. 1998). It is also known that ROS can activate the mitogen-activated protein kinase (MAPK) pathway (Guyton et al. 1996; Gupta et al. 1999),

and evidence has been presented for alteration of critical amino acids in proteins, and changes in structure and function (Thannickal and Fanburg 2000). Thus, oxidative modification of signaling proteins by ROS may be a plausible mechanism for the activation of this pathway. However, the precise molecular targets and the nature of the reactive oxidants responsible in vivo are unknown, though some of the observed effects can be replicated in vitro or ex vivo by specific species such as hydrogen peroxide (H_2O_2) (Son et al. 2011).

3 ROS Activate MMPs and HIF

Matrix metalloproteinases (MMPs) are a large family of ubiquitous extracellular endopeptidases, first reported by Gross and Lapierre (1962), that play important roles in physiology and pathology (Stefano and Herrero 2016; Mittal et al. 2016), proliferation and development (Small and Crawford 2016), transcriptional regulation (Sternlicht and Werb 2001), differentiation (Barkho et al. 2008), tissue remodeling (Hulboy et al. 1997), wound healing (Lazaro et al. 2016), angiogenesis (Vu et al. 1998; Pepper 2001; Rundhaug 2005), hematopoiesis (Pruijt et al. 1999), cancer progression (Coussens et al. 2002), and apoptosis (Vu et al. 1998).

Hao et al. have demonstrated that adrenoceptor-dependent ROS generation in rat small mesenteric arteries is decreased in the presence of MMP inhibitors, suggesting the involvement of MMP on ROS generation (Hao et al. 2006). There are also a number of studies highlighting a role for ROS in the activation of various MMPs. Thus, Zhan et al. have shown that increases of ROS activate MMP-2 in MCF-7 cells (Zhang et al. 2002). Lu and Wahl have shown that ROS activate MMP-1 through NF-kB activation in lipopolysaccharide-activated human primary monocytes (Lu and Wahl 2005).

Many genes are involved in oxygen-regulated gene expression (Ratcliffe et al. 1998). The hypoxic-inducible factor-1 (HIF-1) is expressed in all cell types and functions as a master regulator of oxygen homeostasis by playing critical roles in both embryonic development and postnatal physiology (Semenza 2004). The expression of over 70 genes is known to be activated at the transcriptional level by HIF-1 (Semenza 2004). Under normal oxygen conditions, HIF α-subunits is degraded by a process that requires the von Hippel-Lindau (VHL) tumor-suppressor protein in the presence of oxygen (Kaelin 2002). VHL is a recognition motif and E3 ubiquitin ligase (Kaelin 2002). Under hypoxia and acidic conditions, VHL is transported into nucleus (Mekhail et al. 2004). However, interestingly under hypoxic and normal pH conditions, this transportation does not occur, in contrast to effects seen under low-pH conditions (Mekhail et al. 2004). Zhao et al. have reported that active HIF-1 induced the reprogramming of glucose metabolism from oxidative phosphorylation to anaerobic glycolysis, leading to a decrease in cytotoxic ROS levels (Zhao et al. 2014). It has also been shown that in addition to hypoxia, H_2O_2 and menadione activate HIF-1α, but not HIF-1β in HeLa, A549, HCT116, and HepG2 cells (Jung et al. 2008). Interestingly, ROS work differently on HIF-1 pathway in the context of cancer and ischemia (Qutub and Popel 2008). It is well established that

angiotensin II (Ang II) is related to HIF-1 stabilization. Ang II stimulation causes H_2O_2 production via NADPH oxidases (NOXs) 1 and 4 (Dikalov et al. 2008). This Ang II-mediated H_2O_2 production stabilizes HIF-1α through a decrease in ascorbate levels, and decrease in hydroxylation of proline residue 402, which leads to decreased HIF prolyl-hydroxylase activity (Pagé et al. 2008). Mitochondria may be involved in HIF-1α stabilization. Hagen demonstrated the hypothesis that mitochondria play a critical role in the regulation of HIF-1α by controlling intracellular oxygen concentrations (Hagen 2012). Bonello et al. demonstrated that NF-κB is involved in HIF-1 activation by the observation that the HIF-1α promoter contains a functional NF-κB-binding site, and NF-κB modulates the expression of the HIF-1 target gene (Bonello et al. 2007). Modestly increased ROS lengthen the life span in *Caenorhabditis elegans*. Hwang et al. have demonstrated that ROS activate AMP-activated kinase (AMPK) and HIF-1 promotes longevity by increasing immunity (Hwang et al. 2014). They demonstrated that internal ROS levels are reduced by AMPK, while being amplified by HIF-1 when animals are stimulated to have increased ROS levels. Thus, balancing ROS at optimal levels appears to be crucial for organismal health and longevity (Hwang et al. 2014).

4 NF-κB, NADPH Oxidases (NOX), and Reactive Oxidants Control GATA

GATA proteins, a family of transcription factors named after their capacity to bind to the DNA sequence "GATA," are essential transduction factors in development, growth, and survival (Shivdasani et al. 1997; Lentjes et al. 2016). GATA1/2/3 are required for differentiation of mesoderm and ectoderm-derived tissues, including the hematopoietic and central nervous system, whereas GATA4/5/6 are implicated in development and differentiation of endoderm- and mesoderm-derived tissues such as induction of differentiation of embryonic stem cells, cardiovascular embryogenesis, and guidance of epithelial cell differentiation in the adult (Lentjes et al. 2016). A recent study has suggested that NF-κB controls GATA (Fang et al. 2016), with Das et al. reporting that inhibition of NF-κB activity prevents GATA-3 expression (Das et al. 2001). BCL3 is a proto-oncogene product and controls nuclear translocation of the NF-κB subunit p50, but not of p65 (Heissmeyer et al. 1999; Naumann et al. 1993; Thornburg et al. 2003). Suppression of BCL3 results in a decrease in GATA3 (Corn et al. 2005), consistent with NF-κB-modulating GATA3 activation.

NADPH oxidases (NOXs) also work as signal transduction regulators (reviewed in Jiang et al. 2011), with the oxidants formed by these enzyme complexes [superoxide radicals ($O_2^{\bullet -}$) and H_2O_2] reported to regulate GATA through redox-dependent c-Jun protein activation (Jiang et al. 2011; Murray et al. 2013). Interestingly, NOX2-deficient T cells appear to have decreased GATA-3 expression (Shatynski et al. 2012). Hyperglycemia can cause systolic dysfunction and a higher expression of cardiac troponin-I (cTnI) in cardiomyocytes, with this reported to occur through oxidant-mediated enhancement of MEK/ERK-induced GATA-4 phosphorylation and accumulation in the cell nucleus (Ku et al. 2011). It has also been reported that GATA is

controlled by reactive oxidants generated by leptin synthesis (Ghantous et al. 2016). These reports are consistent with direct effects of reactive oxidants on GATA.

5 Amino Acid Modification in Proteins by Reactive Oxidants in Signaling and Aging

Modification of proteins by oxidants has been implicated as an important factor in aging and has been reviewed (Thannickal and Fanburg 2000; Stadtman 2006; Hawkins et al. 2009; Dean et al. 1997; Davies 2016), and how oxidants can modify protein systems and multiple potential mechanisms have been described. These include (1) modification of critical active site and functional side chains (e.g., cysteines, Cys) by oxidation; (2) formation of (reversible) *intramolecular* disulfide linkages; (3) protein dimerization by formation of (reversible) *intermolecular* disulfide linkages; (4) formation of (irreversible) dityrosine and other covalent cross-links; (5) unfolding resulting from side-chain modifications and increased hydrophilicity; and (6) induction of protein backbone cleavage (fragmentation). These modifications can be induced by both radicals (one-electron oxidants) and two-electron oxidants [e.g., peroxynitrous acid (ONOOH), hypochlorous acid (HOCl), and related species, ozone, singlet oxygen, photochemical reactions, peroxides] (Dean et al. 1997; Davies 2016). Metal ion-catalyzed oxidation of proteins by "Fenton-like" chemistry has been shown to generate protein carbonyls, both on the side chain and backbone of proteins, by a variety of oxidative pathways (Thannickal and Fanburg 2000; Dean et al. 1997; Davies 2016), but these species are not unique to metal ion-catalyzed reactions, and are also formed, in variable yields, by other oxidants. Carbonyl groups can not only be introduced into proteins by direct oxidation of amino acids, but also via secondary oxidation products of lipid peroxidation (reactive aldehydes, e.g., the formation of malondialdehyde-lysine adducts), and by the oxidation products of reducing sugars (e.g., glucose and dicarbonyls, which give species such as carboxymethyllysine and hydroimidazolones) (Wadley et al. 2016). Metal ion-catalyzed oxidation of lysine (Lys) gives α-aminoadipic semialdehyde, arginine (Arg) gives glutamic semialdehyde, proline (Pro) gives glutamic semialdehyde and 2-pyrrolidone, and threonine (Thr) gives 2-amino-3-ketobutyric acid. Direct oxidation of proteins by radicals can also yield highly reactive carbonyl derivatives resulting from the cleavage of peptide bonds by the α-amidation pathway, by radical attack at the α-carbon and subsequent oxygen radical chemistry, or from backbone hydroperoxides (reviewed in Dean et al. 1997; Davies 2016). Less backbone fragmentation is observed with most two-electron oxidants. Carbonyl groups may be introduced into proteins by adduction of reactive aldehydes derived from the metal-catalyzed oxidation of polyunsaturated fatty acids. These lipoxidation products include di-aldehydes such as malondialdehyde (MDA), which reacts with Lys residues; α,β-unsaturated aldehydes [4-hydroxy-2-nonenal (HNE), acrolein], which can undergo Michael-addition reactions between their electrophilic C=C double bonds and the sulfhydryl group of Cys; the ε-amino group of Lys or the imidazole group of His residues; and γ-ketoaldehydes (levuglandins, isoketals, neuroketals), which react with Lys residues. The chemical modification of protein by reactive carbonyl compounds

derived from lipid peroxidation reactions results in the formation of advanced lipoxidation end products (ALEs). Carbonyl groups can also be generated by secondary reaction of the primary amino group of Lys residues with reactive carbonyl derivatives (ketoamines, ketoaldehydes, deoxyosones), produced by the reaction of reducing sugars or their oxidation products with Lys, Arg, His, and Cys residues on proteins (glycation/glycoxidation reactions), eventually leading to the formation of advanced glycation end products (AGEs) (Dalle-Donne et al. 2006).

6 Mitochondria as Source of Intracellular Energy and a Source of Intracellular Oxidants

Mitochondria are source of intracellular energy, via OXPHOS and ATP production that occurs via the electron transport chain (ETC) located in the inner membrane of mitochondria. Electrons are transported through the ETC from metabolic substrates (that eventually get converted to carbon dioxide), and with ADP converted to ATP at the expense of reduction of O_2 to water. During this oxidative phosphorylation process, 2–3% of the electrons leak from the ETC, with these predominantly resulting in reaction with O_2 to give $O_2^{\bullet -}$. These relatively unreactive species undergo rapid enzymatic or spontaneous dismutation to H_2O_2, which in turn can give rise to

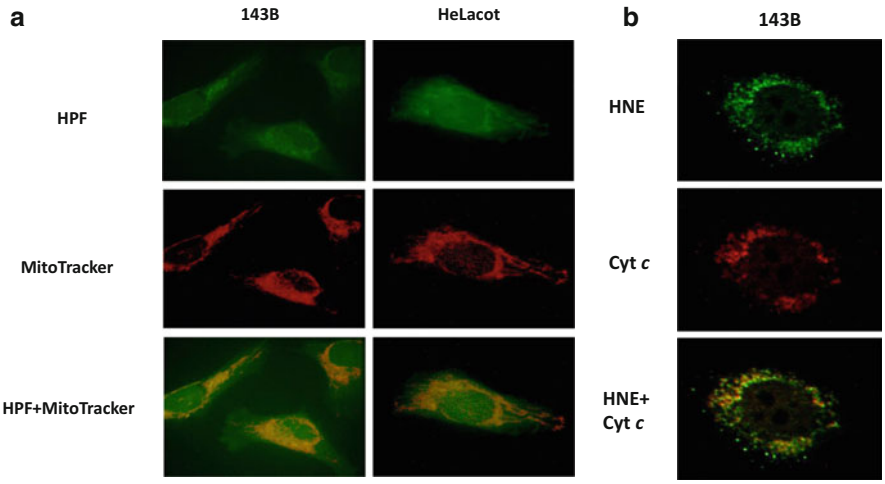

Fig. 1 Intracellular ROS are generated by mitochondria, and lipid peroxidation occurs around mitochondria. (**a**) Representative images of live human 143B and HeLacot cells in which ROS were visualized using HPF (*green*). To examine the location of mitochondria, the same cells were stained with MitoTracker Red CMXRos. Merged double images of HPF and MitoTracker fluorescence were constructed to identify ROS localization in mitochondria. (**b**) 143B cells were fixed and a major lipid peroxidation product, HNE, was immunocytochemically stained. To establish the localization of HNE (*green*), double staining was performed using anti-cytochrome *c* (Cyt *c*), which is present in the mitochondria (*red*). Most HNE was localized in mitochondria, as seen in the merged image (*yellow*). Figure reproduced from Indo et al. (2007)

additional reactive species (e.g., HOCl, peroxynitrous acid, singlet oxygen); this mixture is commonly referred to as "ROS" (see review Indo et al. 2015). Mitochondria are a major *intracellular* source of ROS as shown in Fig. 1a, which is supported by the detection of lipid peroxidation localized around mitochondria as shown in Fig. 1b, similar to that observed in Fig. 1a (Indo et al. 2007). Together, these data support a key role of mitochondrial ROS in apoptosis, aging, antioxidant actions, and signaling processes (see reviews Indo et al. 2015; Majima et al. 2016).

7 Fluorescent Dyes for Detection of ROS Localization

Our early studies indicated that ROS generated from mitochondria cause apoptosis (Majima et al. 1998), with this conclusion derived from studies using 2-[6-(4′-hydroxy)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (HPF), which detects hydroxyl radicals and peroxynitrous acid (Indo et al. 2007; Majima et al. 2011a). This dye has a neutral charge and can provide information regarding the site of ROS generation by use of a laser confocal microscope (Indo et al. 2007). When other dyes such as 2′,7′-dichlorodihydrofluorescin diacetate (DCF), and dihydroethidium (DHE), were employed, mitochondrial ROS generation could not be observed, whereas dihydrorhodamine 123 (DHR) gave clear images of mitochondrial ROS generation (Indo et al. 2007). DHR is an oxidation-sensitive lipophilic dye that enters cells, and when oxidized by mitochondrial ROS gives the positively charged and fluorescent product rhodamine 123 that accumulates and localizes in the mitochondria (Johnson et al. 1980). MitoB is a boronate probe that is also positively charged and accumulates and localizes in the mitochondria (Cochemé et al. 2011). MitoB accumulates in mitochondria, because the surface of mitochondria is negatively charged, −150–180 mV, and is oxidized by H_2O_2 (Kamo et al. 1979; Perry et al. 2011). However, it is possible that the dye can be oxidized before entering and localizing in the mitochondria (Indo et al. 2007). Kalyanaraman have reported that DHE, and its analogue Mito-SOX®, which like MitoB localizes in the mitochondria owing to the presence of a triphenylphosphonium group (TPP^+), cannot detect mitochondrial $O_2^{\bullet -}$ efficiently (Kalyanaraman 2011).

8 Can Mitochondrial ROS Be Released into the Cytoplasm to Perform Signal Transduction?

Generation of $O_2^{\bullet -}$ from mitochondria via the OXPHOS pathway has been long known (Boveris and Cadenas 1975; Takeshige and Minakami 1979). Although there is clear evidence that most of the $O_2^{\bullet -}$ produced is localized inside mitochondria (Fig. 1; Indo et al. 2007), Majima et al. have also provided evidence that mitochondrial ROS can result in apoptosis using MnSOD cellular transfections. Thus, cells transfected with MnSOD vectors resulted in less ROS production, reduced lipid oxidation, and decreased apoptosis (Majima et al. 1998). However, the significance of mitochondrial ROS release has been more difficult to determine, and although it is

known that ROS can initiate various signal transduction pathways, the role of mitochondrial ROS in initiating signal transductions in the cell cytosol has been the subject of controversy. This subject has been dealt with in several reviews (Zhang and Gutterman 2007; Finkel 2012; Cosentino-Gomes et al. 2012; Holmström and Finkel 2014; Chandel 2014, 2015; Shadel and Horvath 2015; Itoh et al. 2015).

Water has been reported to only equilibrate slowly across cellular membranes, with most water transported through aquaporin water channels (Goodman 2002; Tradtrantip et al. 2009). These data indicate that $O_2^{\bullet -}$, due to its charge and ionic nature, and also H_2O_2 are unlikely to diffuse rapidly through the mitochondrial membrane, and opening of VDAC may be necessary for $O_2^{\bullet -}$ release into the cytosol. However, opening of VDAC is controlled by a number of proteins including Bad and Bak (McCommis and Baines 2012), and these can initiate apoptosis. Thus, the major portion of mitochondrial $O_2^{\bullet -}$, which is produced on the matrix site of the inner membrane, is unlikely to exit the mitochondria. $O_2^{\bullet -}$ has been shown to cross the outer mitochondrial membrane via anion channels (Han et al. 2003), but $O_2^{\bullet -}$ passage through VDAC may be a late event in signal transduction, as the opening of VDAC is a defining event in apoptosis.

Mitochondrial ROS might play a number of roles in homeostasis in cellular physiology and pathology, and it is clear that maintenance of homeostasis is of critical importance to life. Signal transduction is therefore likely to be important in the protection of cells from adverse and unexpected events. There is good evidence that ROS can markedly affect and alter cellular environments, and that ROS formation can lead to apoptosis, necrosis, or autophagy (Majima et al. 2009). However, ROS formation may also act as a pre-conditioner that helps prevent or ameliorate secondary or subsequent oxidative stress events. This may occur via up- or down-regulation of cell signaling pathways, and some of these processes may be driven, either directly or indirectly, via mitochondrial ROS formation and its downstream consequences. This may be regulated by the mitochondrial membrane, and also membrane pore opening regulated by ROS.

9 Evidence of Mitochondrial ROS as the Signal Transduction Initiator

Mitochondrial ROS may act on signal transduction pathways via different routes or mechanisms. Mitochondrial ROS might work to initiate a signal transduction in cells to prevent further oxidative stress and injury. Changes in MnSOD gene expression, which exists in mitochondria and removes mitochondrial $O_2^{\bullet -}$ in the mitochondrial matrix, are a key tool in investigating the relevance and role of mitochondrial ROS in signal transduction pathways. Thus, transfection with MnSOD vectors and examination of subsequent changes can be a powerful indicator of the role of mitochondrial $O_2^{\bullet -}$, with cells transfected with MnSOD vector potentially giving different results dependent on the amount and role of mitochondrial $O_2^{\bullet -}$. To illustrate this

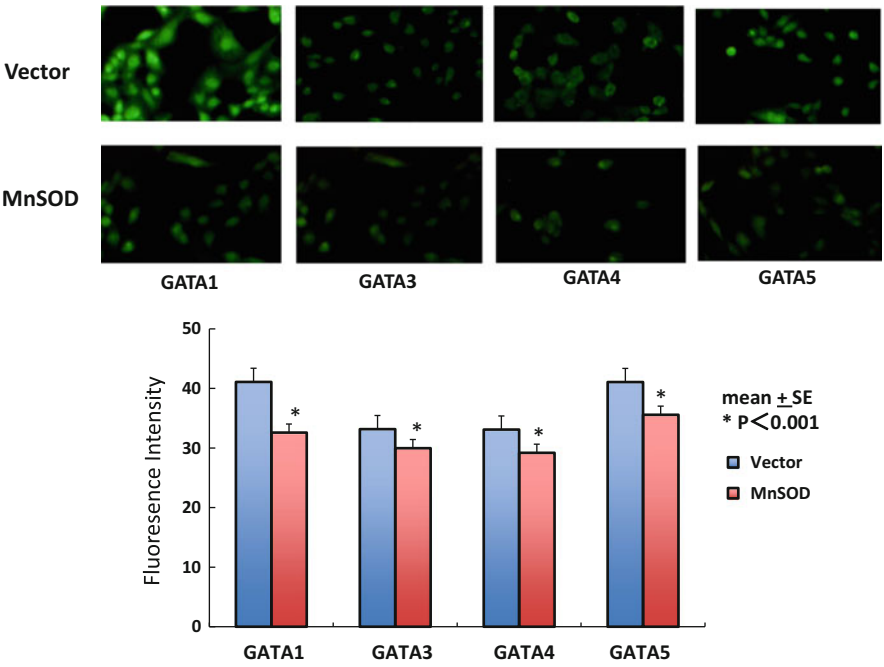

Fig. 2 Immunofluorescent staining for GATA proteins. Representative images of rat gastric mucosal tumor cells (RGK1) stained with antibodies specifically directed against GATA proteins using GATA antibodies: GATA1(N1) rat monoclonal antibody, GATA3(HG3-31) mouse monoclonal antibody, GATA4(C-20) goat antibody, and GATA5(M-20) goat antibody with appropriate Alexa Fluor 488-conjugated second antibodies. The vector control-transfected cells are shown in the top panel, with MnSOD vector-transfected cells shown in the bottom panel. Bioimages of GATA proteins were obtained using a CSU-10 confocal laser scanning unit (Yokogawa Electric Co., Tokyo, Japan) coupled to an IX90 inverted microscope with a UPlanAPO X20 objective lens (Olympus Optical Co., Tokyo, Japan) and a C5810-01 color chilled 3CCD camera (Hamamatsu Photonics, Hamamatsu, Japan). Alexa Fluor 488-conjugated second antibodies were excited at 488 nm and the emissions were filtered using a 515-nm barrier filter. The intensity of the laser beam, the exposure time of the 3CCD camera, and the gain of the amplifier were held at 500 μW, 4.0 s, and 18 dB, respectively, to allow quantitative comparisons of the relative fluorescence intensity of the cells between groups. Cells were chosen on a random basis and scanned at more than three points for analysis. The graph represents the average fluorescence intensity per cell in the vector control and MnSOD-transfected cells after immunostaining with GATA1, GATA3, GATA4, and GATA5 using IPLab Spectrum version 3.0 software (Scanalytics Inc., Fairfax, VA) with some modification of the program by one of the authors (H.J.M.). Statistical analysis was performed by Student t-test. Continuous variables were expressed as mean values ± standard error. A p value of less than 0.001 was considered to be statistically significant

approach, we have compared mouse gastric tumor cells RGK1 transfected with vector alone, or MnSOD vector. Western analysis, and immunoblotting for anti-GATA 1, 3, 4, and 5 proteins, reveals that the MnSOD transfectants have significantly decreased amounts of the GATA protein (Fig. 2), suggesting that a decrease in the

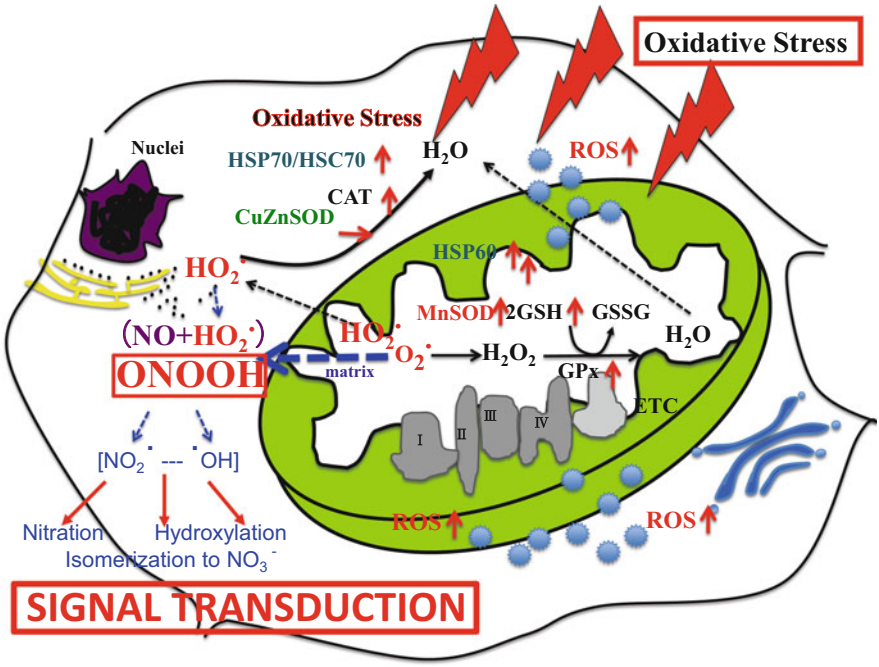

Fig. 3 Scheme showing mitochondrial generation of ROS and the resulting cellular consequences. $O_2^{\bullet-}$ generated from mitochondria moves to the cytosol where it can react with $NO^{\bullet}$ to produce ONOOH, which leads to the formation of $NO_2^{\bullet}$ and $HO^{\bullet}$. It is noted that the rate constant of $O_2^{\bullet-}$ and $HO_2^{\bullet}$ is extremely low compared to $HO^{\bullet}$ (see Table 1). Therefore, $HO_2^{\bullet}$ could have sufficient lifetime to diffuse and cross the mitochondrial membrane and exert effects on the cytosol. MnSOD decreases the amount of $O_2^{\bullet-}$, therefore decreasing further production of $NO_2^{\bullet}$ and $HO^{\bullet}$ (reproduced from Majima et al. 2016)

levels of mitochondrial $O_2^{\bullet-}$diminished GATA expression. This provides good evidence for an influence of mitochondrial ROS on signal transduction in the cell cytosol (see scheme in Fig. 3). The exact nature of the ROS involved is unknown, but potential candidates that can induce this signal transduction include the hydroperoxyl radical ($HOO^{\bullet}$; protonated superoxide) or H_2O_2, as these species are more likely to have a sufficiently long lifetime due to the low rate constant for reaction with biological substrates, and diffusion radius to cross the mitochondrial membrane and exert effects in the cytosol. Table 1 shows the rate constants for the reaction of the equilibrium mixture of $AscH_2/AscH^-/Asc^{2-}$ at pH 7.4 (Buettner and Jurkiewicz 1996). As shown in Table 1, the rate constant of $HOO^{\bullet}$ is extremely low. It is noted that the excess production of H_2O_2 by MnSOD could be quickly detoxified by GPX by reducing it to water in mitochondria (Davies 2000). Further studies along these lines to explore the role of mitochondrial $O_2^{\bullet-}$ in cell signaling processes are clearly warranted.

The roles of ROS on cell metabolism, homeostasis, and cell physiology and pathology have not been clarified yet. Helmut Sies established the principle of "oxidative stress" in 1985, as described elsewhere (Majima et al. 2016). Further roles of

Table 1 Second-order rate constants for the reaction of the free radicals with ascorbate (from Indo et al. 2015, amended from Buettner and Jurkiewicz 1996)

Rate Constants	
Radical	$k_{obs}(M^{-1}s^{-1})$
$^{\bullet}OH$	1.1×10^{10}
$RO^{\bullet}$ (tert-butyl alkoxyl radical)	1.6×10^{9}
$ROO^{\bullet}$ (alkyl peroxyl radical)	$1\text{-}2 \times 10^{6}$
$GS^{\bullet}$ (glutathionyl radical)	6×10^{8}
$O_2^{\bullet -}$	1×10^{5}

mitochondrial ROS, effects of antioxidants in vivo and in vitro, and the mitochondrial superoxide theory for oxidative stress, diseases, and aging are discussed elsewhere (Majima et al. 2016). Mitochondria could be a target in oxidative diseases, aging, and neurodegenerative disorders (Majima et al. 2011b).

Acknowledgments This work was supported in part by a Grant-in-Aid for Scientific Research (C) (No. 22592093 to H.J.M.) and the Strategic Promotion Program for Basic Nuclear Research (to H.J.M.) of the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry (BRAIN) (to H.J.M.) by the Ministry of Agriculture, Forestry and Fisheries of Japan. M.J.D. gratefully acknowledges financial support from the Novo Nordisk Foundation (Laureate Research Grant NNF13OC0004294).

Conflict of Interest

No potential conflicts of interest were disclosed.

References

Andersson SG, Zomorodipour A, Andersson JO, Sicheritz-Pontén T, Alsmark UC, Podowski RM, Näslund AK, Eriksson AS, Winkler HH, Kurland CG (1998) The genome sequence of Rickettsia prowazekii and the origin of mitochondria. Nature 396:133–140. doi:10.1038/24094

Arnold GL, Anbar AD, Barling J, Lyons TW (2004) Molybdenum isotope evidence for widespread anoxia in mid-Proterozoic oceans. Science 304:87–90

Barkho BZ, Munoz AE, Li X, Li L, Cunningham LA, Zhao X (2008) Endogenous matrix metalloproteinase (MMP)-3 and MMP-9 promote the differentiation and migration of adult neural progenitor cells in response to chemokines. Stem Cells 26:3139–3149

Bonello S, Zähringer C, BelAiba RS, Djordjevic T, Hess J, Michiels C, Kietzmann T, Görlach A (2007) Reactive oxygen species activate the HIF-1alpha promoter via a functional NFkappaB site. Arterioscler Thromb Vasc Biol 7:755–761

Boveris A, Cadenas E (1975) Mitochondrial production of superoxide anions and its relationship to the antimycin insensitive respiration. FEBS Lett 54:311–314

Bowie A, O'Neill LA (2000) Oxidative stress and nuclear factor-kappaB activation: a reassessment of the evidence in the light of recent discoveries. Biochem Pharmacol 9:13–23

Buettner GR, Jurkiewicz BA (1996) Catalytic metals, ascorbate and free radicals: combinations to avoid. Radiat Res 145:532–541

Chandel NS (2014) Mitochondria as signaling organelles. BMC Biol 12:34. doi:10.1186/1741-7007-12-34

Chandel NS (2015) Evolution of mitochondria as signaling organelles. Cell Metab 22:204–206

Cochemé HM, Quin C, McQuaker SJ, Cabreiro F, Logan A, Prime TA, Abakumova I, Patel JV, Fearnley IM, James AM, Porteous CM, Smith RA, Saeed S, Carré JE, Singer M, Gems D, Hartley RC, Partridge L, Murphy MP (2011) Measurement of H2O2 within living Drosophila during aging using a ratiometric mass spectrometry probe targeted to the mitochondrial matrix. Cell Metab 13:340–350

Corn RA, Hunter C, Liou HC, Siebenlist U, Boothby MR (2005) Opposing roles for RelB and Bcl-3 in regulation of T-box expressed in T cells, GATA-3, and the effect of differentiation. J Immunol 175:2102–2110

Cosentino-Gomes D, Rocco-Machado N, Meyer-Fernandes JR (2012) Cell signaling through protein kinase C oxidation and activation. Int J Mol Sci 13:10697–10721

Coussens LM, Fingleton B, Matrisian LM (2002) Matrix metalloproteinase inhibitors and cancer: trials and tribulations. Science 295:2387–2392

Dalle-Donne I, Aldini G, Carini M, Colombo R, Rossi R, Milzani A (2006) Protein carbonylation, cellular dysfunction, and disease progression. J Cell Mol Med 10:389–406

Das J, Chen CH, Yang L, Cohn L, Ray P, Ray A (2001) A critical role for NF-kappa B in GATA3 expression and TH2 differentiation in allergic airway inflammation. Nat Immunol 2:45–50

Davies KJ (2000) Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems. IUBMB Life 50:279–289

Davies MJ (2016) Protein oxidation and peroxidation. Biochem J 473:805–825

Dean RT, Fu S, Stocker R, Davies MJ (1997) Biochemistry and pathology of radical-mediated protein oxidation. Biochem J 324:1–18

Dikalov SI, Dikalova AE, Bikineyeva AT, Schmidt HH, Harrison DG, Griendling KK (2008) Distinct roles of Nox1 and Nox4 in basal and angiotensin II-stimulated superoxide and hydrogen peroxide production. Free Radic Biol Med 45:1340–1351

DiMauro S, Schon EA (2006) The mitochondrial respiratory chain and its disorders. In: DiMauro S, Hirano M, Schon EA (eds) Mitochondrial medicine. Informa, Abington, pp 7–26

Dirsch VM, Gerbes AL, Vollmar AM (1998) Ajoene, a compound of garlic, induces apoptosis in human promyeloleukemic cells, accompanied by generation of reactive oxygen species and activation of nuclear factor kappaB. Mol Pharmacol 53:402–407

Fang P, Shi HY, Wu XM, Zhang YH, Zhong YJ, Deng WJ, Zhang YP, Xie M (2016) Targeted inhibition of GATA-6 attenuates airway inflammation and remodeling by regulating caveolin-1 through TLR2/MyD88/NF-κB in murine model of asthma. Mol Immunol 75:144–150

Finkel T (2012) Signal transduction by mitochondrial oxidants. J Biol Chem 287:4434–4440

Ganesh Yerra V, Negi G, Sharma SS, Kumar A (2013) Potential therapeutic effects of the simultaneous targeting of the Nrf2 and NF-κB pathways in diabetic neuropathy. Redox Biol 1:394–397

Ghantous CM, Kobeissy FH, Soudani N, Rahman FA, Al-Hariri M, Itani HA, Sabra R, Zeidan A (2016) Mechanical stretch-induced vascular hypertrophy occurs through modulation of leptin synthesis-mediated ROS formation and GATA-4 nuclear translocation. Front Pharmacol 6:240. doi:10.3389/fphar.2015.00240, eCollection 2015

Gloire G, Piette J (2009) Redox regulation of nuclear post-translational modifications during NF-kappaB activation. Antioxid Redox Signal 11:2209–2222

Gloire G, Legrand-Poels S, Piette J (2006) NF-kappaB activation by reactive oxygen species: fifteen years later. Biochem Pharmacol 72:1493–1505
Goodman BE (2002) Transport of small molecules across cell membranes: water channels and urea transporters. Adv Physiol Educ 26:146–157
Gray MW, Burger G, Lang BF (1999) Mitochondrial evolution. Science 283:1476–1481
Gross J, Lapierre CM (1962) Collagenolytic activity in amphibian tissues: a tissue culture assay. Proc Natl Acad Sci U S A 48:1014–1022
Gupta A, Rosenberger SF, Bowden GT (1999) Increased ROS levels contribute to elevated transcription factor and MAP kinase activities in malignantly progressed mouse keratinocyte cell lines. Carcinogenesis 20:2063–2073
Guyton KZ, Liu Y, Gorospe M, Xu Q, Holbrook NJ (1996) Activation of mitogen-activated protein kinase by H2O2. Role in cell survival following oxidant injury. J Biol Chem 271:4138–4142
Hagen T (2012) Oxygen versus reactive oxygen in the regulation of HIF-1α: the balance tips. Biomed Res Int 2012:436981. doi:10.1155/2012/436981
Halliwell B, Gutteridge JMC (2007a) Oxygen is a toxic gas – an introduction to oxygen toxicity and reactive species. In: Halliwell B, Gutteridge JMC (eds) Free radicals in biology and medicine, 4th edn. Oxford University Press, Oxford, pp 1–29
Halliwell B, Gutteridge JMC (2007b) Antioxidant defences: endogeneous and diet derived. In: Halliwell B, Gutteridge JMC (eds) Free radicals in biology and medicine, 4th edn. Oxford University Press, Oxford, pp 79–186
Han D, Antunes F, Canali R, Rettori D, Cadenas E (2003) Voltage-dependent anion channels control the release of the superoxide anion from mitochondria to cytosol. J Biol Chem 278:5557–5563
Hao L, Nishimura T, Wo H, Fernandez-Patron C (2006) Vascular responses to alpha1-adrenergic receptors in small rat mesenteric arteries depend on mitochondrial reactive oxygen species. Arterioscler Thromb Vasc Biol 26:819–825
Hawkins CL, Morgan PE, Davies MJ (2009) Quantification of protein modification by oxidants. Free Radic Biol Med 46:965–988
Heissmeyer V, Krappmann D, Wulczyn FG, Scheidereit C (1999) NF-kappaB p105 is a target of IkappaB kinases and controls signal induction of Bcl-3-p50 complexes. EMBO J 18:4766–4778
Herscovitch M, Comb W, Ennis T, Coleman K, Yong S, Armstead B, Kalaitzidis D, Chandani S, Gilmore TD (2008) Intermolecular disulfide bond formation in the NEMO dimer requires Cys54 and Cys347. Biochem Biophys Res Commun 367:103–108
Holmström KM, Finkel T (2014) Cellular mechanisms and physiological consequences of redox-dependent signalling. Nat Rev Mol Cell Biol 15:411–421
Hulboy DL, Rudolph LA, Matrisian LM (1997) Matrix metalloproteinases as mediators of reproductive function. Mol Hum Reprod 3:27–45
Hwang AB, Ryu EA, Artan M, Chang HW, Kabir MH, Nam HJ, Lee D, Yang JS, Kim S, Mair WB, Lee C, Lee SS, Lee SJ (2014) Feedback regulation via AMPK and HIF-1 mediates ROS-dependent longevity in *Caenorhabditis elegans*. Proc Natl Acad Sci U S A 111:E4458–E4467
Indo HP, Davidson M, Yen HC, Suenaga S, Tomita K, Nishii T, Higuchi M, Koga Y, Ozawa T, Majima HJ (2007) Evidence of ROS generation by mitochondria in cells with impaired electron transport chain and mitochondrial DNA damage. Mitochondrion 7:106–118
Indo HP, Yen H-C, Nakanishi I, Matsumoto K, Tamura M, Nagano Y, Matsui H, Gusev O, Cornette R, Okuda T, Minamiyama Y, Ichikawa H, Suenaga S, Oki M, Sato T, Ozawa T, St Clair DK, Majima HJ (2015) A mitochondrial superoxide theory for oxidative stress diseases and aging. J Clin Biochem Nutr 56:1–7
Itoh K, Tong KI, Yamamoto M (2004) Molecular mechanism activating Nrf2-Keap1 pathway in regulation of adaptive response to electrophiles. Free Radic Biol Med 36:1208–1213
Itoh K, Ye P, Matsumiya T, Tanji K, Ozaki T (2015) Emerging functional cross-talk between the Keap1-Nrf2 system and mitochondria. J Clin Biochem Nutr 56:91–97
Jamaluddin M, Wang S, Boldogh I, Tian B, Brasier AR (2007) TNFalpha-induced NF-kappaB/RelA Ser(276) phosphorylation and enhanceosome formation is mediated by an ROS-dependent PKAc pathway. Cell Signal 19:1419–1433

Jiang F, Zhang Y, Dusting GJ (2011) NADPH oxidase-mediated redox signaling: roles in cellular stress response, stress tolerance, and tissue repair. Pharmacol Rev 63:218–242

Johnson LV, Walsh ML, Chen LB (1980) Localization of mitochondria in living cells with rhodamine 123. Proc Natl Acad Sci U S A 77:990–994

Jung SN, Yang WK, Kim J, Kim HS, Kim EJ, Yun H, Park H, Kim SS, Choe W, Kang I, Ha J (2008) Reactive oxygen species stabilize hypoxia-inducible factor-1 alpha protein and stimulate transcriptional activity via AMP-activated protein kinase in DU145 human prostate cancer cells. Carcinogenesis 29:713–721

Kaelin WG Jr (2002) Molecular basis of the VHL hereditary cancer syndrome. Nat Rev Cancer 2:673–682

Kalyanaraman B (2011) Oxidative chemistry of fluorescent dyes: implications in the detection of reactive oxygen and nitrogen species. Biochem Soc Trans 39:1221–1225

Kamata H, Manabe T, Oka S, Kamata K, Hirata H (2002) Hydrogen peroxide activates IkappaB kinases through phosphorylation of serine residues in the activation loops. FEBS Lett 519:231–237

Kamo N, Muratsugu M, Hongoh R, Kobatake Y (1979) Membrane potential of mitochondria measured with an electrode sensitive to tetraphenyl phosphonium and relationship between proton electrochemical potential and phosphorylation potential in steady state. J Membr Biol 49:105–121

Kerr RA (2005) Earth science. The story of O_2. Science 308:1730–1732

Kobayashi M, Yamamoto M (2006) Nrf2-Keap1 regulation of cellular defense mechanisms against electrophiles and reactive oxygen species. Adv Enzyme Regul 46:113–140

Ku PM, Chen LJ, Liang JR, Cheng KC, Li YX, Cheng JT (2011) Molecular role of GATA binding protein 4 (GATA-4) in hyperglycemia-induced reduction of cardiac contractility. Cardiovasc Diabetol 10:57. doi:10.1186/1475-2840-10-57

Lazaro JL, Izzo V, Meaume S, Davies AH, Lobmann R, Uccioli L (2016) Elevated levels of matrix metalloproteinases and chronic wound healing: an updated review of clinical evidence. J Wound Care 25:277–287

Lentjes MH, Niessen HE, Akiyama Y, de Bruïne AP, Melotte V, van Engeland M (2016) The emerging role of GATA transcription factors in development and disease. Expert Rev Mol Med 18:e3

Li N, Karin M (1999) Is NF-kappaB the sensor of oxidative stress? FASEB J 13:1137–1143

Liu J, Yoshida Y, Yamashita U (2008) DNA-binding activity of NFkappaB and phosphorylation of p65 are induced by N-acetylcysteine through phosphatidylinositol (PI) 3-kinase. Mol Immunol 45:3984–3989

Lu Y, Wahl LM (2005) Oxidative stress augments the production of matrix metalloproteinase-1, cyclooxygenase-2, and prostaglandin E2 through enhancement of NF-kappa B activity in lipopolysaccharide-activated human primary monocytes. J Immunol 175:5423–5429

Majima HJ, Oberley TD, Furukawa K, Mattson MP, Yen HC, Szweda LI, St Clair DK (1998) Prevention of mitochondrial injury by manganese superoxide dismutase reveals a primary mechanism for alkaline-induced cell death. J Biol Chem 273:8217–8224

Majima HJ, Indo HP, Tomita K, Iwashita Y, Suzuki H, Masuda D, Shimazu T, Tanigaki F, Umemura S, Yano S, Fukui K, Higashibata A, Yamazaki TQ, Kameyama M, Suenaga S, Sato T, Yen H-C, Gusev O, Okuda T, Matsui H, Ozawa T, Ishioka N (2009) Bio-assessment of risk in long-term manned space exploration – cell death factors in space radiation and/or microgravity: a review. Biol Sci Space 23:43–53

Majima HJ, Indo HP, Suenaga S et al (2011a) Mitochondria as source of free radicals. In: Naito Y, Suematsu M, Yoshikawa T (eds) Free radical biology in digestive diseases, Front Gastrointest Res, vol 29. Karger, Basel, pp 12–22

Majima HJ, Indo HP, Suenaga S, Matsui H, Yen HC, Ozawa T (2011b) Mitochondria as possible pharmaceutical targets for the effects of vitamin E and its homologues in oxidative stress-related diseases. Curr Pharm Des 17:2190–2195

Majima HJ, Indo HP, Nakanishi I, Suenaga S, Matsumoto K, Matsui H, Minamiyama Y, Ichikawa H, Yen H-C, Hawkins CL, Davies MJ, Ozawa T, St Clair DK (2016) Chasing great paths of helmut sies “oxidative stress”. Arch Biochem Biophys 595:54–60

McCommis KS, Baines CP (2012) The role of VDAC in cell death: friend or foe? Biochim Biophys Acta 1818:1444–1450
Mekhail K, Gunaratnam L, Bonicalzi ME, Lee S (2004) HIF activation by pH-dependent nucleolar sequestration of VHL. Nat Cell Biol 6:642–647
Mittal R, Patel AP, Debs LH, Nguyen D, Patel K, Grati M, Mittal J, Yan D, Chapagain P, Liu XZ (2016) Intricate functions of matrix metalloproteinases in physiological and pathological conditions. J Cell Physiol 231:2599–2621
Morgan MJ, Liu ZG (2011) Crosstalk of reactive oxygen species and NF-κB signaling. Cell Res 21:103–115
Murray TV, Smyrnias I, Shah AM, Brewer AC (2013) NADPH oxidase 4 regulates cardiomyocyte differentiation via redox activation of c-Jun protein and the cis-regulation of GATA-4 gene transcription. J Biol Chem 288:15745–15759
Naumann M, Wulczyn FG, Scheidereit C (1993) The NF-kappa B precursor p105 and the proto-oncogene product Bcl-3 are I kappa B molecules and control nuclear translocation of NF-kappa B. EMBO J 12:213–222
Pagé EL, Chan DA, Giaccia AJ, Levine M, Richard DE (2008) Hypoxia-inducible factor-1alpha stabilization in nonhypoxic conditions: role of oxidation and intracellular ascorbate depletion. Mol Biol Cell 19:86–94
Peng HB, Libby P, Liao JK (1995) Induction and stabilization of I kappa B alpha by nitric oxide mediates inhibition of NF-kappa B. J Biol Chem 270:14214–14219
Pepper MS (2001) Role of the matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis. Arterioscler Thromb Vasc Biol 21:1104–1117
Perry SW, Norman JP, Barbieri J, Brown EB, Gelbard HA (2011) Mitochondrial membrane potential probes and the proton gradient: a practical usage guide. Biotechniques 50:98–115
Pruijt JF, Fibbe WE, Laterveer L, Pieters RA, Lindley IJ, Paemen L, Masure S, Willemze R, Opdenakker G (1999) Prevention of interleukin-8-induced mobilization of hematopoietic progenitor cells in rhesus monkeys by inhibitory antibodies against the metalloproteinase gelatinase B (MMP-9). Proc Natl Acad Sci U S A 96:10863–10868
Qutub AA, Popel AS (2008) Reactive oxygen species regulate hypoxia-inducible factor 1alpha differentially in cancer and ischemia. Mol Cell Biol 28:5106–5119
Ratcliffe PJ, O'Rourke JF, Maxwell PH, Pugh CW (1998) Oxygen sensing, hypoxia-inducible factor-1 and the regulation of mammalian gene expression. J Exp Biol 201:1153–1162
Rundhaug JE (2005) Matrix metalloproteinases and angiogenesis. J Cell Mol Med 9:267–285
Salway JG (2004) Metabolism of one molecule of glucose yields 31 (or should be 38?) molecules of ATP. In: Salway JG (ed) Metabolism at a glance, 3rd edn. Blackwell, Malden, pp 20–21
Schieven GL, Kirihara JM, Myers DE, Ledbetter JA, Uckun FM (1993) Reactive oxygen intermediates activate NF-kappa B in a tyrosine kinase-dependent mechanism and in combination with vanadate activate the p56lck and p59fyn tyrosine kinases in human lymphocytes. Blood 82:1212–1220
Schoonbroodt S, Piette J (2000) Oxidative stress interference with the nuclear factor-kappa B activation pathways. Biochem Pharmacol 60:1075–1083
Semenza GL (2004) Hydroxylation of HIF-1: oxygen sensing at the molecular level. Physiology (Bethesda) 19:176–182
Shadel GS, Horvath TL (2015) Mitochondrial ROS signaling in organismal homeostasis. Cell 163:560–569
Shatynski KE, Chen H, Kwon J, Williams MS (2012) Decreased STAT5 phosphorylation and GATA-3 expression in NOX2-deficient T cells: role in T helper development. Eur J Immunol 42:3202–3211
Shivdasani RA, Fujiwara Y, McDevitt MA, Orkin SH (1997) A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development. EMBO J 16:3965–3973
Small CD, Crawford BD (2016) Matrix metalloproteinases in neural development: a phylogenetically diverse perspective. Neural Regen Res 11:357–362

Son Y, Cheong YK, Kim NH, Chung HT, Kang DG, Pae HO (2011) Mitogen-activated protein kinases and reactive oxygen species: how can ROS activate MAPK pathways? J Signal Transduct 792639. doi:10.1155/2011/792639

Stadtman ER (2006) Protein oxidation and aging. Free Radic Res 40:1250–1258

Stadtman ER, Berlett BS (1991) Fenton chemistry. Amino acid oxidation. J Biol Chem 266:17201–17211

Stefano ME, Herrero MT (2016) The multifaceted role of metalloproteinases in physiological and pathological conditions in embryonic and adult brains. Prog Neurobiol. doi:10.1016/j.pneurobio.2016.08.002

Sternlicht MD, Werb Z (2001) How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol 17:463–516

Takada Y, Mukhopadhyay A, Kundu GC, Mahabeleshwar GH, Singh S, Aggarwal BB (2003) Hydrogen peroxide activates NF-kappa B through tyrosine phosphorylation of I kappa B alpha and serine phosphorylation of p65: evidence for the involvement of I kappa B alpha kinase and Syk protein-tyrosine kinase. J Biol Chem 278:24233–24241

Takeshige K, Minakami S (1979) NADH- and NADPH-dependent formation of superoxide anions by bovine heart submitochondrial particles and NADH-ubiquinone reductase preparation. Biochem J 180:129–135

Thannickal VJ, Fanburg BL (2000) Reactive oxygen species in cell signaling. Am J Physiol Lung Cell Mol Physiol 279:L1005–L1028

Thornburg NJ, Pathmanathan R, Raab-Traub N (2003) Activation of nuclear factor-kappaB p50 homodimer/Bcl-3 complexes in nasopharyngeal carcinoma. Cancer Res 63:8293–8301

Tradtrantip L, Tajima M, Li L, Verkman AS (2009) Aquaporin water channels in transepithelial fluid transport. J Med Invest 56 Suppl:179–184

Vu TH, Shipley JM, Bergers G, Berger JE, Helms JA, Hanahan D, Shapiro SD, Senior RM, Werb Z (1998) MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. Cell 93:411–422

Wadley AJ, Turner JE, Aldred S (2016) Factors influencing post-exercise plasma protein carbonyl concentration. Free Radic Res 50:375–384

Wang Z, Wu M (2015) An integrated phylogenomic approach toward pinpointing the origin of mitochondria. Sci Rep 5:7949. doi:10.1038/srep07949

Zhang DX, Gutterman DD (2007) Mitochondrial reactive oxygen species-mediated signaling in endothelial cells. Am J Physiol Heart Circ Physiol 292:H2023–H2031

Zhang HJ, Zhao W, Venkataraman S, Robbins ME, Buettner GR, Kregel KC, Oberley LW (2002) Activation of matrix metalloproteinase-2 by overexpression of manganese superoxide dismutase in human breast cancer MCF-7 cells involves reactive oxygen species. J Biol Chem 277:20919–20926

Zhao T, Zhu Y, Morinibu A, Kobayashi M, Shinomiya K, Itasaka S, Yoshimura M, Guo G, Hiraoka M, Harada H (2014) HIF-1-mediated metabolic reprogramming reduces ROS levels and facilitates the metastatic colonization of cancers in lungs. Sci Rep 4:3793. doi:10.1038/srep03793

MITO-Porter for Mitochondrial Delivery and Mitochondrial Functional Analysis

Yuma Yamada and Hideyoshi Harashima

Contents

Abstract

Mitochondria are attractive organelles that have the potential to contribute greatly to medical therapy, the maintenance of beauty and health, and the development of the life sciences. Therefore, it would be expected that the further development of mitochondrial drug delivery systems (DDSs) would exert a significant impact on the medical and life sciences. To achieve such an

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H. Singh, S.-S. Sheu (eds.), *Pharmacology of Mitochondria*,
Handbook of Experimental Pharmacology 240, DOI 10.1007/164_2016_4

innovative objective, it will be necessary to deliver various cargoes to mitochondria in living cells. However, only a limited number of approaches are available for accomplishing this. We recently proposed a new concept for mitochondrial delivery, a MITO-Porter, a liposome-based carrier that introduces macromolecular cargoes into mitochondria via membrane fusion. To date, we have demonstrated the utility of mitochondrial therapeutic strategy by MITO-Porter using animal models of diseases. We also showed that the mitochondrial delivery of antisense oligo-RNA by the MITO-Porter results in mitochondrial RNA knockdown and has a functional impact on mitochondria. Here, we summarize the current state of mitochondrial DDS focusing on our research and show some examples of mitochondrial functional regulations using mitochondrial DDS.

Keywords
Membrane fusion • Mitochondria • Mitochondrial drug delivery • Mitochondrial function • Mitochondrial gene therapy • Mitochondrial medicine development • MITO-Porter

1 Significance of Targeting Mitochondria and the Current Status of Mitochondrial DDS Development

Why is it important to target mitochondria rather than the nucleus, cytosol, or other organelles? Mitochondria carry out various essential functions including ATP production, regulating apoptosis, mitochondrial biosynthesis, and have their own genome, mitochondrial DNA (mtDNA). In recent years, mitochondrial dysfunctions have been implicated in a variety of diseases including neurodegenerative diseases, cardiac infarction, and mitochondrial inherited diseases (Schapira 2006; Tuppen et al. 2010). It is now well accepted that mutations and defects in mtDNA form the basis of a variety of diseases (Wallace 1999; Goto et al. 1990; Shanske et al. 1990; Shoffner et al. 1990). Therefore, the answer to the previous question is because mitochondria represent promising organelles for various fields including the life sciences, drug discovery, and gene therapy.

To achieve such innovative technology and mitochondrial therapy, a mitochondrial drug delivery system (DDS) will be required. During this past decade, numerous mitochondrial DDS developments have been reported (Zhang et al. 2011; Yamada and Harashima 2008; Biswas and Torchilin 2014; Weissig 2011; Kajimoto et al. 2014); however, only a limited number of approaches are available for mitochondrial therapy. However, these strategies face many problems, including cell internalization, size limitations and the physicochemical properties of the cargoes, modification of a functional device, and the denaturation of the cargoes.

To overcome these problems, the use of nanotechnology has attracted considerable attention, and research directed at the development of nanomachines for

mitochondrial delivery is currently underway (Kajimoto et al. 2014). What do you imagine a "nanomachine" would be? In a Japanese manga entitled *The Monster of the 38th Parallel* and the US movie entitled "Fantastic Voyage," humans boarded nanomachines to make voyages inside the human body. Although these stories were scientific fiction, even in the real world, medical nanomachines have been developed and have been applied to treating various diseases. A liposome is a lipid bilayer membrane vesicle, originally discovered by Bangham, A.D., and Horne, R.W. in 1964 (Bangham and Horne 1964), and has attracted attention as a successful current nanomachine for pharmaceuticals and cosmetics. Doxil is world's first liposomal formulation in which doxorubicin, an anticancer agent, is encapsulated. It accumulates at increased levels in cancer cells, permitting its therapeutic effect to be enhanced, by regulating biodistribution in the human body (Oku et al. 1994).

To reach mitochondria in living cells, the development of more advanced innovative nanomachines are required to regulate intracellular trafficking. In this chapter, I will mainly discuss the development of the MITO-Porter, which is a liposomal mitochondrial DDS developed in our laboratory, and the validation of mitochondrial functional regulations by the MITO-Porter system.

2 Construction of a MITO-Porter

Figure 1a shows a schematic image of mitochondrial delivery by the MITO-Porter, which delivers the encapsulated cargoes to mitochondria via membrane fusion (Yamada et al. 2008; Sato et al. 2014). The MITO-Porter, which is modified with a cell penetrating peptide, octaarginine (R8), is first internalized into the cytosol via macropinocytosis. The MITO-Porter then binds to mitochondria via electrostatic interactions between the cationic R8 and negatively charged mitochondria. Finally, the cargoes are delivered to mitochondria via membrane fusion. Mitochondria actively repeat fusion and fission to share bio macromolecules in living cells. Therefore, we expected that a strategy involving membrane fusion via a MITO-Porter system would permit the delivery of a cargo to mitochondria, independent of its size and physical properties.

We first screened for mitochondrial fusogenic liposomes using isolated rat liver mitochondria by varying the lipid composition of a panel of liposomes. As a result, we succeeded in identifying liposomes with a high mitochondrial fusogenic activity among more than 100 different liposomes and referred to these liposomes as MITO-Porters (Fig. 1b). The successful MITO-Porter had the following lipid composition: 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE)/sphingomyelin (SM)/stearylated R8 (STR-R8) [9:2:1, molar ratio] or DOPE/phosphatidic acid (PA)/STR-R8 [9:2:1, molar ratio] (Yamada et al. 2008, 2012a; Yamada and Harashima 2014).

To evaluate mitochondrial delivery by the MITO-Porter in living cells, we observed intracellular trafficking using confocal laser scanning microscopy (CLSM). We confirmed that the green fluorescent protein (GFP) that was encapsulated in the MITO-Porter was co-localized with mitochondria (Fig. 1c)

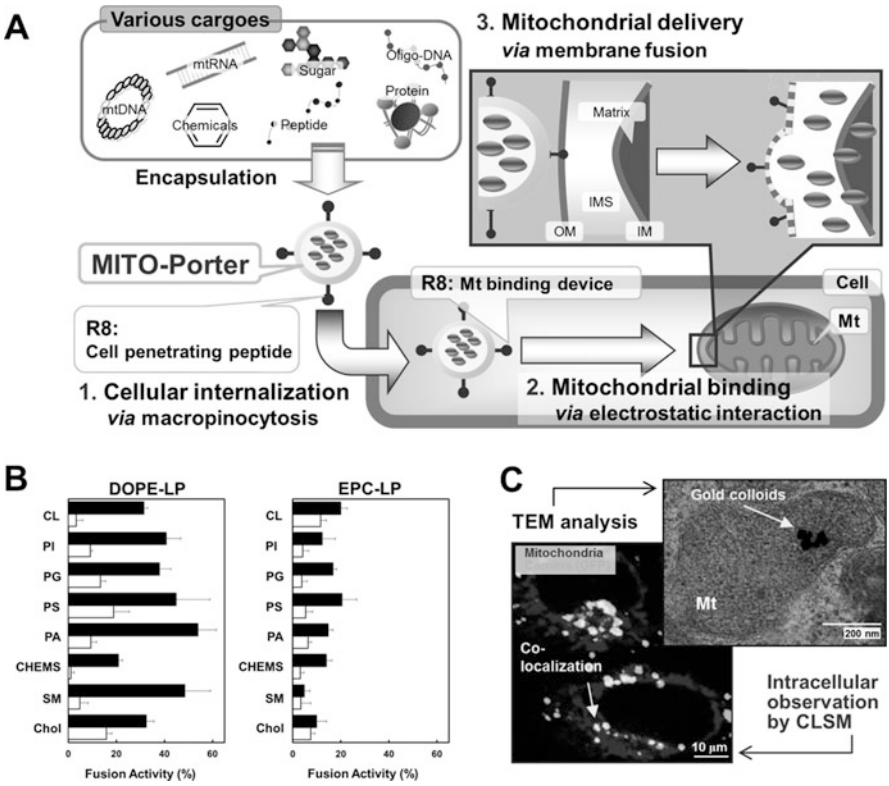

Fig. 1 Schematic image of mitochondrial delivery by MITO-Porter (**a**). IM, inner membrane; IMS, intermembrane space; OM, outer membrane; R8, octaarginine. Screening of fusogenic lipid compositions with the mitochondrial membrane (**b**). Fusion activities (%) of DOPE-LP and EPC-LP. *Closed bars*, R8-LP; *open bars*, unmodified liposome. Intracellular observations of the MITO-Porter after staining mitochondria and transmission electron microscopy (TEM) analysis, indicating that gold colloids encapsulated in MITO-Porter were delivered to mitochondria (**c**). Mt, mitochondria. These figures are used with permission from Elsevier (Sato et al. 2014). For interpretation to color in this figure, the reader is referred to the article (Sato et al. 2014)

(Yamada et al. 2008). On the other hand, low mitochondrial fusogenic liposomes (e.g., liposomes containing egg yolk phosphatidylcholine (EPC)) showed no co-localization with mitochondria. We also performed electron microscopy analyses and observed that the MITO-Porter successfully delivered colloidal gold particles as model macromolecules into mitochondria (Fig. 1c) (Yamada et al. 2008). These results verify that the MITO-Porter is capable of delivering macromolecules into mitochondria of living cells.

So far, we described research outcomes regarding the development of a MITO-Porter system and the mitochondrial delivery of macromolecule in living cells. We also performed other studies to establish the MITO-Porter, including the construction of a dual function (DF) MITO-Porter, which possesses mitochondria-fusogenic

inner and endosome-fusogenic outer envelopes (Yamada et al. 2011, 2012b; Yamada and Harashima 2012, 2015), selective delivery by a mitochondrial targeting device (Kawamura et al. 2013a, b; Yamada and Harashima 2013; Tawaraya et al. 2014), and characteristics and pharmaceutical formulation (Yamada et al. 2011, 2012a, b; Yamada and Harashima 2015; Yamada et al. in press). Based on established technologies, our research then focused on the development of a mitochondrial medicine and regulating mitochondrial gene expression by a MITO-Porter via collaboration with experts in various fields. In the following section, we summarize our latest research outcomes regarding these themes.

3 Research Directed Toward Mitochondrial Medicine Development

The delivery of pharmacological agents to mitochondria is considered to be an important issue in the development of mitochondrial medicines. Each drug must be sorted to the appropriate region of a mitochondrion depending on the pharmacodynamic action of the molecule. For example, apoptosis-inducing and apoptosis-inhibiting drugs should be delivered to the outer membrane, where the main events related to apoptosis are triggered. These delivery systems would be expected to be useful for cancer therapy and therapy for cardiac infractions caused by excessive apoptosis. In the case of respiratory chain-related proteins and coenzymes, they should be delivered to the intermembrane space and the inner membrane region, where the key components of the electron transfer system are found. To achieve these, as a researcher of mitochondrial DDS, it is necessary to validate the proof of concept (POC) of therapeutic strategies by mitochondrial DDS and demonstrate the utility of mitochondrial targeting based on the experimental results.

3.1 Validation of POC of Therapeutic Strategy by Mitochondrial DDS Using Model Culture Cells

We first attempted to validate the POC of a therapeutic strategy using a MITO-Porter in living cells in an in vitro experiment that involved the mitochondrial delivery superoxide dismutase (SOD), an antioxidant enzyme, and bongkrekic acid (BKA), an anti-apoptosis chemical.

In an evaluation of the antioxidant effect conferred by delivering SOD to mitochondria using the MITO-Porter, we collaborated with Prof. Igarashi, R., and Prof. Takenaga, M., in the St. Marianna University School of Medicine, who developed phosphatidylcholine-conjugated SOD (PC-SOD) (Igarashi et al. 1992). We succeeded in incorporating PC-SOD into the lipid envelope of a MITO-Porter (SOD-MITO-Porter), which is made up of positively charged particles with diameters of around 150 nm. Intracellular fluorescence microscopy observations showed that the green fluorescent-labeled SOD-MITO-Porter was effectively internalized into cells (Fig. 2A(a)). We also confirmed that the SOD-MITO-Porter

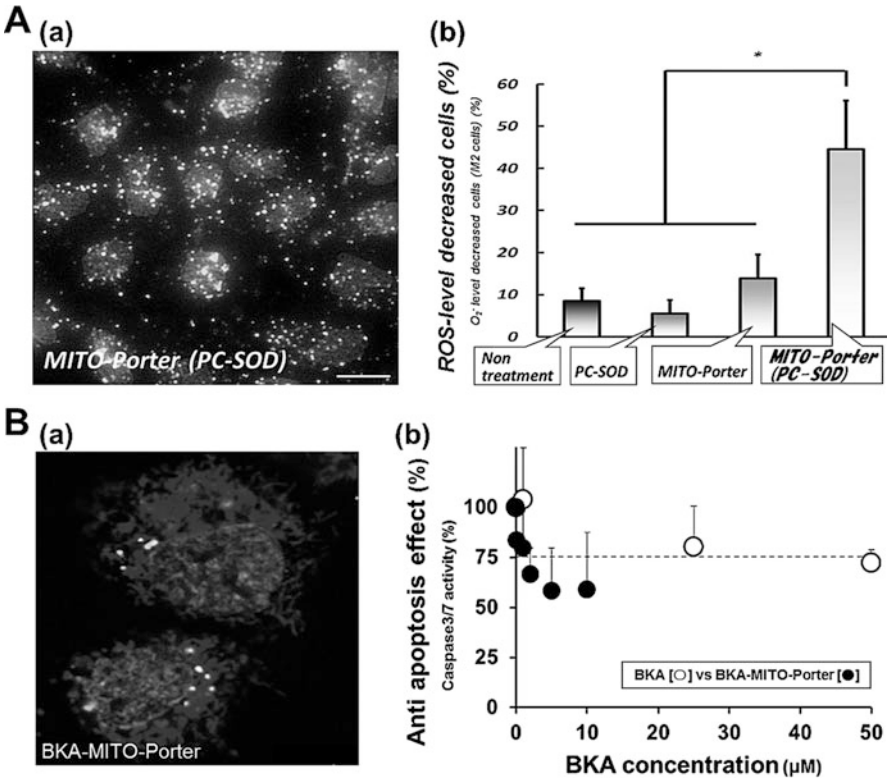

Fig. 2 Intracellular observation and therapeutic effects of the SOD-MITO-Porter (**A**). (*a*) PC-SOD in cells was visualized using wide-field fluorescence microscopy. Scale bar, 20 μm. (*b*) ROS-level decreased cells were investigated. Data are represented as the mean ± S.D. ($n = 3$). *Significant difference ($p < 0.01$) was calculated by one-way ANOVA. Intracellular observations and therapeutic effect of the BKA-MITO-Porter (**B**). (*a*) Intracellular observation of BKA-MITO-Porter using CLSM. Scale bar, 10 μm. (*b*) Comparison of anti-apoptosis between BKA-MITO-Porter and naked BKA. The closed and open circles represent values corresponding to caspase 3/caspase 7 activity (%), when the BKA-MITO-Porter and naked BKA were used. Data are represented by the mean ± SD ($n = 4$). These figures are reproduced from the references (Furukawa et al. 2011; Yamada et al. 2013a). For interpretation to color in this figure, the reader is referred to the article (Furukawa et al. 2011; Yamada et al. 2013a)

decreased reactive oxygen species (ROS) level of cells more effectively than naked SOD (Fig. 2A(b)) (Furukawa et al. 2011).

We also attempted the mitochondrial delivery of BKA, in an attempt to develop an innovative medicine for the treatment of apoptosis-related diseases. We succeeded in packaging the highly hydrophobic BKA in the lipid envelope of a MITO-Porter, a BKA-MITO-Porter. Intracellular observations showed that the BKA-MITO-Porter was co-localized with mitochondria (Fig.2B(a)). Moreover, we confirmed that the MITO-Porter enhanced the anti-apoptosis effect of naked

BKA (Fig. 2B(b)) (Yamada et al. 2013a). These results indicate the POC of a therapeutic strategy using MITO-Porter in living cells in an in vitro experiment.

3.2 Demonstration of Mitochondrial Therapeutic Strategy Using Disease Model Animal

To accelerate the development of mitochondrial medicine, our challenge was to demonstrate the utility of MITO-Porter using model animals in in vivo experiments regarding "construction of tissue targeting DDS for mitochondrial delivery (Yamada et al. 2013b, 2014, 2015a)" and "validation of mitochondrial therapeutic strategy using diseases model animals (Yamada et al. 2015b)." Here, we would like to introduce our latest published work entitled "Evaluation of antioxidant effect conferred by mitochondrial delivery of coenzyme CoQ_{10} (CoQ_{10}) using ischemic/reperfusion (I/R) injury model mouse (Yamada et al. 2015b)." Figure 3A shows a schematic image of the prevention of a hepatic I/R injury by the mitochondrial delivery of CoQ_{10}, an antioxidant chemical, using a MITO-Porter system. Under an ideal scenario, the MITO-Porter encapsulating CoQ_{10} reaches the liver tissue via systemic injection, and the carrier is then internalized into hepatocytes. In the cytosol, the carrier delivers CoQ_{10} to mitochondria via membrane fusion, resulting in the development of a pharmacological effect of CoQ_{10} in mitochondria. In this experiment, we first constructed a CoQ_{10}-MITO-Porter and investigated its accumulation in liver mitochondria and then evaluated hepatoprotective effect from I/R injury.

3.3 Construction of CoQ_{10}-MITO-Porter and Investigation of Liver Mitochondria Accumulation

Before the construction of the CoQ_{10}-MITO-Porter, we investigated the accumulation of conventional MITO-Porter in the liver mitochondria. This particular particle was surface-modified with a high density of R8. It has been reported that R8 functions as a cellular uptake device via macropinocytosis (Futaki et al. 2001; Khalil et al. 2006) and as a mitochondrial targeting device via electrostatic interactions (Yamada and Harashima 2008; Yamada et al. 2008). Moreover, it has been reported that R8-modified liposomes effectively accumulate in the liver when administered via tail vein injection (Khalil et al. 2011). Therefore, we expected that the MITO-Porter would also accumulate in the liver and target mitochondria.

We first performed histological observations of liver tissue. In this experiment, we administered the fluorescent-labeled MITO-Porter to mice via a tail vein injection and observed the liver tissue using fluorescent imaging. As a result, we confirmed that numerous strong fluorescent signals corresponding to the MITO-Porter could be detected in liver cells, while only a weak signal derived from a polyethylene glycol (PEG)-modified liposome (PEG-LP) as a control carrier was

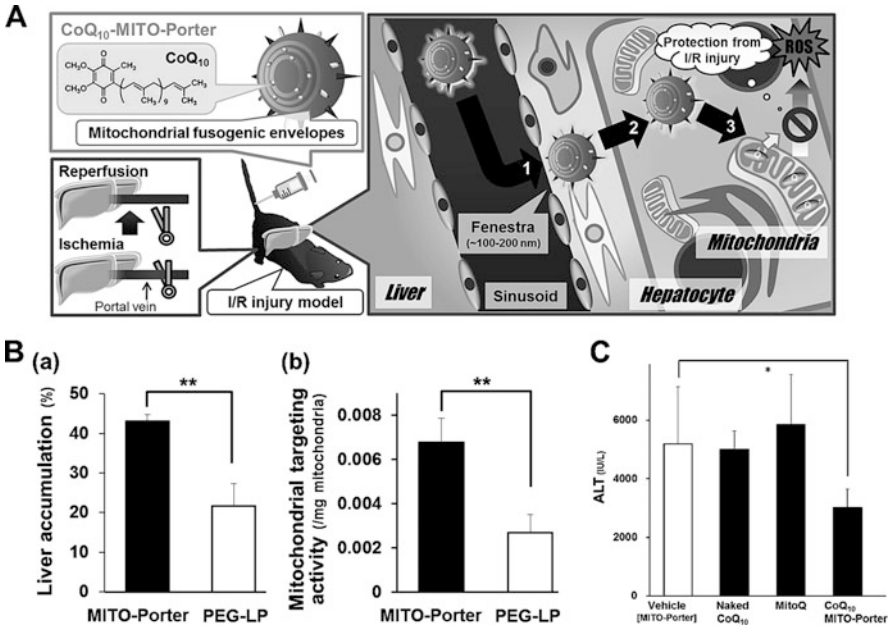

Fig. 3 Schematic image of the prevention of the hepatic I/R injury by the mitochondrial delivery of CoQ_{10} using the MITO-Porter system (**A**). Quantification of mitochondrial accumulation in the liver following the systemic injection of the CoQ_{10}-MITO-Porter (**B**). Quantification of carrier accumulation in the liver (*a*) and liver mitochondria (*b*) by RI. MITO-Porter and PEG-LP labeled with [^{3}H]-CHE were systemically injected into the tail vein of mice. The values for liver accumulation and mitochondrial targeting activity were calculated from the [^{3}H] content in the liver and liver mitochondrial lysate at 1 h after injection. Bars indicate means ± S.D. ($n = 3$–5). **Significant differences ($p < 0.01$) between MITO-Porter and PEG-LP were calculated by an unpaired Student's *t*-test. Evaluation of hepatoprotective effect from an I/R injury (**C**). Mice were administered with samples containing CoQ_{10}. MITO-Porter uncontaining CoQ_{10} was used as a vehicle. Serum ALT activities were measured after hepatic I/R injury. Data are represented as the mean ± S.D. ($n = 4$). *Significant difference between vehicle and others ($p < 0.05$ by one-way ANOVA). These figure are used with permission from Elsevier (Yamada et al. 2015b)

observed (Yamada et al. 2015b). We then quantified the accumulation of radio isotope (RI)-labeled form of the carrier in liver mitochondria after systemic injection. In this experiment, the livers were harvested after systemic injection and homogenized to obtain the mitochondria-enriched fraction, followed by RI detection. As a result, the MITO-Porter accumulated in the liver and the mitochondria much more efficiently than a control carrier, PEG-LP (Yamada et al. 2015b) (Fig. 3B). These results indicate that the MITO-Porter that was validated for mitochondrial delivery in cultured cells was also able to reach the liver via systemic injection, followed by mitochondrial targeting in the liver.

We next attempted to construct a CoQ_{10}-MITO-Porter. We succeeded in packaging the highly hydrophobic CoQ_{10} in a lipid envelope of a MITO-Porter containing PEG-lipids by an EtOH dilution method. Using this method, it is

relatively easy to control the size of the CoQ_{10} containing carriers by simply varying the concentration of EtOH. We then investigated the size effect of the carriers on liver accumulation after systemic administration using fluorescent imaging. As a result, we observed that numerous strong fluorescent signals had accumulated in liver cells in the case of a small sized CoQ_{10}-MITO-Porter. On the other hand, only very weak signals in the liver were observed in the case of the large sized carrier (Yamada et al. 2015b). Thus, we concluded that controlling the size of the CoQ_{10}-MITO-Porter is an important factor in the accumulation of the carriers in the liver after systemic injection. In the following experiments, we used a small sized CoQ_{10}-MITO-Porter.

3.4 Evaluation of Hepatoprotective Effect from I/R Injury

To evaluate the therapeutic effect of the nanoparticles, we administrated the CoQ_{10}-MITO-Porter to mice, followed by inducing a hepatic I/R injury, and then measured serum alanine aminotransferase (ALT) levels. In this experiment, it was expected that the delivery of CoQ_{10} to mitochondria in livers of the I/R injury-induced mice would protect the liver from mitochondria-derived ROS, resulting in a decrease in ALT levels (a marker of a liver injury). As a result, we confirmed that the CoQ_{10}-MITO-Porter resulted in a significant decrease in ALT levels compared with naked CoQ_{10} and other carriers (Yamada et al. 2015b) (Fig. 3C).

In addition, to validate the therapeutic effect resulting from the delivery of CoQ_{10} to mice liver mitochondria by the MITO-Porter via membrane fusion, we prepared control carriers, which were CoQ_{10}-EPC-LP with low binding and fusion activities and CoQ_{10}-R8-EPC-LP with high binding and low fusion activities. As a result, the CoQ_{10}-MITO-Porter with high binding and fusion activities resulted in a significant decrease in serum ALT levels (Yamada et al. 2015b). On the other hand, the control carriers showed no detectable therapeutic effect. Based on these results, we concluded that the mitochondrial membrane fusion activity of the CoQ_{10}-MITO-Porter is an important factor in producing a therapeutic effect.

4 Validation of Mitochondrial Gene Regulations by Mitochondrial Delivery of Functional Cargoes Using MITO-Porter

4.1 Needs for MITO-Porter to Achieve Successful Mitochondrial Gene Regulation

To achieve mitochondrial gene therapy and diagnosis, therapeutic agents need to be delivered into the innermost mitochondrial space, the mitochondrial matrix, which contains the mtDNA pool. To validate that this is possible, we utilized propidium iodide, PI, an impermeable fluorescence-dye for nucleic acids, as a probe to detect mtDNA, and encapsulated it in the MITO-Porter. As a result, we confirmed that the

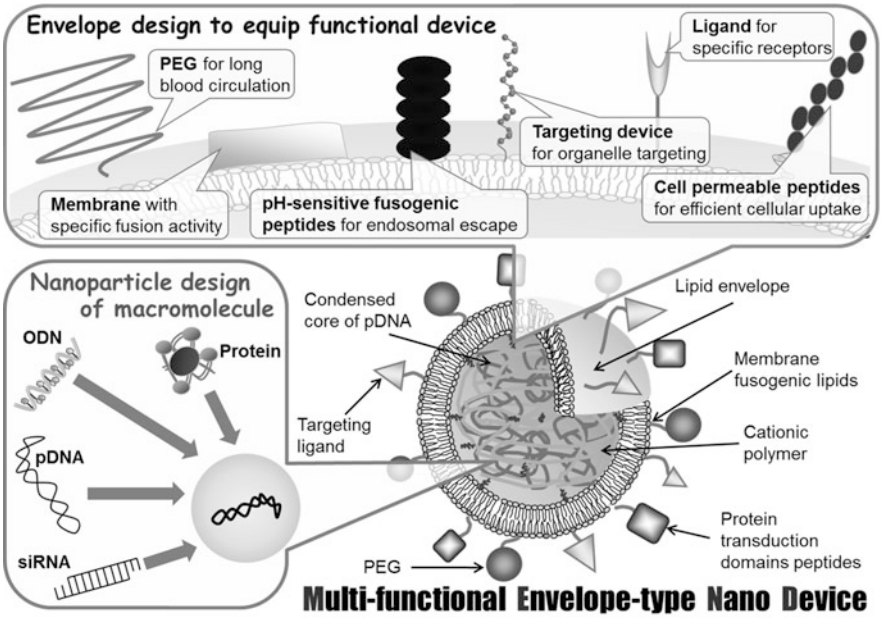

Fig. 4 Schematic representation of a multifunctional envelope-type nano-device (MEND) (Yamada et al. 2012a). This figure is used with permission from Elsevier. The MEND consists of condensed pDNA molecules, coated with a lipid envelope modified with functional devices. Various cargoes can be efficiently encapsulated in MEND

MITO-Porter delivered its cargo to the mitochondrial matrix, as evidenced by the result that PI forms conjugates with mtDNA which emits a fluorescent light (Yasuzaki et al. 2010).

In another point, the efficient packaging of therapeutic macromolecules including proteins and nucleic acids in the carrier is a very important issue. Sufficient amounts of cargoes need to be delivered to mitochondria, in order to effectively control mitochondrial function. Figure 4 shows a multifunctional envelope-type nano-device (MEND), a nonviral vector developed in our laboratory, in which various macromolecules are packaged efficiently (Kajimoto et al. 2014; Yamada et al. 2012a; Kogure et al. 2004). In this case, we packaged therapeutic macromolecules in the MITO-Porter system based on the MEND concept and validated mitochondrial gene therapeutic strategy, including the mitochondrial delivery of proteins and nucleic acids.

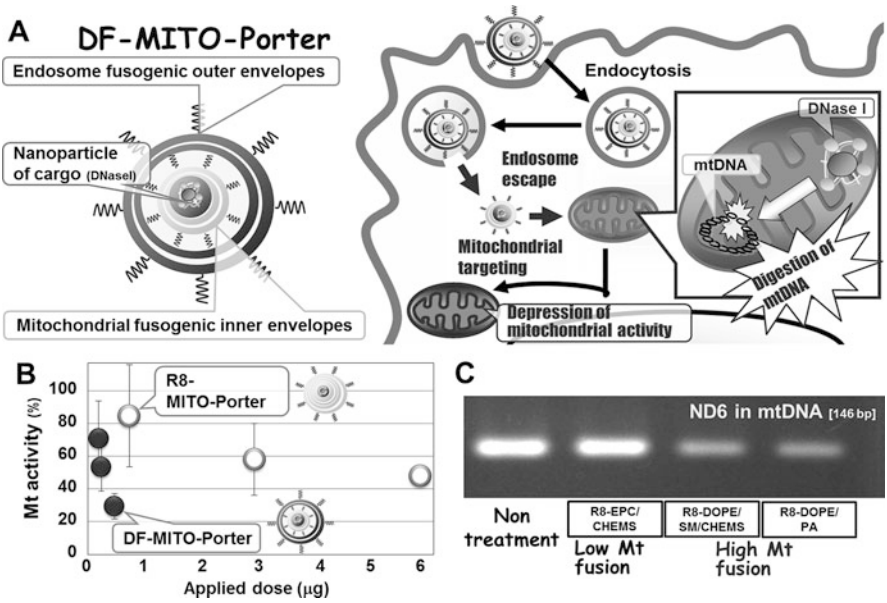

Fig. 5 Summary of experimental results to validate mitochondrial genome targeting by a DF-MITO-Porter. (**a**) Schematic image of mitochondrial genome targeting DF-MITO-Porter encapsulating DNase I protein. (**b**) Comparison of mitochondrial activity after DNase I delivery between the DF-MITO-Porter and a conventional MITO-Porter. The *closed* and *open circles* represent the values corresponding to mitochondrial activity (%), when the DF-MITO-Porter and conventional MITO-Porter were used. Data are represented as the mean ± S.D. ($n = 3$–4). (**c**) Evaluation for the levels of mtDNA after the mitochondrial delivery of DNase I. DNase I proteins encapsulated in the DF-MITO-Porter (lanes 3, 4) or the control carrier with low mitochondrial fusion activity (lane 2) was incubated with HeLa cells. Cellular DNA was collected and then subjected to PCR for the mitochondrial NADH dehydrogenase 6 (ND6) gene (146 bp) derived from mtDNA. These figures are reproduced from reference Yamada and Harashima (2012)

4.2 Validation of Mitochondrial Genome Targeting by a MITO-Porter Encapsulating a Bioactive Molecule, DNase I Protein

To validate mitochondrial genome targeting by the MITO-Porter system, we attempted to deliver the DNase I protein into mitochondria in living cells and evaluated this by measuring the digestion of mtDNA and mitochondrial activity (Fig. 5a). For this validation, we developed a DF-MITO-Porter, which is made up of mitochondria-fusogenic inner and endosome-fusogenic outer envelopes. Intracellular observations showed that the DF-MITO-Porter was more efficient than a conventional MITO-Porter for mitochondrial delivery (Yamada et al. 2011). The construction of the DF-MITO-Porter encapsulating DNase I involves the following three steps: construction of nanoparticles containing the DNase I protein, coating the nanoparticles with a mitochondria-fusogenic envelope, and further coating with an endosome-fusogenic envelope. For this preparation, the particle diameter was around 150 nm, and the zeta potential was around plus 30 mV (Yamada et al. 2011).

We evaluated mitochondrial activity after delivery of the DNase I protein by measuring the activity of mitochondrial dehydrogenase. In this experiment, mitochondrial activity would be inversely proportional to the efficiency of the mitochondrial delivery of DNase I. As a result, the use of the DF-MITO-Porter resulted in a significant decrease in mitochondrial activity compared with control carriers with a low mitochondrial fusion activity (Yamada and Harashima 2012). Moreover, we confirmed that the DF-MITO-Porter was more efficient than the conventional MITO-Porter in mitochondrial delivery (Yamada and Harashima 2012) (Fig. 5b). We also evaluated mtDNA-levels within the cells after the delivery of the DNase I protein using PCR, followed by EtBr staining after separation by electrophoresis. The results indicated that the use of the DF-MITO-Porter resulted in decrease mtDNA-levels, whereas the carriers with low fusion properties had only a negligible effect on mtDNA-levels (Yamada et al. 2011; Yamada and Harashima 2012) (Fig. 5c). These results show that a DF-MITO-Porter achieved mitochondrial genome targeting of bioactive molecules in living cells.

4.3 Mitochondrial Delivery of Nucleic Acids and Gene Silencing

In the area of research regarding mitochondrial gene expression regulation, we attempted mitochondrial gene silencing by the mitochondrial delivery of nucleic acids. In this experiment, we performed the mitochondrial delivery of antisense RNA targeted to endogenous mitochondrial mRNA using the MITO-Porter. We chose an mRNA that codes for COX2 as a target. COX2 is one of the endogenous mitochondrial proteins related to the maintenance of mitochondrial membrane potential. If the knockdown of the COX2 mRNA was successful, the expression levels of the target mitochondrial protein would be decreased, followed by a decrease in mitochondrial membrane potential.

Figure 6A provides information on the quantification of mRNA levels coding COX2 after transfection by the MITO-Porter. As a result, the mRNA levels of COX2 were decreased as the result of the mitochondrial delivery of antisense RNA (Furukawa et al. 2015) (Fig. 6A). We also quantified the expression levels of the target protein using immunostaining and confirmed that the use of antisense RNA resulted in a significant decrease in the level of expression of the target protein (Furukawa et al. 2015) (Fig. 6B).

We then evaluated mitochondrial membrane potential using JC-1. JC-1 emits a red fluorescence in mitochondria when the mitochondria have a high membrane potential, while the fluorescence changes to green in the cytosol when the potential is low. As a result, in the case of nontreated and mock-treated cells, red fluorescent mitochondria were observed. On the other hand, we observed a green color in the cytosol as result of the mitochondrial delivery of antisense RNA (Fig. 6C) (please refer to reference (Furukawa et al. 2015) for an interpretation of the color in this figure). These results indicate that MITO-Porter system is able to regulate mitochondrial gene expression and mitochondrial functions.

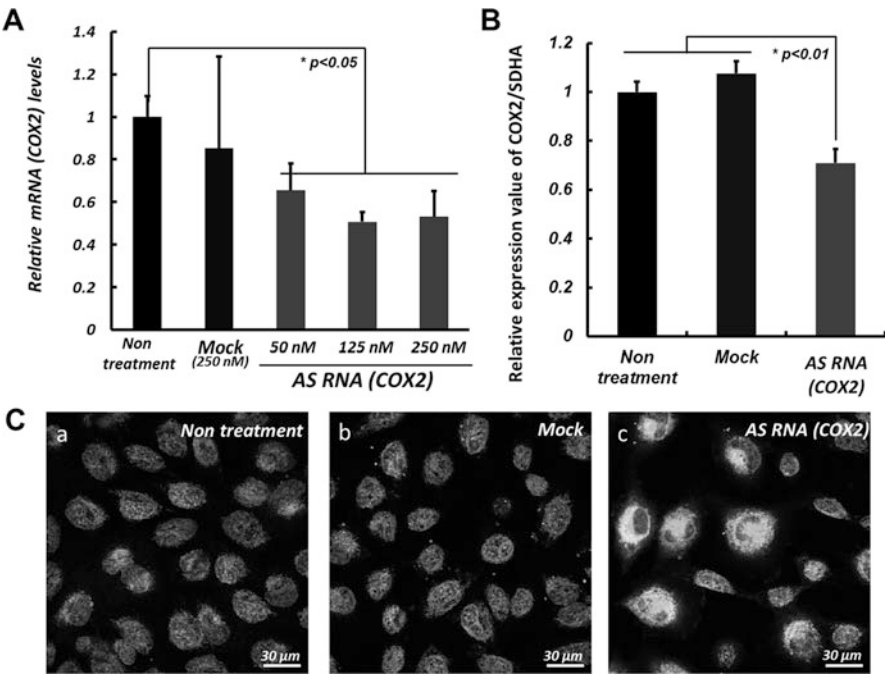

Fig. 6 Summary of experimental results for regulating mitochondrial gene expression levels using MITO-Porter system. (**A**) Quantification of target mRNA levels was performed by qRT-PCR. Bars indicate the mean with SEM ($n = 3$–4). *Significant differences were calculated by one-way ANOVA ($p < 0.05$). COX, cytochrome c oxidase subunit II. (**B**) Evaluation of relative COX2/SDHA protein expression levels in mitochondrial protein knockdown using immunostaining. Bars are the mean with SEM ($n = 64$). *Significant differences were calculated by one-way ANOVA ($p < 0.01$). (**C**) Visualization of mitochondrial membrane potential using JC-1. After 48 h transfection of MITO-Porter system (nontreatment (*a*), Mock (*b*), AS RNA (COX2) (*c*)), HeLa cells were incubated with JC-1 and observed by CLSM. These figures are reproduced from reference Furukawa et al. (2015). For interpretation to color in this figure, the reader is referred to the article (Furukawa et al. 2015)

5 Perspective: DDS and Mitochondria

In this chapter, we focused on our current efforts regarding mitochondrial delivery including the development of a MITO-Porter for mitochondrial delivery via membrane fusion and validation that mitochondrial function can be regulated by the MITO-Porter system. We currently conduct trials to establish a viable mitochondrial therapeutic strategy. Our ultimate goal is to carry out successful mitochondrial therapy using a mitochondrial DDS. To reach this ultimate goal, we believe that mitochondrial DDS requires collaboration with experts in various fields of research (e.g., genetics, molecular biology, pathology, and so on).

To date, we have collaborated with numerous researchers in various fields and clinicians. Such a collaboration provides us with many fruitful interactions that would be impossible for a single research group to achieve. Therefore, we established a private delivery system of mitochondrial DDS to accelerate collaborations all over the world. It is our hope that these collaborations will open new research areas in mitochondrial DDS and will have a significant impact on the medical and life sciences.

Acknowledgments This work was supported, in part, by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO), Japan, and by a Grant-in-Aid for Young Scientists (A) and Scientific Research (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japanese Government (MEXT), the Mochida Memorial Foundation for Medical and Pharmaceutical Research, and the Uehara Memorial Foundation. We also thank Dr. Milton Feather for his helpful advice in writing the manuscript.

References

Bangham AD, Horne RW (1964) Negative staining of phospholipids and their structural modification by surface-active agents as observed in the electron microscope. J Mol Biol 8:660–668

Biswas S, Torchilin VP (2014) Nanopreparations for organelle-specific delivery in cancer. Adv Drug Deliv Rev 66:26–41

Furukawa R, Yamada Y, Takenaga M, Igarashi R, Harashima H (2011) Octaarginine-modified liposomes enhance the anti-oxidant effect of Lecithinized superoxide dismutase by increasing its cellular uptake. Biochem Biophys Res Commun 404(3):796–801

Furukawa R, Yamada Y, Kawamura E, Harashima H (2015) Mitochondrial delivery of antisense RNA by MITO-Porter results in mitochondrial RNA knockdown, and has a functional impact on mitochondria. Biomaterials 57:107–115

Futaki S, Suzuki T, Ohashi W, Yagami T, Tanaka S, Ueda K, Sugiura Y (2001) Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. J Biol Chem 276(8):5836–5840

Goto Y, Nonaka I, Horai S (1990) A mutation in the tRNA(Leu)(UUR) gene associated with the MELAS subgroup of mitochondrial encephalomyopathies. Nature 348(6302):651–653

Igarashi R, Hoshino J, Takenaga M, Kawai S, Morizawa Y, Yasuda A, Otani M, Mizushima Y (1992) Lecithinization of superoxide dismutase potentiates its protective effect against Forssman antiserum-induced elevation in guinea pig airway resistance. J Pharmacol Exp Ther 262(3):1214–1219

Kajimoto K, Sato Y, Nakamura T, Yamada Y, Harashima H (2014) Multifunctional envelope-type nano device for controlled intracellular trafficking and selective targeting in vivo. J Control Release 190C:593–606

Kawamura E, Yamada Y, Harashima H (2013a) Mitochondrial targeting functional peptides as potential devices for the mitochondrial delivery of a DF-MITO-Porter. Mitochondrion 13 (6):610–614

Kawamura E, Yamada Y, Yasuzaki Y, Hyodo M, Harashima H (2013b) Intracellular observation of nanocarriers modified with a mitochondrial targeting signal peptide. J Biosci Bioeng 116 (5):634–637

Khalil IA, Kogure K, Futaki S, Harashima H (2006) High density of octaarginine stimulates macropinocytosis leading to efficient intracellular trafficking for gene expression. J Biol Chem 281(6):3544–3551

Khalil IA, Hayashi Y, Mizuno R, Harashima H (2011) Octaarginine- and pH sensitive fusogenic peptide-modified nanoparticles for liver gene delivery. J Control Release 156(3):374–380

Kogure K, Moriguchi R, Sasaki K, Ueno M, Futaki S, Harashima H (2004) Development of a non-viral multifunctional envelope-type nano device by a novel lipid film hydration method. J Control Release 98(2):317–323

Oku N, Doi K, Namba Y, Okada S (1994) Therapeutic effect of adriamycin encapsulated in long-circulating liposomes on Meth-A-sarcoma-bearing mice. Int J Cancer 58(3):415–419

Sato Y, Nakamura T, Yamada Y, Akita H, Harashima H (2014) Multifunctional enveloped nanodevices (MENDs). Adv Genet 88:139–204

Schapira AH (2006) Mitochondrial disease. Lancet 368(9529):70–82

Shanske S, Moraes CT, Lombes A, Miranda AF, Bonilla E, Lewis P, Whelan MA, Ellsworth CA, DiMauro S (1990) Widespread tissue distribution of mitochondrial DNA deletions in Kearns-Sayre syndrome. Neurology 40(1):24–28

Shoffner JM, Lott MT, Lezza AM, Seibel P, Ballinger SW, Wallace DC (1990) Myoclonic epilepsy and ragged-red fiber disease (MERRF) is associated with a mitochondrial DNA tRNA(Lys) mutation. Cell 61(6):931–937

Tawaraya Y, Hyodo M, Ara MN, Yamada Y, Harashima H (2014) RNA aptamers for targeting mitochondria using a mitochondria-based SELEX method. Biol Pharm Bull 37(8):1411–1415

Tuppen HA, Blakely EL, Turnbull DM, Taylor RW (2010) Mitochondrial DNA mutations and human disease. Biochim Biophys Acta 1797(2):113–128

Wallace DC (1999) Mitochondrial diseases in man and mouse. Science 283(5407):1482–1488

Weissig V (2011) From serendipity to mitochondria-targeted nanocarriers. Pharm Res 28 (11):2657–2668

Yamada Y, Harashima H (2008) Mitochondrial drug delivery systems for macromolecule and their therapeutic application to mitochondrial diseases. Adv Drug Deliv Rev 60(13–14):1439–1462

Yamada Y, Harashima H (2012) Delivery of bioactive molecules to the mitochondrial genome using a membrane-fusing, liposome-based carrier, DF-MITO-Porter. Biomaterials 33 (5):1589–1595

Yamada Y, Harashima H (2013) Enhancement in selective mitochondrial association by direct modification of a mitochondrial targeting signal peptide on a liposomal based nanocarrier. Mitochondrion 13(5):526–532

Yamada Y, Harashima H (2014) A method for screening mitochondrial fusogenic envelopes for use in mitochondrial drug delivery. Methods Mol Biol 1141:57–66

Yamada Y, Harashima H (2015) Targeting the mitochondrial genome via a dual function MITO-Porter: evaluation of mtDNA levels and mitochondrial function. Methods Mol Biol 1265:123–133

Yamada Y, Akita H, Kamiya H, Kogure K, Yamamoto T, Shinohara Y, Yamashita K, Kobayashi H, Kikuchi H, Harashima H (2008) MITO-Porter: a liposome-based carrier system for delivery of macromolecules into mitochondria via membrane fusion. Biochim Biophys Acta 1778(2):423–432

Yamada Y, Furukawa R, Yasuzaki Y, Harashima H (2011) Dual function MITO-Porter, a nano carrier integrating both efficient cytoplasmic delivery and mitochondrial macromolecule delivery. Mol Ther 19(8):1449–1456

Yamada Y, Akita H, Harashima H (2012a) Multifunctional envelope-type nano device (MEND) for organelle targeting via a stepwise membrane fusion process. Methods Enzymol 509:301–326

Yamada Y, Kawamura E, Harashima H (2012b) Mitochondrial-targeted DNA delivery using a DF-MITO-Porter, an innovative nano carrier with cytoplasmic and mitochondrial fusogenic envelopes. J Nanopart Res 14(8):1013–1027

Yamada Y, Nakamura K, Furukawa R, Kawamura E, Moriwaki T, Matsumoto K, Okuda K, Shindo M, Harashima H (2013a) Mitochondrial delivery of bongkrekic acid using a MITO-porter prevents the induction of apoptosis in human HeLa cells. J Pharm Sci 102(3):1008–1015

Yamada Y, Hashida M, Hayashi Y, Tabata M, Hyodo M, Ara MN, Ohga N, Hida K, Harashima H (2013b) An approach to transgene expression in liver endothelial cells using a liposome-based gene vector coated with hyaluronic acid. J Pharm Sci 102(9):3119–3127

Yamada Y, Tabata M, Yasuzaki Y, Nomura M, Shibata A, Ibayashi Y, Taniguchi Y, Sasaki S, Harashima H (2014) A nanocarrier system for the delivery of nucleic acids targeted to a pancreatic beta cell line. Biomaterials 35(24):6430–6438

Yamada Y, Hashida M, Harashima H (2015a) Hyaluronic acid controls the uptake pathway and intracellular trafficking of an octaarginine-modified gene vector in CD44 positive- and CD44 negative-cells. Biomaterials 52:189–198

Yamada Y, Nakamura K, Abe J, Hyodo M, Haga S, Ozaki M, Harashima H (2015b) Mitochondrial delivery of Coenzyme Qvia systemic administration using a MITO-Porter prevents ischemia/reperfusion injury in the mouse liver. J Control Release 213:86–95

Yamada Y, Fukuda Y, Harashima H (in press) An analysis of membrane fusion between mitochondrial double membranes and MITO-Porter, mitochondrial fusogenic vesicles. Mitochondrion

Yasuzaki Y, Yamada Y, Harashima H (2010) Mitochondrial matrix delivery using MITO-Porter, a liposome-based carrier that specifies fusion with mitochondrial membranes. Biochem Biophys Res Commun 397(2):181–186

Zhang E, Zhang C, Su Y, Cheng T, Shi C (2011) Newly developed strategies for multifunctional mitochondria-targeted agents in cancer therapy. Drug Discov Today 16(3–4):140–146

Toxicity of Antiepileptic Drugs to Mitochondria

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Contents

The author has nothing to declare.

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H. Singh, S.-S. Sheu (eds.), *Pharmacology of Mitochondria*,
Handbook of Experimental Pharmacology 240, DOI 10.1007/164_2016_2

Abstract

Some of the side and beneficial effects of antiepileptic drugs (AEDs) are mediated via the influence on mitochondria. This is of particular importance in patients requiring AED treatment for mitochondrial epilepsy. AED treatment in patients with mitochondrial disorders should rely on the known influences of AEDs on these organelles. AEDs may influence various mitochondrial functions or structures in a beneficial or detrimental way. There are AEDs in which the toxic effect outweighs the beneficial effect, such as valproic acid (VPA), carbamazepine (CBZ), phenytoin (PHT), or phenobarbital (PB). There are, however, also AEDs in which the beneficial effect on mitochondria outweighs the mitochondrion-toxic effect, such as gabapentin (GBT), lamotrigine (LTG), levetiracetam (LEV), or zonisamide (ZNS). In the majority of the AEDs, however, information about their influence of mitochondria is lacking. In clinical practice mitochondrial epilepsy should be initially treated with AEDs with low mitochondrion-toxic potential. Only in cases of ineffectivity or severe mitochondrial epilepsy, mitochondrion-toxic AEDs should be given. This applies for AEDs given orally or intravenously.

Keywords

Antiepileptic drugs • Apoptosis • Epilepsy • Membrane potential • Mitochondrion • Oxidative stress • Reactive oxygen species • Respiratory chain • Side effects • Toxicity

1 Introduction

All prescribed drugs in medicine have side effects, depending on the dosage, toxicity, and individual tolerability (Ray 2014). This is also the case for antiepileptic drugs (AEDs) (Finsterer and Zarrouk-Mahjoub 2012). Some of these side effects result from toxicity of AEDs to mitochondria. Various AEDs have been reported to influence function, biogenesis, kinetics, and survival of mitochondria. Toxicity of AEDs to mitochondria has particular implications for the treatment of epilepsy in mitochondrial disorders. The following chapter deals with the mitochondrion toxicity of AEDs and its consequences for the treatment of epilepsy in patients with mitochondrial disorders (mitochondrial epilepsy).

2 Effects of AEDs on Mitochondria

Generally, AEDs may have a beneficial effect, may induce adverse reactions, or may have no effect on mitochondria (Table 1). The effect of AEDs on mitochondria may be evident on the molecular level, on the clinical level, or on both. On the clinical level, the beneficial or adverse effects of an AED are particularly seen in patients with a mitochondrial disorder. In some of the AEDs, predominantly adverse reactions have been reported, in some predominantly beneficial reactions,

Table 1 Adverse and beneficial effects of AEDs on mitochondria

AED		Adverse effects	Beneficial effects
VPA	Valproic acid	+++	+
PHT	Phenytoin (diphenylhydantoin)	+++	+
CBZ	Carbamazepine	++	+
PB	Phenobarbital	++	+
ZNS	Zonisamide	+	+++
LTG	Lamotrigine	+	++
LEV	Levetiracetam	+	++
GBP	Gabapentin	+	++
TPM	Topiramate	+	+
ESM	Ethosuximide	+	+
OXC	Oxcarbazepine	++	–
FBM	Felbamate	+	–
e.g. NZP	Benzodiazepines	–	+
PRM	Primidone	–	+
TGB	Tiagabine	–	+
VGB	Vigabatrin	–	+
PGB	Pregabalin	–	+
LAC	Lacosamide	–	–
RFM	Rufinamide	–	–
PER	Perampanel	–	–

but in most both (Table 1). In the following section, the effects of the various AEDs on mitochondria are discussed in detail.

2.1 Valproic Acid

2.1.1 Adverse Effects

Molecular Level

In a study of the effect of antidepressants on the respiratory chain in mitochondria from pig neurons, valproic acid (VPA) inhibited complex I and complex IV of the respiratory chain (Hroudova and Fisar 2010). In rats fed with VPA during 75 days, a 30% decrease of the respiration rate in liver mitochondria was observed (Ponchaut et al. 1992). In this study VPA induced loss of cytochrome-c oxidase, which correlated with the simultaneous reduction of cytochrome aa3 (Ponchaut et al. 1992). In a HepG2 in vitro model, VPA decreased O_2 rates and $\Delta\psi$, depleted ATP levels, increased reactive oxygen species (ROS), decreased superoxide dismutase (SOD), and increased cell death (Komulainen et al. 2015). In hepatocyte-like cells differentiated from pluripotent stem cells deriving from fibroblasts from Alpers-Huttenlocher disease (AHD) patients, VPA induced mitochondrion-dependent apoptosis due to activated caspase-9 and cytochrome-c release from the intermembrane space (Li et al. 2015). Additionally, soluble and

oligomeric OPA1, which keeps cristae junctions tight, was reduced in these cells (Li et al. 2015). In this study VPA also reduced the expression of POLG1, the mtDNA content, and the ATP production and caused abnormal mitochondrial ultrastructure, SOD flashes, and frequent spontaneous bursts of SOD generation (Li et al. 2015). VPA toxicity to these cells can be prevented by cyclosporine A, which inhibits the opening of the mitochondrial permeability pore (Li et al. 2015). In a study of Wistar rats, VPA induced apoptosis, mitochondrial swelling, and cristolysis of mitochondria in uterine stroma cells (Cansu et al. 2010). In this study apoptosis was also increased in ovaries (Cansu et al. 2010). In another in vitro study, VPA stabilised a specific acetyl modification of the p53 tumour suppressor protein, resulting in increased pro-apoptotic activity of the mitochondrial membrane (Chen et al. 2011). VPA also affects the morphology of mitochondria. In a study of rat liver and kidney mitochondria, conformational changes of respiratory chain proteins and structural abnormalities of the inner mitochondrial membrane were found (Rumbach et al. 1986).

Clinical Level

On the clinical level, mitochondrial toxicity of VPA has been documented particularly in mitochondrial disorder patients. In a newborn with AHD, VPA treatment was associated with severe liver failure and cortical blindness (Castro-Cago et al. 1999). Development of liver failure 3 months after administration of VPA was also observed in a 3-year-old girl with complex IV deficiency with fatal outcome (Chabrol et al. 1994). Fatal liver failure was additionally described in a 30-year-old female with chronic progressive external ophthalmoplegia (CPEO) who received VPA for status epilepticus and died 4 months after starting VPA (Krähenbühl et al. 2000). In a patient with mitochondrial encephalopathy lactic acidosis and stroke-like episodes (MELAS) syndrome, VPA aggravated epilepsy, which did not resolve before the discontinuation of VPA (Hsu et al. 2012). VPA also aggravated epilepsia partialis continua in a male with MELAS syndrome (Lin and Thajeb 2007). In a single patient with MELAS syndrome due to the m.3243A > G mutation, VPA induced convulsions (Lam et al. 1997). In a boy with AHD, administration of VPA was followed by development of epilepsia partialis continua, hepatic failure, and death (Rasmussen et al. 2000). VPA caused hepatotoxicity in several other patients with AHD (Bicknese et al. 1992; Kayihan et al. 2000; Pronicka et al. 2011; Saneto et al. 2010; Schaller et al. 2011; Schwabe et al. 1997; Simonati et al. 2003; Uusimaa et al. 2008; Wolf et al. 2009). VPA-induced liver failure was also reported in patients with mitochondrial recessive ataxia syndrome (MIRAS) (Hakonen et al. 2010). The reason why the liver is the predominant target of VPA toxicity in mitochondrial disorders remains speculative.

2.1.2 Beneficial Effects

VPA may not only exhibit adverse reactions in mitochondrial disorder and non-mitochondrial disorder patients but may also produce some beneficial effects (Barnerias et al. 2010). In a study of SH-SY5Y cells, VPA together with lithium

exhibited a protective effect against oxidative stress-induced cell death (Lai et al. 2006). In another study VPA together with lithium protected against methamphetamine-induced reduction of cytochrome-c activity and of the mitochondrial anti-apoptotic Bcl-2/Bax ratio and enhanced mitochondrial functions (Bachmann et al. 2009). In this study long-term application of VPA to SH-SY5Y cells increased the cell respiration rate and $\Delta\psi$ (Bachmann et al. 2009). In the rotenone rat model of Parkinson's disease, VPA exhibited a neuroprotective effect by inhibiting histone deacetylases (Monti et al. 2010). In a study of 3-nitropropionic acid-induced convulsions in mice, VPA reversed the mitochondrion-toxic effect of this agent, which inhibits complex II of the respiratory chain (Urbanska et al. 1998). In HepG2 cells, VPA increased the release of interferon-γ and TNF-α, an effect which increased with exposure time (Neuman et al. 2013). In fibroblasts of patients carrying POLG1 mutations, VPA increased the expression of POLG1 and of regulators of the mitochondrial biogenesis (Sitarz et al. 2014). This effect was more pronounced in mutated cells as compared to normal cells (Sitarz et al. 2014).

2.2 Phenytoin

2.2.1 Adverse Effects

Molecular Level

In rat hepatocytes, PHT, an aromatic AED and heterocyclic amide, elevated ROS formation, reduced intracellular reduced glutathione, increased intracellular oxidised glutathione, and enhanced lipid peroxidation and mitochondrial damage (Eghbal et al. 2014). In a murine hepatic microsomal system, PHT decreased state-3 respiration, ATP synthesis, and $\Delta\psi$, increased state-4 respiration, impaired Ca++− uptake and release, and inhibited Ca++− induced swelling (Santos et al. 2008a). Mitochondrial dysfunction was higher as compared to phenobarbital (PB) and carbamazepine (CBZ) (Santos et al. 2008a). The hepatotoxic effect of PHT was mediated by oxidative stress induced by PHT metabolites (Santos et al. 2008b).

Clinical Level

One month after intravenous PHT for treating status epilepticus in a 16-year-old female with MELAS syndrome due to the m.3243A > G mutation, the patient developed intestinal pseudo-obstruction. Pseudo-obstruction resolved not earlier than after discontinuation of PHT (Chiyonobu et al. 2008). Decreased cerebrospinal fluid (CSF) folate levels were found in a patient with Kearns–Sayre syndrome after administration of PHT (Allen et al. 1983). Folate deficiency resolved after replacing PHT by another AED (Allen et al. 1983).

2.2.2 Beneficial Effect

Intraperitoneal injection of PHT in an animal model of epilepsy resulted in enhanced SOD activity, decreased cerebral malondialdehyde, a biomarker of

oxidative stress, and decreased monoamine oxidase A + B activity (Bogdanov et al. 2009).

2.3 Carbamazepine

2.3.1 Adverse Effects

In a murine hepatic microsomal system, CBZ, an aromatic AED, decreased state-3 respiration, ATP synthesis, and $\Delta\psi$, increased state-4 respiration, impaired Ca++− uptake and release, and inhibited Ca++− induced swelling of mitochondria (Santos et al. 2008a). The potential to disrupt mitochondrial functions was lower than with PHT and PB (Santos et al. 2008a). The hepatotoxic effect of CBZ was mediated by oxidative stress induced by metabolites of these drugs (Santos et al. 2008b). In a study of five children with a mitochondrial disorder, CBZ reduced the ATP production (Berger et al. 2010).

2.3.2 Beneficial Effects

In animal studies CBZ has been shown to protect against rotenone-induced striatal neuronal dysfunction (Costa et al. 2008). Rotenone selectively inhibits complex I of the respiratory chain (Costa et al. 2010). The beneficial effect occurred only in the presence of γ-aminobutyric acid (GABA) (Costa et al. 2008). Rotenone can also prevent the back flow of electrons from complex II/III to complex I.

2.4 Phenobarbital

2.4.1 Adverse Effects

PB decreased state-3 respiration, ATP synthesis, and $\Delta\psi$, increased state-4-respiration, impaired Ca++− uptake or release, and inhibited Ca++− induced swelling in a murine hepatic microsomal system (Santos et al. 2008a). The potential to induce mitochondrial dysfunction was higher compared to CBZ but lower compared to PHT (Santos et al. 2008a). The hepatotoxic effect of PB was mediated by oxidative stress induced by metabolites of these drugs (Santos et al. 2008b).

2.4.2 Beneficial Effects

In a study of 3-nitropropionic acid-induced convulsions in mice, PB reversed the mitochondrion-toxic effect of this agent (Urbanska et al. 1998). In a study of five children with a mitochondrial disorder, PB, an aromatic AED, had a mild stimulating effect on ATP production (Berger et al. 2010).

2.5 Zonisamide

2.5.1 Adverse Effects

In an in vitro study using the dansyl amide competition binding assay, ZNS inhibited the mitochondrial carbonic anhydrase (De Simone et al. 2005).

2.5.2 Beneficial Effects

In a study of 15 patients with Parkinson's disease, ZNS exhibited a protective effect on mitochondria (Grover et al. 2013). ZNS has been also shown to exhibit an antioxidative effect (Condello et al. 2013). In a study of human neuroblastoma SH-SY5Y cells differentiated towards a neuronal phenotype, ZNS significantly reduced the intracellular ROS production (Condello et al. 2013). In addition, ZNS restored $\Delta\psi$ (Condello et al. 2013). In this study ZNS exhibited an anti-apoptotic effect, as demonstrated by flow cytometric analysis, and attenuated rotenone-increased caspase-3-activity (Condello et al. 2013). ZNS counteracted apoptotic signalling mechanisms mainly by its antioxidative effects. In a study of astroglial C6 cells, ZNS increased glutathione levels in astroglial C6 cells, thus enhancing the antioxidative capacity (Asanuma et al. 2010). Glutathione levels were also increased by ZNS-induced enhancement of the astroglial cysteine transport system or astroglial proliferation via production and secretion of S100b (Asanuma et al. 2010). Additionally, ZNS reduced rotenone-induced mitochondrial toxicity against complex I (Costa et al. 2010). ZNS is the AED of which its beneficial effects on mitochondria strongly outweigh its adverse effects.

2.6 Lamotrigine

2.6.1 Adverse Effects

In mitochondrial disorder patients, LTG may not only have an effect on the central nervous system but also on the myocardium. In a 67-year-old male with a non-syndromic mitochondrial disorder manifesting with malignant hyperthermia susceptibility, epilepsy, and hypertrophic cardiomyopathy, LTG was made responsible for subaortic valve stenosis, which resolved immediately upon switching from LTG to LEV (Stöllberger et al. 2011). In a patient with late-onset very long-chain acyl-CoA dehydrogenase deficiency (VLCAD) with complex partial seizures, administration of LTG resulted in deterioration of epilepsy (Shchelochkov et al. 2009).

2.6.2 Beneficial Effects

In 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mice, the mouse model of Parkinson's disease, LTG maintained the dopamine transporter density, mitigated apoptosis of dopaminergic neurons in the substantia nigra, and promoted sprouting of striatal dendrites (Lagrue et al. 2007). In a study of five children with a mitochondrial disorder, LTG increased ATP production from pyruvate, maleate, and succinate (Berger et al. 2010). In this study LTG also decreased the cortical and

amygdaloid choline acetyltransferase activity, thus reducing malonate-induced destruction of basal forebrain cholinergic neurons (Connop et al. 1997). In a study of rats exposed to 3-nitropropionic acid, which reduces oxidative defence and impairs function of complexes I, II, and IV of the respiratory chain, administration of LTG restored mitochondrial oxidative defence mechanisms and respiratory chain complex activities (Kumar et al. 2012).

2.7 Levetiracetam

2.7.1 Adverse Effects

In an animal model of prolonged status epilepticus, LEV did not prevent mitochondrial dysfunction and neuronal death (Gibbs and Cock 2007).

2.7.2 Beneficial Effects

In male Wistar rats, LEV has been shown to exhibit a neuroprotective effect by modifying the expression of xCT and iNOS in connection with lipid peroxidation, by enhancing the increased basal endogenous antioxidant ability in the hippocampus and by decreasing the basal concentration of glutamate (Ueda et al. 2009). In C17.2 neuronal cells, LEV increased $\Delta\psi$ in the presence of nerve growth factor (Rogers et al. 2014). In a mitochondrial disorder patient carrying the m.13042G > A mutation, LEV exhibited a long-term beneficial effect on myoclonus (Schinwelski et al. 2015). In a patient with POLG-related mitochondrial epilepsy, LEV together with low glycemic index treatment (a variant of the ketogenic diet) had a long-term beneficial effect on the seizure frequency (Martikainen et al. 2012).

2.8 Gabapentin

Whether GBT inhibits the mitochondrial branched-chain aminotransferase, which reversibly catalyses transamination of the essential branched-chain amino acids leucine, isoleucine, and valin, remains a matter of debate (Goto et al. 2005; Hutson et al. 2001). In a study of rats exposed to 3-nitropropionic acid, which reduces oxidative defence and impairs function of complexes I, II, and IV of the respiratory chain, administration of GBT restored mitochondrial oxidative defence mechanisms and respiratory chain complex activities (Kumar et al. 2012). In a transgenic mouse model, GBT-lactam has been shown to exhibit a neuroprotective effect by opening mitochondrial ATP-dependent K-channels (Zucker et al. 2004). GBT has been shown to inhibit mitochondrial forms of the brain branched-chain aminotransferase in rats resulting in reduced synthesis of glutamate from branched-chain amino acids (Goldlust et al. 1995).

2.9 Oxcarbazepine

Exposure of rat hippocampal neurons towards OXC increased the production of ROS, decreased ATP production, and decreased $\Delta\psi$ (Araújo et al. 2004). OXC caused swelling and degeneration of hippocampal neurons and was more toxic than CBZ on the molecular level (Araújo et al. 2004). In a study of Wistar rats, OXC induced apoptosis in ovaries (Cansu et al. 2010).

2.10 Topiramate

TPM exhibits adverse and beneficial effects on mitochondria. Whether TPM is a strong or weak inhibitor of the mitochondrial carbonic anhydrase is a matter of debate (De Simone et al. 2005; Dodgson et al. 2000). In the pilocarpine rat model, TPM exhibited a neuroprotective effect being attributable to inhibition of the mitochondrial transition pore (Kudin et al. 2004). In the pilocarpine model, temporal epilepsy is induced by administration of pilocarpine to rats.

2.11 Ethosuximide

In a study of the Mg-ATPase and Na,K-ATPase in subcellular fractions of Sprague–Dawley rat cerebral cortices, ESM, a heterocyclic amide, reduced the activity of the mitochondrial Na,K-ATPase (Gilbert et al. 1974). Intraperitoneal injection of ESM in an animal model of epilepsy resulted in enhanced SOD activity, decrease of cerebral malondialdehyde content, and decrease of monoamine oxidase A + B (Bogdanov et al. 2009). In a patient with MELAS syndrome due to the m.3243A > G mutation, ESM was effective against slow-spike wave activity, consistent with Lennox–Gastaut syndrome and electroencephalographic status epilepticus disregarding the adverse effects reported above (Demarest et al. 2014).

2.12 Felbamate

Little information about the effect of FBM on mitochondria is available from the literature, and FBM has been only rarely given to patients with a mitochondrial disorder. In a review about the interactions of AEDs, FBM was reported to inhibit the mitochondrial β-oxidation in addition to inhibition of some specific isoforms of cytochrome-P (CYP) (Riva et al. 1996).

2.13 Benzodiazepines

Benzodiazepines used as antiepileptic drugs include diazepam, lorazepam, clobazam, clorazepate, and clonazepam. In a study of 3-nitropropionic acid-

induced convulsions in mice, diazepam reversed the mitochondrion-toxic effect of this agent (Urbanska et al. 1998). In single patients with MERRF syndrome, clonazepam has been shown beneficial for myoclonus (Iwanga et al. 1992).

2.14 Primidone

Only few data about the effect of PRM on mitochondria are available. In an animal model of epilepsy, intraperitoneal injection of PRM, a heterocyclic amide, resulted in enhanced SOD activity, decrease of cerebral malondialdehyde content, and decrease of monoamine oxidase A + B (Bogdanov et al. 2009).

2.15 Tiagabine

Only few data about the effect of TGB on mitochondria are available. In an animal model of Huntington's disease, TGB reversed 3-nitropropionic acid-induced increase of lipid peroxidation, nitrate levels, and total RNA levels and reduction of glutathione, adrenal ascorbic acid levels, and succinate dehydrogenase levels (Dhir et al. 2008). In cell cultures of rat cortical astrocytes, TGB exhibited a protective effect against oxidative stress (Cardile et al. 2000).

2.16 Vigabatrin

In normal mice VGB, an inhibitor of the γ-aminobutyric acid transaminase, increases the number and area of mitochondria (Vogel et al. 2015). VGB has been shown to have a beneficial effect on infantile spasms and hypsarrhythmia in infants carrying the m.8783T > G mutation (Desguerre et al. 2003). Administration of VGB to livers of rat fetuses resulted in increased protein and lipid peroxidation in these animals (Cengiz et al. 2005).

2.17 Pregabalin

In cell cultures and rats, PGB has been shown to antagonise the neurotoxic effects of copper (Marmolino and Manto 2010). In vitro copper decreases survival of neurons, enhances the production of nitric oxide (NO), and activates the transcription of NO synthase and of PGC1a, a key regulator of mitochondrial biogenesis (Marmolino and Manto 2010). In vivo copper impairs NMDA-mediated regulation of glutamate, increases the production of NO, and enhances motor cortex excitability (Marmolino and Manto 2010).

2.18 Antiepileptic Drugs

AEDs which have not been reported so far as mitochondrion toxic, respectively, as having a beneficial effect on mitochondria include lacosamide, rufinamide, and perampanel. Whether the absence of reports describing effects of these AEDs on mitochondria is due to missing investigations of these effects or has to be interpreted as an indicator of drug safety remains speculative.

3 Clinical Implications

The effect of AEDs on mitochondria, irrespective if it is beneficial or adverse, has a number of clinical implications. First, AEDs with exclusive or predominant mitochondrial toxicity (e.g. VPA, PHT, CBZ, PB) should, if possible, not be used as first-line agents for the treatment of mitochondrial epilepsy. AEDs with a potentially beneficial therapeutic effect on mitochondria (e.g. ZNS, LTG, LEV, GBT) should be preferred. This applies with restrictions since there are a number of reports showing that mitochondrion-toxic AEDs, such as VPA, can be well tolerated as an antiepileptic treatment by single patients with mitochondrial epilepsy. A beneficial antiepileptic effect despite mitochondrion toxicity could be due to an AED dosage too low to induce the mitochondrion-toxic effect, the uniqueness of each patient with individual tolerability of a drug, and the beneficial effect of an AED which may outweigh the adverse effect or due to compensation of the adverse effect of an AED by the beneficial effect of concomitantly applied AEDs. A toxic effect could be also absent simply because the patient does not adhere to the proposed treatment. A beneficial and harmful effect of the same AED to mitochondria may be explained by the dosage applied, the individual tolerability, the antioxidative capacity of the organelle, and the heteroplasmy rate of mtDNA mutations. Second, AEDs should not be combined with AEDs such that mitochondrion-toxic effects potentiate. In case a combination of AEDs is necessary to control seizures in a mitochondrial disorder patient, combinations of AEDs with known mitochondrion-toxic effect (e.g. VPA with PHT, CBZ, or PB) should be avoided. Again, this applies with restrictions since individual mitochondrial disorder patients may well tolerate even a combination of mitochondrion-toxic AEDs. Third, a number of AEDs applied in the daily routine are not tested for their potential mitochondrion toxicity (e.g. LAC, RFN, PER). In such case the particular AED may be effective or ineffective with regard to the antiepileptic effect, but may exhibit a previously unrecognised mitochondrion-toxic effect with the result that adverse reactions or deterioration of the underlying mitochondrial disorder occurs, which prompt discontinuation of a certain AED treatment. The variable tolerability of AEDs in different patients may result from the fact that AEDs modulate multiple transmitter systems (Costa et al. 2008) and that these systems may be variably affected in individual mitochondrial disorder patients.

AEDs with a high known potential of mitochondrion toxicity include VPA, CBZ, PHT, and PB. Though VPA is considered the AED of choice in progressive

myoclonic epilepsy, it should be used with caution if it is due to a mitochondrial disorder, in particular myoclonic epilepsy with ragged red fibre (MERRF) syndrome (Mancuso et al. 2006). As an alternative LEV can be tried in this indication (Ponchaut et al. 1992). AEDs with low potential of mitochondrion toxicity include LEV, LTG, GBT, and ZNS. Since LTG seems to exhibit only minor mitochondrion-toxic effects in mitochondrial disorder patients but has a high antiepileptic effectiveness, LTG can be recommended as a useful AED for epilepsy in mitochondrial disorder patients. If AEDs with low mitochondrion-toxic potential are ineffective in mitochondrial epilepsy, AEDs with high mitochondrion-toxic potential must be tried disregarding the possibility that they deteriorate the underlying mitochondrial disorder or epilepsy. Particularly in epileptic states or epilepsia partialis continua, single AEDs or combinations of AEDs with high mitochondrion-toxic potential must be tried to evaluate their antiepileptic effect. Since the number of AEDs which can be given intravenously is limited to VPA, LEV, LAC, PHT, PB, and benzodiazepines, VPA, PHT, and PB must be tried in severe cases of mitochondrial epilepsy as soon as it becomes evident that less mitochondrion-toxic AEDs are ineffective.

References

Allen RJ, DiMauro S, Coulter DL et al (1983) Kearns-Sayre syndrome with reduced plasma and cerebrospinal fluid folate. Ann Neurol 13:679–682

Araújo IM, Ambrósio AF, Leal EC et al (2004) Neurotoxicity induced by antiepileptic drugs in cultured hippocampal neurons: a comparative study between carbamazepine, oxcarbazepine, and two new putative antiepileptic drugs, BIA 2–024 and BIA 2–093. Epilepsia 45:1498–1505

Asanuma M, Miyazaki I, Diaz-Corrales FJ et al (2010) Neuroprotective effects of zonisamide target astrocyte. Ann Neurol 67:239–249

Bachmann RF, Wang Y, Yuan P et al (2009) Common effects of lithium and valproate on mitochondrial functions: protection against methamphetamine-induced mitochondrial damage. Int J Neuropsychopharmacol 12:805–822

Barnerias C, Saudubray JM, Touati G et al (2010) Pyruvate dehydrogenase complex deficiency: four neurological phenotypes with differing pathogenesis. Dev Med Child Neurol 52:e1–e9

Berger I, Segal I, Shmueli D et al (2010) The effect of antiepileptic drugs on mitochondrial activity: a pilot study. J Child Neurol 25:541–545

Bicknese AR, May W, Hickey WF et al (1992) Early childhood hepatocerebral degeneration misdiagnosed as valproate hepatotoxicity. Ann Neurol 32:767–775

Bogdanov GN, Mishchenko DV, Kotel'nikova RA, Frog ES, Faĭngol'd II, Tat'ianenko LV, Dorokhotova OV, Nartsissov IR (2009) Anticonvulsants as bioantioxidants under stress conditions. Biomed Khim 55:519–524

Cansu A, Erdogan D, Serdaroglu A, Take G, Coskun ZK, Gurgen SG (2010) Histologic and morphologic effects of valproic acid and oxcarbazepine on rat uterine and ovarian cells. Epilepsia 51:98–107

Cardile V, Pavone A, Renis M, Russo A, Perciavalle V (2000) Biological effects of tiagabine on primary cortical astrocyte cultures of rat. Neurosci Lett 288:49–52

Castro-Cago M, González-Conde V, Fernández-Seara MJ et al (1999) Early mitochondrial encephalomyopathy due to complex IV deficiency consistent with Alpers-Huttenlocher syndrome: report of two cases. Rev Neurol 29:912–917

Cengiz M, Yüksel A, Ozaydin A, Ozkiliç A, Cetinel O, Seven M (2005) The effects of vigabatrin on rat liver antioxidant status. Drug Metabol Drug Interact 21:109–115

Chabrol B, Mancini J, Chretien D et al (1994) Valproate-induced hepatic failure in a case of cytochrome c oxidase deficiency. Eur J Pediatr 153:133–135

Chen X, Wong JY, Wong P, Radany EH (2011) Low-dose valproic acid enhances radiosensitivity of prostate cancer through acetylated p53-dependent modulation of mitochondrial membrane potential and apoptosis. Mol Cancer Res 9:448–461

Chiyonobu T, Noda R, Yoshida M et al (2008) Intestinal pseudo-obstruction in a patient with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) associated with phenytoin therapy. Brain Dev 30:430–433

Condello S, Currò M, Ferlazzo N, Costa G, Visalli G, Caccamo D, Pisani LR, Costa C, Calabresi P, Ientile R, Pisani F (2013) Protective effects of zonisamide against rotenone-induced neurotoxicity. Neurochem Res 38:2631–2639

Connop BP, Boegman RJ, Beninger RJ et al (1997) Malonate-induced degeneration of basal forebrain cholinergic neurons: attenuation by lamotrigine, MK-801, and 7-nitroindazole. J Neurochem 68:1191–1199

Costa C, Tozzi A, Luchetti E et al (2010) Electrophysiological actions of zonisamide on striatal neurons: Selective neuroprotection against complex I mitochondrial dysfunction. Exp Neurol 221:217–224

Costa C, Belcastro V, Tozzi A et al (2008) Electrophysiology and pharmacology of striatal neuronal dysfunction induced by mitochondrial complex I inhibition. J Neurosci 28:8040–8052

Demarest ST, Whitehead MT, Turnacioglu S, Pearl PL, Gropman AL (2014) Phenotypic analysis of epilepsy in the mitochondrial encephalomyopathy, lactic acidosis, and strokelike episodes-associated mitochondrial DNA A3243G mutation. J Child Neurol 29:1249–1256

Desguerre I, Pinton F, Nabbout R, Moutard ML, N'Guyen S, Marsac C, Ponsot G, Dulac O (2003) Infantile spasms with basal ganglia MRI hypersignal may reveal mitochondrial disorder due to T8993G MT DNA mutation. Neuropediatrics 34:265–269

De Simone G, Di Fiore A, Menchise V et al (2005) Carbonic anhydrase inhibitors. Zonisamide is an effective inhibitor of the cytosolic isozyme II and mitochondrial isozyme V: solution and X-ray crystallographic studies. Bioorg Med Chem Lett 15:2315–2320

Dhir A, Akula KK, Kulkarni SK (2008) Tiagabine, a GABA uptake inhibitor, attenuates 3-nitropropionic acid-induced alterations in various behavioral and biochemical parameters in rats. Prog Neuropsychopharmacol Biol Psychiatry 32:835–843

Dodgson SJ, Shank RP, Maryanoff BE (2000) Topiramate as an inhibitor of carbonic anhydrase isoenzymes. Epilepsia 41(suppl 1):S35–S39

Eghbal MA, Taziki S, Sattari MR (2014) Mechanisms of phenytoin-induced toxicity in freshly isolated rat hepatocytes and the protective effects of taurine and/or melatonin. J Biochem Mol Toxicol 28:111–118

Finsterer J, Zarrouk-Mahjoub S (2012) Mitochondrial toxicity of antiepileptic drugs and their tolerability in mitochondrial disorders. Expert Opin Drug Metab Toxicol 8:71–79

Gibbs JE, Cock HR (2007) Administration of levetiracetam after prolonged status epilepticus does not protect from mitochondrial dysfunction in a rodent model. Epilepsy Res 73:208–212

Gilbert JC, Scott AK, Wyllie MG (1974) Proceedings: effects of ethosuximide on adenosine triphosphatase activities of some subcellular fractions prepared from rat cerebral cortex. Br J Pharmacol 50:452P–453P

Goldlust A, Su TZ, Welty DF, Taylor CP, Oxender DL (1995) Effects of anticonvulsant drug gabapentin on the enzymes in metabolic pathways of glutamate and GABA. Epilepsy Res 22:1–11

Goto M, Miyahara I, Hirotsu K et al (2005) Structural determinants for branched-chain aminotransferase isozyme-specific inhibition by the anticonvulsant drug gabapentin. J Biol Chem 280:37246–37256

Grover ND, Limaye RP, Gokhale DV, Patil TR (2013) Zonisamide: a review of the clinical and experimental evidence for its use in Parkinson's disease. Indian J Pharmacol 45:547–555
Hakonen AH, Isohanni P, Rantamäki M et al (2010) Mitochondrial recessive ataxia syndrome (MIRAS) and valproate toxicity. Duodecim 126:1552–1559
Hroudova J, Fisar Z (2010) Activities of respiratory chain complexes and citrate synthase influenced by pharmacologically different antidepressants and mood stabilizers. Neuro Endocrinol Lett 31:336–342
Hsu YC, Yang FC, Perng CL et al (2012) Adult-onset of mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) syndrome presenting as acute meningoencephalitis: a case report. J Emerg Med 43(3):e163–e166
Hutson SM, Lieth E, LaNoue KF (2001) Function of leucine in excitatory neurotransmitter metabolism in the central nervous system. J Nutr 131:846S–850S
Iwanga K, Mori K, Inoue M, Yoshimura T, Tanno Y (1992) Myoclonus epilepsy associated with ragged-red fibers--report of a patient with negative myoclonus. Rinsho Shinkeigaku 32:870–873
Kayihan N, Nennesmo I, Ericzon BG et al (2000) Fatal deterioration of neurological disease after orthotopic liver transplantation for valproic acid-induced liver damage. Pediatr Transplant 4:211–214
Komulainen T, Lodge T, Hinttala R, Bolszak M, Pietilä M, Koivunen P, Hakkola J, Poulton J, Morten KJ, Uusimaa J (2015) Sodium valproate induces mitochondrial respiration dysfunction in HepG2 in vitro cell model. Toxicology 331:47–56
Krähenbühl S, Brandner S, Kleinle S et al (2000) Mitochondrial diseases represent a risk factor for valproate-induced fulminant liver failure. Liver 20:346–348
Kudin AP, Debska-Vielhaber G, Vielhaber S et al (2004) The mechanism of neuroprotection by topiramate in an animal model of epilepsy. Epilepsia 45:1478–1487
Kumar P, Kalonia H, Kumar A (2012) Possible GABAergic mechanism in the neuroprotective effect of gabapentin and lamotrigine against 3-nitropropionic acid induced neurotoxicity. Eur J Pharmacol 674:265–274
Lagrue E, Chalon S, Bodard S et al (2007) Lamotrigine is neuroprotective in the energy deficiency model of MPTP intoxicated mice. Pediatr Res 62:14–19
Lai JS, Zhao C, Warsh JJ et al (2006) Cytoprotection by lithium and valproate varies between cell types and cellular stresses. Eur J Pharmacol 539:18–26
Lam CW, Lau CH, Williams JC et al (1997) Mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) triggered by valproate therapy. Eur J Pediatr 156:562–564
Li S, Guo J, Ying Z, Chen S, Yang L, Chen K, Long Q, Qin D, Pei D, Liu X (2015) Valproic acid-induced hepatotoxicity in Alpers syndrome is associated with mitochondrial permeability transition pore opening-dependent apoptotic sensitivity in an induced pluripotent stem cell model. Hepatology 61:1730–1739
Lin CM, Thajeb P (2007) Valproic acid aggravates epilepsy due to MELAS in a patient with an A3243G mutation of mitochondrial DNA. Metab Brain Dis 22:105–109
Mancuso M, Galli R, Pizzanelli C et al (2006) Antimyoclonic effect of levetiracetam in MERRF syndrome. J Neurol Sci 243:97–99
Marmolino D, Manto M (2010) Pregabalin antagonizes copper-induced toxicity in the brain: in vitro and in vivo studies. Neurosignals 18:210–222
Martikainen MH, Päivärinta M, Jääskeläinen S, Majamaa K (2012) Successful treatment of POLG-related mitochondrial epilepsy with antiepileptic drugs and low glycaemic index diet. Epileptic Disord 14:438–441
Monti B, Gatta V, Piretti F, Raffaelli SS, Virgili M, Contestabile A (2010) Valproic acid is neuroprotective in the rotenone rat model of Parkinson's disease: involvement of alpha-synuclein. Neurotox Res 17:130–141
Neuman MG, Nanau RM, Shekh-Ahmad T, Yagen B, Bialer M (2013) Valproic acid derivatives signal for apoptosis and repair in vitro. Clin Biochem 46:1532–1537

Ponchaut S, van Hoof F, Veitch K (1992) Cytochrome aa3 depletion is the cause of the deficient mitochondrial respiration induced by chronic valproate administration. Biochem Pharmacol 43:644–647

Pronicka E, Weglewska-Jurkiewicz A, Pronicki M et al (2011) Drug-resistant epilepsia and fulminant valproate liver toxicity. Alpers-Huttenlocher syndrome in two children confirmed post mortem by identification of p.W748S mutation in POLG gene. Med Sci Monit 17:CR203–CR209

Rasmussen M, Sanengen T, Skullerud K et al (2000) Evidence that Alpers-Huttenlocher syndrome could be a mitochondrial disease. J Child Neurol 15:473–477

Ray S (ed) (2014) Side effects of drugs annual. Hardbound, 836, Published: December 2014. ISBN 13: 978-0-444-63407-8

Riva R, Albani F, Contin M et al (1996) Pharmacokinetic interactions between antiepileptic drugs. Clinical considerations. Clin Pharmacokinet 31:470–493

Rogers SK, Shapiro LA, Tobin RP, Tow B, Zuzek A, Mukherjee S, Newell-Rogers MK (2014) Levetiracetam differentially alters CD95 expression of neuronal cells and the mitochondrial membrane potential of immune and neuronal cells in vitro. Front Neurol 5:17

Rumbach L, Mutet C, Cremel G et al (1986) Effects of sodium valproate on mitochondrial membranes: electron paramagnetic resonance and transmembrane protein movement studies. Mol Pharmacol 30:270–273

Saneto RP, Lee IC, Koenig MK et al (2010) POLG DNA testing as an emerging standard of care before instituting valproic acid therapy for pediatric seizure disorders. Seizure 19:140–146

Santos NA, Medina WS, Martins NM et al (2008a) Aromatic antiepileptic drugs and mitochondrial toxicity: effects on mitochondria isolated from rat liver. Toxicol In Vitro 22:1143–1152

Santos NA, Medina WS, Martins NM et al (2008b) Involvement of oxidative stress in the hepatotoxicity induced by aromatic antiepileptic drugs. Toxicol In Vitro 22:1820–1824

Schaller A, Hahn D, Jackson CB et al (2011) Molecular and biochemical characterisation of a novel mutation in POLG associated with Alpers syndrome. BMC Neurol 11:4

Schinwelski M, Kierdaszuk B, Dulski J, Tońska K, Kodroń A, Sitek EJ, Bartnik E, Kamińska A, Kwieciński H, Sławek J (2015) Changing phenotypic expression in a patient with a mitochondrial encephalopathy due to 13042G > A de novo mutation--a 5 year follow up. Metab Brain Dis 30:1083–1085

Schwabe MJ, Dobyns WB, Burke B et al (1997) Valproate-induced liver failure in one of two siblings with Alpers disease. Pediatr Neurol 16:337–343

Shchelochkov O, Wong LJ, Shaibani A et al (2009) Atypical presentation of VLCAD deficiency associated with a novel ACADVL splicing mutation. Muscle Nerve 39:374–382

Simonati A, Filosto M, Savio C et al (2003) Features of cell death in brain and liver, the target tissues of progressive neuronal degeneration of childhood with liver disease (Alpers-Huttenlocher disease). Acta Neuropathol 106:57–65

Sitarz KS, Elliott HR, Karaman BS, Relton C, Chinnery PF, Horvath R (2014) Valproic acid triggers increased mitochondrial biogenesis in POLG-deficient fibroblasts. Mol Genet Metab 112:57–63

Stöllberger C, Höftberger R, Finsterer J (2011) Lamotrigine-trigged obstructive hypertrophic cardiomyopathy, epilepsy and metabolic myopathy. Int J Cardiol 149:e103–e105

Ueda Y, Doi T, Takaki M, Nagatomo K, Nakajima A, Willmore LJ (2009) Levetiracetam enhances endogenous antioxidant in the hippocampus of rats: in vivo evaluation by brain microdialysis combined with ESR spectroscopy. Brain Res 1266:1–7

Urbanska EM, Blaszczak P, Saran T, Kleinrok Z, Turski WA (1998) Mitochondrial toxin 3-nitropropionic acid evokes seizures in mice. Eur J Pharmacol 359:55–58

Uusimaa J, Hinttala R, Rantala H et al (2008) Homozygous W748S mutation in the POLG1 gene in patients with juvenile-onset Alpers syndrome and status epilepticus. Epilepsia 49:1038–1045

Vogel KR, Ainslie GR, Jansen EE, Salomons GS, Gibson KM (2015) Torin 1 partially corrects vigabatrin-induced mitochondrial increase in mouse. Ann Clin Transl Neurol 2:699–706

Wolf NI, Rahman S, Schmitt B et al (2009) Status epilepticus in children with Alpers' disease caused by POLG1 mutations: EEG and MRI features. Epilepsia 50:1596–1607

Zucker B, Ludin DE, Gerds TA, Lücking CH, Landwehrmeyer GB, Feuerstein TJ (2004) Gabapentin-lactam, but not gabapentin, reduces protein aggregates and improves motor performance in a transgenic mouse model of Huntington's disease. Naunyn Schmiedebergs Arch Pharmacol 370:131–139

Erratum to: The Mitochondrial Permeability Transition Pore and ATP Synthase

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Handbook of Experimental Pharmacology, DOI 10.1007/164_2016_5

The publisher regrets to inform that the one of the author name was incorrect. The correct name is Kambiz N. Alavian (wrong: Alavian N. Kambiz).

The updated original online version for this chapter can be found at DOI 10.1007/164_2016_5

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H. Singh, S.-S. Sheu (eds.), *Pharmacology of Mitochondria*,
Handbook of Experimental Pharmacology 240, DOI 10.1007/164_2016_87

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H. Singh, S.-S. Sheu (eds.), *Pharmacology of Mitochondria*,
Handbook of Experimental Pharmacology 240, DOI 10.1007/978-3-319-57313-7