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MURINE HOMEOBOX GENE CONTROL OF EMBRYONIC PATTERNING AND ORGANOGENESIS

ADVANCES IN DEVELOPMENTAL BIOLOGY AND BIOCHEMISTRY

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Preface

It has been nearly two decades since the first homeobox gene was molecular cloned in *Drosophila*. This monumental finding rapidly led to the discovery of additional homeobox genes in essentially every animal species examined. Since that time some twenty years ago, enormous progress has been made in our understanding of the distribution of homeobox genes in the genomes of many species and the common functional role homeobox genes play in cell-type specification and development. The amino acid sequence of the homeodomain, and the presence of other conserved protein domains, has allowed the classification of homeodomain-containing proteins (homeoproteins) into over thirty separate families (e.g. *Hox, Dlx, Msx, Otx, Hmx, Cdx* etc.) with most commonly between 2–10 members per family in mammals. Additionally, recent analysis of different animal genomes has now permitted more accurate and detailed models of the evolution of homeobox gene families, which appear to have expanded largely in step with overall gene number in the evolution of more complex organisms.

With the recent completion of the sequencing of the first arthropod and mammalian genomes a major revelation was the relative paucity of genes necessary to construct complex animal life forms. The parsimonious nature of genes was not so foreign to investigators in the homeobox gene area, where an early question had always been how a single gene could fully direct the morphogenesis and development of a complex tissue, organ or entire body segment. This early and fundamental question on the "master" regulatory ability of homeoproteins to a large part still remains a mystery, in part owing to our limited understanding of the downstream effectors of homeobox gene function.

It would be beyond the scope of any single publication to review all recent developments in what has been learned about homeobox gene structure, function and expression. So here we limit ourselves to what has been learned in mammalian systems, primarily focusing on the mouse, as the mouse remains the vertebrate species of choice for using both forward and reverse genetic approaches to generate either gain- or loss-of-function mutations at will. Yet, a common theme to each of these reviews is the underlying importance of what has been learned about each homeobox gene family in other species, particularly *Drosophila*, and how this has aided our interpretation and understanding of the role these genes play in mice and other mammals, namely human.

A question of central interest in the homeobox gene field has been how homeoproteins which act as DNA binding transcription factors, can with a relatively weak specificity of DNA binding, achieve such specificity of action. The chapter by Featherstone explores the mechanisms through which Hox and other homeoproteins achieve specificity in their role as transcriptional regulators (both activators and

repressors) and how homeoprotein interaction with cofactors (often other homeoproteins) affects both cooperativity and specificity of DNA binding.

Members of the *msh/Msx* homeobox gene family have remained remarkably conserved during evolution relative to other homeobox gene families. The section by Maxson et al. explores this evolutionary conservation at the functional level by describing the role of the *Msx* genes in the convergence of both the control of cell proliferation and differentiation and hence pattern via extracellular signals. This chapter also details the role of the *Msx* genes in development of the mammalian skull and goes further to integrate them into an emerging homeobox gene developmental cascade whereby expression of the *Msx* genes is controlled by other homeoproteins and the Msx proteins themselves control the expression of yet other homeobox genes.

An example of the role of homeobox genes in patterning specific regions of the body in a wide range of species is described in the chapter by Lohnes and colleagues where they review what is known about the *Drosophila caudal* homologs in mice (*Cdx1* and *Cdx2*) and other species and their conserved role in patterning the posterior end of the embryo and in gastrulation. Additional functions the *Cdx1/2* genes have evolved include the control of vertebral patterning that is intertwined with their control of the early phase of *Hox* gene expression. How the *Cdx* genes themselves are regulated is also explored and the *wingless/Wnt* family of cell–cell signaling molecules is implicated along with retinoic acid, which has also been shown to directly regulate expression of certain members of the *Hox* gene complex.

The chapter by Levi and colleagues describes the role of two murine *Dlx* genes in craniofacial and limb development. Homologs of *Drosophila Distal-less*, the murine genes have been shown to play an evolutionary conserved role in appendage outgrowth similar to what was seen in their fly counterparts, thus further linking the developmental programs utilized by mammalian limbs and *Drosophila* appendages (antennae, labium, legs and wings). In a similar manner the chapter by Meijlink et al. explores the contribution of the *Prx, Alx* and *Shox* genes to craniofacial and appendicular (limb) morphogenesis. These three mammalian families are highly similar to the *Drosophila aristaless* gene, which is involved in both embryonic development and pattern formation in appendages and head segments and which furthermore overlaps in expression with the *Distal-less* gene in the developing fly head and distal tip of fly appendages.

Probably the best-characterized and most widely studied family of homeobox genes is the *Hox* genes. The chapter by Iulianella and Trainor focus on the role of *Hox* genes in their anterior domain of function and explore their contribution to patterning of the cranial neural crest and head. The authors review the interplay of multiple extracellular signaling systems in neural induction and go on to describe how multiple regulators of *Hox* gene expression are now known, which include retinoic acid and its associated nuclear receptors, Krox20, kreisler, Fgfs and Hox proteins themselves. With regard to patterning parts of the anterior end of the embryo in diverse species, the section by Simeone et al. review the role of the *Otx* genes in murine brain development. In *Drosophila* the *Otx* homolog *orthodenticle (otd)* is responsible for patterning the antennal segment, which gives rise to the eye

and the antenna, as well as sections of the fly brain. This chapter also reviews what is known about the function of neural signaling centers such as the anterior visceral endoderm and their impact on homeobox gene expression.

Almost two decades have passed since the molecular cloning of the first homeobox gene and during that interval great advances have been made in our understanding of homeobox gene structure, expression, function and evolution in mammals. At the same time many old questions remain resistant to rapid solutions, such as a full understanding of the nature and number of different homeobox upstream regulators (both at the DNA and protein level) how they integrate their function with other neighboring enhancers and how they restrict themselves from acting on genes that often lie between them and their normal homeobox responsive gene. Likewise the issue of post-translational modification of homeoproteins and homeoprotein cofactors (proteins or otherwise), their diversity and how they modulate homeoprotein function are only beginning to be understood in a handful of cases. Finally how, when and what homeoproteins control in terms of target genes is still in its infancy. Hopefully the emergence of promising new tools in the areas of genomics and proteomics combined with ongoing advances in molecular genetics and bioinformatics will help us better address many of these questions in the near future.

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HOX proteins and their co-factors in transcriptional regulation

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1. Introduction

The homeobox was first identified in segmentation and *Hox* genes of the fruit fly (McGinnis et al., 1984b; Scott and Weiner, 1984). The conceptual translation of the homeobox into a peptidic homeodomain revealed homologies with the helix-turn-helix DNA-binding domains of prokaryotic transcriptional regulators (Laughon and Scott, 1984; McGinnis et al., 1984a), a finding consistent with the predicted role of *Hox* genes as master regulators of antero-posterior (AP) patterning (Garcia-Bellido, 1977). The subsequent two decades of research have amply supported a transcriptional function for the products of a large variety of homeobox-containing genes. Nonetheless, insight into the molecular mechanisms of transcriptional regulation by HOX proteins themselves has lagged behind that of other homeoproteins such as mammalian Oct family members, and Mat**a**1 and Matα2 of yeast. In part, this may have been because many researchers interested in *Hox* gene function took a developmental perspective. Probably, more important was the difficulty in establishing robust and biologically relevant experimental conditions for addressing this issue. These problems included the paucity of known regulatory targets, relatively indiscriminate DNA-binding activity, and poor transcriptional output in classical transfection assays. However, growing evidence anchors HOX proteins firmly within the paradigms established for better-studied transcription factors. Thus, *Hox* gene products localize to the nucleus, bind DNA (particularly well in the presence of certain homeodomain partners), harbor transcriptional activation and repression domains, recruit co-regulators with chromatin modifying activity, and act through discrete recognition sites on naturally occurring enhancers in downstream target genes. Perhaps less orthodox is the observation that transcriptional repression by HOX proteins may involve multiple binding sites (up to 41 binding sites for Ultrabithorax (UBX) in the *Antennapedia* (*Antp*) promoter) (Appel and Sakonju, 1993) over large stretches of DNA (Biggin and McGinnis, 1997). Despite these advances, we are far from a full understanding of some fundamental processes: How is regulatory "input" provided by HOX proteins integrated with that of other transcription factors? How do the various *Hox* gene products differentially regulate target gene expression? To what extent are these the same question? This review examines the molecular mechanisms by which HOX proteins regulate transcription, with an emphasis on how they achieve specificity.

1.1. Hox genes and their products

Insects have a single *Hox* cluster (Fig. 1). In *Drosophila*, this cluster has been split between the three genes of the bithorax complex (BX-C), *Ubx*, *abdominal-A* (*abd-A*), and *Abdominal-B* (*Abd-B*), and the five of the Antennapedia complex (ANT-C), *labial* (*lab*), *proboscipedia* (*pb*), *Deformed* (*Dfd*), *Sex combs reduced* (*Scr*), and *Antp*. However, from an evolutionary and genetic perspective, this is a single cluster that has been physically divided. By contrast, in the mouse and human genomes, there are 39 *Hox* genes distributed over four clusters designated A through D (Fig. 1).

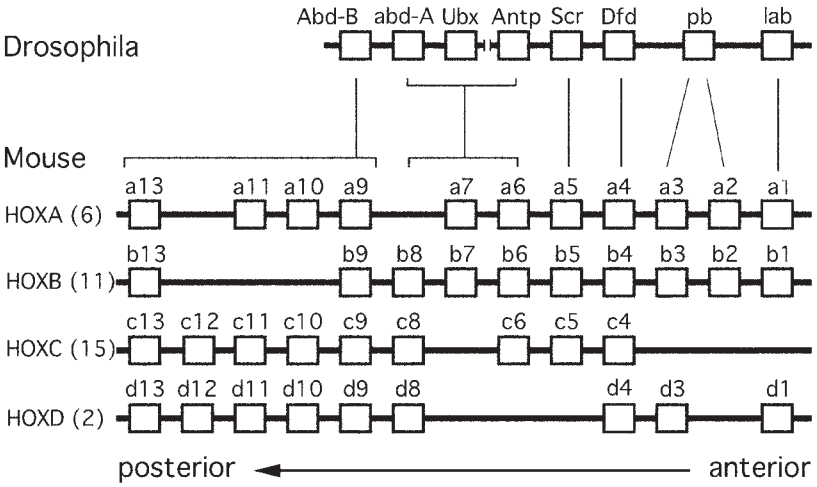

Fig. 1. Organization of fly and mouse *Hox* complexes. The eight fly *Hox* genes are diagrammed on top. The split between BX-C and ANT-C is indicated by a gap. The murine genes are given below, with cluster names on the left, and chromosome number in brackets. Orthologous genes are connected by vertical lines. The arrow indicates colinear *Hox* gene expression along the antero-posterior axis of flies and mice.

As a result of cluster duplication and gene loss, there are thirteen paralog groups, though no single cluster retains all thirteen. Each *Hox* gene is designated by locus and paralog number. For example, *Hoxd4* is in paralog group 4 of the D cluster (Duboule et al., 1990). The vertebrate clusters are clearly related to that of insects, pointing to an ancient evolutionary origin for this genomic organization (Slack et al., 1993; Ferrier and Holland, 2001).

For the majority of mammalian *Hox* genes, a single protein product has been described, though there are exceptions (Baron et al., 1987; LaRosa and Gudas, 1988; Ali and Bienz, 1991; Kömüves et al., 2000). Mammalian HOX proteins are relatively small, with molecular weights in the range of 25,000 to 49,000. The homeobox generally falls within the second of two coding exons, placing the homeodomain in the C-terminal half of the protein (Fig. 2A). The situation in flies can be more complex with, for example, multiple alternative splice products for *Ubx* (Kornfeld et al., 1989).

HOX proteins from paralog groups 1 to 8—all but *Abd-B* in flies (Fig. 1)—share a short motif with the consensus YPWM located N-terminal to the homeodomain and required for co-operative DNA-binding with the PBC family of homeodomain proteins (Fig. 2A). In paralogs 9 and 10, the function of the YPWM is replaced by another tryptophan-containing motif, ANW (Chang et al., 1996). In addition to the YPWM/ANW motif and homeodomain, the extreme N-terminus also shows some conservation among HOX proteins (McGinnis et al., 1990; Rambaldi et al., 1994).

Hox genes are active in a broad range of organs, but in a tissue they are spatially restricted in a manner consonant with their function in AP patterning. In mammals,

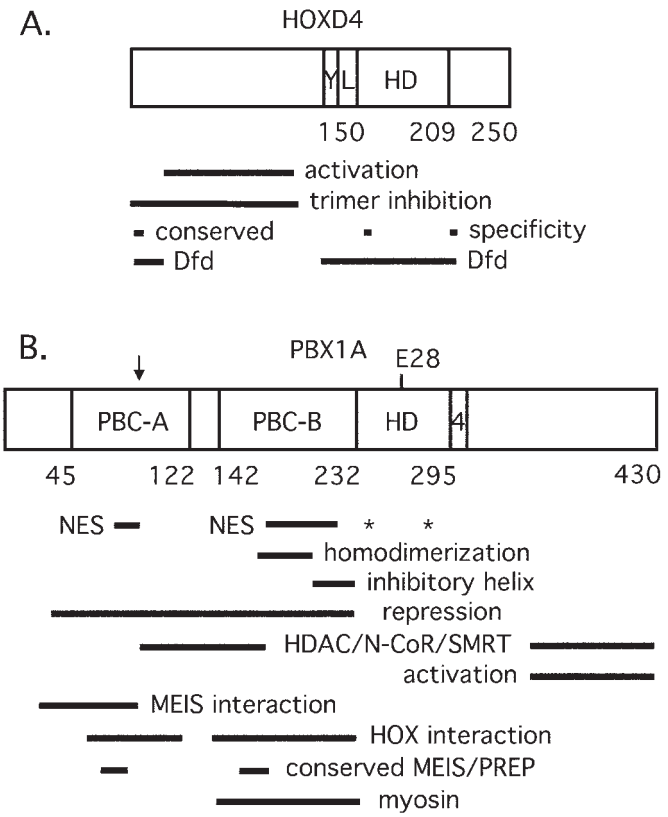

Fig. 2. Structure of HOX and PBX proteins.(A). Domains of murine HOXD4. Scale drawing indicates the linker (L) separating the YPWM motif (Y) from the homeodomain (HD). Thick black lines denote positions of the activation domain (activation), a region that inhibits trimer formation with PBX and MEIS partners (trimer inhibition), an extreme N-terminal domain conserved among many HOX proteins (conserved), regions just C-terminal to the homeodomain and in the N-terminal arm that define the specificity of DFD function (specificity), and two domains conserved among DFD and fourth paralog HOX proteins (Dfd). (B). Domains of human PBX1A. Scale drawing indicates the PBC-A and PBC-B domains, the homeodomain (HD), the fourth helix (4) which forms upon DNA-binding, and the glutamate at position 28 of the homeodomain that plays a key role in contacting the N-terminus. Thick black lines denote positions of presumptive NES; two NLS (asterisks); a region that mediates homodimerization; a domain that inhibits monomeric DNA-binding, cooperative DNA-binding with HOX partners, and nuclear localization (inhibitory helix); a transcriptional repression domain (repression); regions that interact with HDACs 1 and 3, N-CoR and/or SMRT (HDAC/N-CoR/SMRT); a weak C-terminal transcriptional activation domain (activation); regions required for interaction with MEIS/PREP (MEIS interaction) and HOX (HOX interaction) (Knoepfler et al., 1997; Shanmugam et al., 1999); two stretches conserved in the MEIS/PREP HR1 and HR2 domains (conserved MEIS/PREP), and the site of interaction with non-muscle myosin II heavy chain B (myosin). Vertical arrow, residue 89—site of fusion to E2A resulting from some t(1;19) translocation events. See text for additional references. Amino acid numbers are given below the drawings.

Hox genes are expressed in the central nervous system within, and posterior to, the hindbrain, as well as in neural crest and its derivatives, the somitic column, lateral plate mesoderm, limbs, genital tubercle, and regions of the gut and urogenital tract (Duboule, 1992; Krumlauf, 1994; Zákány and Duboule, 1999; Trainor and Krumlauf, 2001). Although their functions in the adult are less studied, they also play important roles in hematopoiesis, hair shaft production, and mammary gland maturation (Godwin and Capecchi, 1998; Chen and Capecchi, 1999; Antonchuk et al., 2002)

In vertebrates and flies, the order of *Hox* genes along the chromosome reflects their spatial expression domains (Fig. 1), a phenomenon termed colinearity (Lewis, 1978; Duboule, 1998). This process divides the embryo into AP domains or compartments of differential *Hox* gene expression. In vertebrates, these domains tend to be nested, resulting in the co-expression of increasing numbers of *Hox* genes in more posterior regions of the embryo. The embryonic hindbrain is segmented along its AP axis into eight rhombomeres. For those *Hox* genes active in the hindbrain, anterior expression borders fall at the boundaries between rhombomeres, often with a two-segment periodicity (Krumlauf, 1994). Thus, a third group paralog like *Hoxa3* is expressed up to the boundary between rhombomeres 4 and 5 (r4/5), while the fourth group gene *Hoxd4* has a limit at r6/7. Such restricted spatial (and temporal) expression is critical to numerous AP patterning events, including vertebral morphogenesis, the suppression of legs in the fly abdomen, and vulval development in the worm. Clearly, even the same target gene must be differentially regulated along the AP axis to achieve such effects. Since this is dependent, at least in part, on variations in HOX amino acid sequence (see Section 3.4), how these substitutions affect transcriptional control is of great interest.

1.2. The TALE class of homeodomain proteins

The most intensively studied HOX partners are members of the three amino acid loop extension (TALE) family of homeodomain proteins (Table 1). PBX (vertebrates), extradenticle (EXD, flies) and CEH-20 (*C. elegans*) are three members of the PBC-group of TALE proteins. Up to five *Pbx* genes may be present in the vertebrate genome (Pöpperl et al., 2000; Wagner et al., 2001). At least two of these, *Pbx1* and *Pbx3*, generate C-terminal isoforms due to alternative splicing (Kamps et al., 1990; Nourse et al., 1990; Monica et al., 1991). Comparison of the primary amino acid sequence reveals two blocks of homology N-terminal to the homeodomain designated PBC-A and PBC-B (Fig. 2B) (Bürglin and Ruvkin, 1992; Bürglin, 1998). Spatial and tissue *Pbx* expression domains are widespread, extending beyond those of the *Hox* genes, indicative of *Hox*-independent functions in transcriptional regulation (Roberts et al., 1995; Schnabel et al., 2001). Indeed, the phenotype of *Pbx1* null mutant mice reveals defects not only in the skeleton, which comes under *Hox* control, but also in the spleen and pancreas whose development is independent of clustered *Hox* genes (DiMartino et al., 2001; Selleri et al., 2001; Kim et al., 2002).

Table 1
Tale class proteins

Sub-family name	Protein name	Species[a]
PBC	PBX1	human
	PBX2	human
	PBX3	human
	PBX4	mouse
	lazarus	zebrafish
	EXD	fly
	CEH-20	nematode
MEIS[b]	MEIS1	mouse
	MEIS2	mouse
	MEIS3	mouse
	HTH	fly
	CEH-25	nematode
PREP[b]	PREP1	human
	PREP2	human

[a]Indicates the species in which the corresponding gene was first described. The mouse and human genomes have a minimum of four *Pbx* and three *Meis* genes, whereas flies and nematodes have a single ortholog for each.
[b]Collectively referred to as MEIS/PREP or MEINOX.

A role for PBC-class proteins in modulating HOX function was first inferred from the phenotype of *exd* null mutant larvae of *Drosophila* (Peifer and Wieschaus, 1990). These animals displayed AP patterning defects characteristic of *Hox* mutants, but without gross changes in the expression patterns of the *Hox* genes examined. The first member of the family, human *Pbx1* (pre-B cell leukemia transcription factor 1), was identified at the t(1;19) chromosomal breakpoint present in 25% of pediatric pre-B cell leukemias (Kamps et al., 1990; Nourse et al., 1990). Subsequent cloning of *exd* revealed it to be the *Pbx* homolog in flies (Flegel, 1993; Rauskolb et al., 1993).

The MEIS/PREP (or MEINOX) group of TALE proteins comprises members of the MEIS and PREP families (Table 1). *Meis1* (murine ecotropic integration site 1) was discovered as the site of retroviral integration leading to myeloid leukemia (Moskow et al., 1995). Orthologs of the three murine *Meis* genes are *homothorax* (*hth*) in flies and *Ceh-25* in *C. elegans* (Moskow et al., 1995; Bürglin, 1997; Rieckhof et al., 1997; Steelman et al., 1997; Bürglin, 1998). The two mammalian *Prep* genes (also known as *Pknox1* and *Pknox2*) are related to the *Knotted-1* gene of plants (Chen et al., 1997; Knoepfler et al., 1997; Berthelsen et al., 1998a; Berthelsen et al., 1998c; Bürglin, 1998; Imoto et al., 2001; Fognani et al., 2002; Haller et al., 2002). Two regions of conservation, HR1 and HR2, have been noted N-terminal to the homeodomain of HTH, MEIS and PREP proteins, and share homology with PBC-A and B (Fig. 2B) (Berthelsen et al., 1998c; Bürglin, 1998). Similar to *Pbx*, *Meis* and *Prep* genes are broadly expressed in development, and are expected to play *Hox*-dependent and independent roles (Ferretti et al., 1999; Haller et al., 2002).

1.3. A summary of co-factor interactions

Versatility in target gene regulation by HOX and TALE class proteins is augmented by multiple interactions. HOX proteins from paralog groups 1 to 10 (Fig. 1) undergo co-operative DNA-binding with PBC family members (Chang et al., 1995; Lu et al., 1995; Neuteboom et al., 1995; Phelan et al., 1995; Pöpperl et al., 1995; Chang et al., 1996; Phelan and Featherstone, 1997). This interaction requires the HOX YPWM motif which makes contact to a hydrophobic pocket in the PBX homeodomain (Lu and Kamps, 1996b; Green et al., 1998; Jabet et al., 1999; Passner et al., 1999; Piper et al., 1999; Sprules et al., 2000). The rate of dissociation from optimal sites on DNA by HOX–PBX heterodimers is at least one order of magnitude slower than for monomeric complexes (Shen et al., 1996; Lu and Kamps, 1997; Phelan and Featherstone, 1997; Shanmugam et al., 1997; Shanmugam et al., 1999). Abd-B class HOX proteins (paralog groups 9 to 13) bind DNA cooperatively with MEIS family members, although only MEIS DNA-binding appears to be stabilized by this interaction (Shen et al., 1997). This does not depend on the HOX ANW motif, but rather on sequences further N-terminal, and on the MEIS homeodomain and/or C-terminus.

Distinct from their interactions with HOX proteins, PBX and MEIS/PREP can also form heterodimeric DNA-binding complexes (Chang et al., 1997b; Berthelsen et al., 1998c). In fact, PBX–MEIS/PREP heterodimers are stable in the absence of DNA-binding, unlike HOX–PBX or HOX–MEIS complexes (Berthelsen et al., 1998c; Jacobs et al., 1999). PBX–MEIS and PBX–PREP interactions are mediated by N-terminal regions comprising PBC-A and HR2 in the respective partners (Chang et al., 1997b; Knoepfler et al., 1997; Berthelsen et al., 1998b; Jacobs et al., 1999; Ryoo et al., 1999; Shanmugam et al., 1999; Haller et al., 2002). Unlike the DNA-binding requirements of PBX–HOX complexes (discussed in Sections 2.3 and 2.4), PBX–MEIS heterodimers can bind to half-sites on DNA with variable spacing (Jacobs et al., 1999). Further complicating this picture, DNA-bound PBX–PBX and MEIS–MEIS homodimers have also been observed (Neuteboom and Murre, 1997; Calvo et al., 1999).

Because non-overlapping domains are used for the formation of HOX–PBX and PBX–MEIS/PREP heterodimers, HOX–PBX–MEIS/PREP (and HOX–EXD–HTH) heterotrimers can also form, and may be stable in the absence of DNA-binding (Berthelsen et al., 1998b; Jacobs et al., 1999; Shanmugam et al., 1999; Shen et al., 1999; Ferretti et al., 2000). At least in some contexts, only the HOX and PBX components need contact DNA in order to form a DNA-bound heterotrimer in which MEIS/PREP is tethered by protein–protein interactions (Berthelsen et al., 1998b) that stabilize the complex (Shanmugam et al., 1999). This is supported by results in transient transfections showing that a DNA-binding-defective MEIS-VP16 fusion protein can be recruited to an enhancer driven by HOX–PBX binding sites (Shanmugam et al., 1999). However, other studies found that DNA-binding by MEIS, PREP or HTH was important for the stability of the heterotrimer on sub-optimal sites (Jacobs et al., 1999; Ryoo et al., 1999; Ferretti et al., 2000; Gebelein et al., 2002). A reciprocal heterotrimer, where PBX is tethered

to a DNA-bound HOX–MEIS heterodimer, has also been described (Shanmugam et al., 1999). Last, we have noted a HOX–PBX–PBX heteromer in which all three proteins are co-operatively bound to DNA (K. Shanmugam and M. F., unpublished observations).

2. Monomeric and heteromeric DNA-binding

2.1. DNA-binding by HOX proteins

The homeodomain is a simple DNA-binding structure of approximately 60 amino acids (Gehring et al., 1994a,b; Wolberger, 1996). It is composed of three alpha helices and a flexible N-terminal arm. Helices 2 and 3 form a helix-turn-helix, with the third helix making base-specific contacts in the major groove. Specificity is also imparted by the N-terminal arm which contacts the minor groove. The core binding site for HOX homeodomain recognition is 5′ TAAT 3′. The first two base pairs (TAAT) are specified by the N-terminal arm, typically by an arginine or lysine at position 3, and arginine at position 5. Exceptions are the products of the labial or first paralog group (Fig. 1), some of which have uncharged residues at position 3. This leads to reduced DNA-binding activity in vitro, at least under some assay conditions (Phelan et al., 1994; Phelan and Featherstone, 1997). Not surprisingly, the N-terminal arm has been implicated in the discrimination of DNA-binding sites (Ekker et al., 1994; Phelan et al., 1994; Zappavigna et al., 1994; Chang et al., 1996; Phelan and Featherstone, 1997), and transcriptional targets (Kuziora and McGinnis, 1991; Lin and McGinnis, 1992; Zeng et al., 1993; Chauvet et al., 2000); however, this may not be a simple correlation (see Section 3.4).

The two 3′ base pairs of the core (TAAT) are specified by helix 3. The invariant asparagine at position 51 within helix 3, found in all homeodomains, contacts the base pair at the third position of the core (TAAT). Homeodomain position 50 confers specificity of DNA-binding by restricting the bases tolerated at the two positions 3′ to the core (5′ TAATNN 3′). Q50 selects against the presence of cytosine residues at these 3′ positions (Hanes and Brent, 1989; Treisman et al., 1989). By contrast, K50 in the bicoid homeodomain favors cytosines at these same sites (Hanes and Brent, 1989; Treisman et al., 1989). All HOX homeodomains have a glutamine at position 50, and so this residue cannot account for selective DNA-binding within the family. While variations at other positions dictate mild differences in binding site specificity (Dessain et al., 1992; Ekker et al., 1992; Ekker et al., 1994), the ability of the homeodomain to discriminate between sites, and between specific and non-specific DNA, is poor (Affolter et al., 1990; Pellerin et al., 1994). This does not mean, however, that HOX monomers do not play regulatory roles in vivo (Pinsonneault et al., 1997; Li and McGinnis, 1999; Li et al., 1999a; Galant et al., 2002). Moreover, there is evidence for co-operative DNA-binding between HOX proteins bound to adjacent and non-adjacent sites (Beachy et al., 1993; Galang and Hauser, 1993).

2.2. DNA-binding by PBC family proteins

The PBC family of homeodomain proteins (PBX, EXD, CEH-20) are members of the TALE class which, as their name suggests, display a three residue insertion in the loop between helices 1 and 2 (designated positions 23a, b and c). A fourth helix C-terminal to the PBX homeodomain is formed upon DNA-binding (Fig. 2B), and packs against helices 1 and 3, thereby stabilizing interactions with DNA (Green and Chambon, 1987; Lu and Kamps, 1996b; Jabet et al., 1999; Passner et al., 1999; Piper et al., 1999; Sprules et al., 2000). Position 50 of PBX/EXD is occupied by glycine whose small R group does not permit direct contacts to the major groove, though water interactions do take place (Passner et al., 1999; Piper et al., 1999).

Even under optimal conditions, monomer DNA-binding activity by PBX or EXD is not strong. Nonetheless, site selection and Dnase I protection experiments defined a PBX/EXD binding site as 5′ TGATTGAT 3′ (Van Dijk et al., 1993; LeBrun and Cleary, 1994). This site is actually composed of tandem repeats of the sequence TGAT, the core PBX/EXD recognition sequence. In some cases, this may have resulted from the use of GST fusion proteins which can dimerize via their GST moieties. However, full-length PBX proteins do bind as homodimers to these closely juxtaposed sites (Neuteboom and Murre, 1997; Calvo et al., 1999), as well as to TGAT cores separated by up to 18 bp (K. Shanmugam and M.F., unpublished results). The homodimer interface (Fig. 2B) maps to the PBX N-terminus, corroborating a role for residues beyond the homeodomain in co-operative EXD binding (Sun et al., 1995). While one group has further localized this interface to the C-terminal PBC-B region (Calvo et al., 1999), another study found that both PBC-A and B were required (K. Shanmugam and M.F., unpublished results). This discrepancy may be explained by the use of adjacent vs. widely separated binding sites, since the PBX N-terminus could change orientation to accommodate differentially spaced cores. The PBC-B domain may be the more robust interaction surface since this region of human PBX1 (residues 89–232) interacts with a peptide spanning PBC-A and B in a yeast two hybrid assay (I. Rambaldi and M.F., unpublished observations).

Monomeric DNA-binding by full-length PBX proteins is essentially undetectable. However, deletion of the PBX N-terminus reveals a modest DNA-binding potential of the monomeric PBX homeodomain (Neuteboom and Murre, 1997; Green et al., 1998; Calvo et al., 1999; Shanmugam et al., 1999), and a domain inhibitory for DNA-binding (Fig. 2B) has been mapped to the C-terminus of the PBC-B region downstream of the homodimer interface (Neuteboom and Murre, 1997; Calvo et al., 1999). This inhibitory domain has been proposed to form an alpha helix that contacts the homeodomain and blocks DNA-binding (Calvo et al., 1999). Upholding the model, mutation of homeodomain residue 28 from glutamic acid to arginine rescues DNA-binding in the presence of the inhibitory domain (Calvo et al., 1999). In addition, the PBX homeodomain and N-terminus physically interact in vitro (Saleh et al., 2000a).

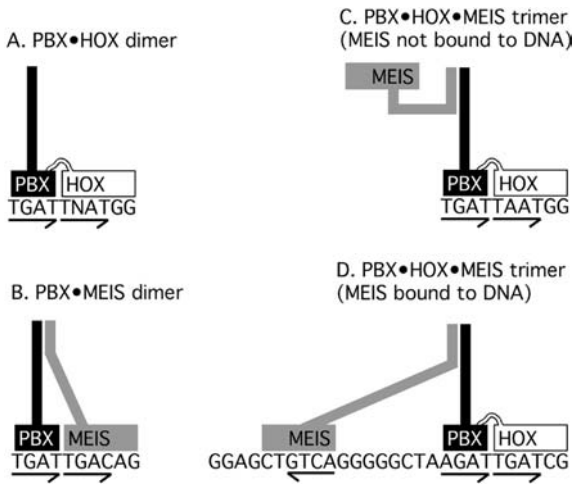

Fig. 3. DNA-binding complexes of HOX proteins and their partners. Four complexes are represented. Homeodomains are given by boxes containing the name of the factor. The N-termini of PBX and MEIS are represented by bars. The HOX linker and YPWM motif are drawn as a white squiggle. Half arrows denote core binding sites. (A) PBX–HOX dimer. Different complexes have preferences for the base at the sixth position, denoted by "N." (B) PBX–MEIS dimer. This complex is shown bound to abutting sites, but can form on widely separated sites in varying orientation. See "D." (C) PBX–HOX–MEIS trimer with MEIS not bound to DNA, such as observed with HOXD4 (Shanmugam et al., 1999). (D) PBX–HOX–MEIS trimer with MEIS bound to DNA, such as observed with HOXB1 on the *Hoxb2* r4 enhancer (Jacobs et al., 1999).

2.3. Co-operative DNA-binding by PBX–HOX heterodimers

Oligonucleotides that included the tandem PBX recognition site (TGATTGAT, Fig. 3A) were found to mediate co-operative DNA-binding by EXD and fly HOX proteins (van Dijk and Murre, 1994). This was followed by similar demonstrations for their mammalian counterparts (Chang et al., 1995; Knoepfler and Kamps, 1995; Neuteboom et al., 1995; Phelan et al., 1995; van Dijk et al., 1995; Lu and Kamps, 1996b; Green et al., 1998). Biochemical and structural analysis have shown that PBX/EXD binds the 5′ TGAT half-site and makes protein contacts to its HOX partner through the HOX YPWM motif (Lu et al., 1995; Knoepfler et al., 1996; Green et al., 1998; Passner et al., 1999; Piper et al., 1999). Mapping studies defined the minimal domains required for co-operativity, these being the HOX and PBX/EXD homeodomains, and the HOX YPWM (Chang et al., 1995; Johnson et al., 1995; Knoepfler and Kamps, 1995; Neuteboom et al., 1995; Phelan et al., 1995; Lu and Kamps, 1996b; Green et al., 1998). This implies that the YPWM directly contacts the PBX homeodomain (Lu and Kamps, 1996b; Peltenburg and Murre, 1997; Green et al., 1998), a model borne out by crystallographic and NMR structure determinations (Jabet et al., 1999; Passner et al., 1999; Piper et al., 1999; Sprules et al., 2000). Roles have also been suggested for a region of UBX C-terminal to the homeodomain (Chan et al., 1994; Galant et al., 2002; Gebelein et al., 2002), residues

N- and C-terminal to the ANTP homeodomain (Jaffe et al., 1997), and N-terminal domains of EXD and PBX (Chan et al., 1994; van Dijk and Murre, 1994). Large regions spanning both PBC-A and B domains are critical for the stability of PBX–HOX interactions (Fig. 2B) (Calvo et al., 1999; Shanmugam et al., 1999) and may be due to direct contact to the HOX homeodomain (Li et al., 1999a).

Upon DNA-binding, residues 53 to 58 adopt an alpha helical character, extending the length of helix 3 and inducing the formation of a fourth helix (Fig. 2B) just C-terminal to the homeodomain (Jabet et al., 1999; Piper et al., 1999; Sprules et al., 2000). A hydrophobic pocket, formed by the loop between helices 1 and 2, helix 3, the bend between helices 3 and 4, and helix 4, accommodates the YPWM motif through a series of van der Waals contacts and hydrogen bonds (Jabet et al., 1999; Passner et al., 1999; Piper et al., 1999; Sprules et al., 2000). The free HOX YPWM motif exists as a prefolded domain whose structure is maintained in complexes with PBX (Passner et al., 1999; Piper et al., 1999; Slupsky et al., 2001). Deletion of PBX helix 4 decreases affinity for the YPWM by 10 fold. This is likely due to destabilizing effects on helix 3, and the loss of a hydrogen bond between the region of the YPWM and Q64 of helix 4 (Sprules et al., 2000). The latter direct interaction could explain why helix 4 appears to play a more important role in interactions with certain HOX proteins (Chang et al., 1997a; Peltenburg and Murre, 1997). One of the few studies to look at the issue has demonstrated the importance of the YPWM domain for HOX function in vivo (Zhao et al., 1996).

2.4. Specificity of DNA-binding by PBX–HOX

The majority of HOX homeodomains recognize highly related sites with similar affinities, though products of the Abd-B class (paralog groups 9 to 13) prefer a TTAT core (Ekker et al., 1994). Somewhat better discrimination is conferred on PBX–HOX heterodimers. HOX proteins bind the 3′ TNAT in the PBX–HOX cooperative binding site (Fig. 3A). Because position 50 in the PBX homeodomain is occupied by glycine, the fifth and sixth positions of the site (TGATTNAT) should be specified by the N-terminal arm of the HOX partner only (Passner et al., 1999; Piper et al., 1999). Moreover, different HOX partners dictate preferential binding to sites that vary at the sixth position. Together, these results suggest that the HOX N-terminal arm plays a more important role in distinguishing the DNA-binding specificity of the PBX–HOX heterodimer than for HOX monomers.

Intriguingly, the N-terminal arm is located just C-terminal to the YPWM motif, with the two domains separated by a linker (Fig. 2A) whose length is characteristic of a given paralog group (Neuteboom et al., 1995; Phelan et al., 1995). Thus, interaction with PBX could effect a conformational change in the HOX N-terminal arm that alters its interaction with DNA. Indeed, a number of studies have implicated the HOX N-terminal arm and upstream linker in conferring DNA-binding specificity on the PBX–HOX complex (Chang et al., 1996; Knoepfler et al., 1996; Shen et al., 1996; Chan et al., 1997; Phelan and Featherstone, 1997; Ryoo and Mann, 1999; Gebelein et al., 2002) and on the specificity of HOX function in vivo

(Kuziora and McGinnis, 1991; Lin and McGinnis, 1992; Zeng et al., 1993; Chauvet et al., 2000; Gebelein et al., 2002).

It is somewhat perplexing, therefore, that structural studies indicate that the HOX N-terminal arm within PBX–HOX heterodimers does not contact the sixth position of the recognition sequence in DNA (Passner et al., 1999; Piper et al., 1999). However, the PBX–HOX complex does result in a widened minor groove in the region of the HOX N-terminal arm (Passner et al., 1999; Piper et al., 1999), which may alter protein–DNA contacts, perhaps influenced by additional partners such as MEIS/PREP. A role for positions 5 and 6 of the co-operative binding site in the specificity of PBX/EXD and HOX function is supported by experiments in vivo. Conversion of a "LAB" PBX–HOX binding site (TGATGGATGG) to a "DFD" binding site (TGATTAATGG) directs reporter gene activity in the fly embryo from the *lab* to the *Dfd* expression domain in a manner dependent on *exd* and *Dfd*. Moreover, similar "TA" sites direct expression with an r6/7 anterior border in the mouse hindbrain, corresponding to the domains of DFD homologs of the fourth paralog group (Chan et al., 1997). While dramatic, the "readout" for these experiments is transcriptional activation, and not DNA-binding. It therefore remains possible that many different PBC–HOX complexes can bind the above elements in vivo, but only one in each case achieves transcriptional activation depending on genetic and cellular context (White et al., 2000). In agreement with this, the same changes to positions 5 and 6 fail to switch responsiveness of a *decapentaplegic* (*dpp*) enhancer from EXD–LAB to EXD–DFD (Grieder et al., 1997). Reciprocally, the above noted EXD–LAB response element can be switched to an EXD–DFD response element without any change in the sequence of the actual binding site. Rather, this is accomplished by association with a 21 bp inverted repeat originally identified in a *Dfd* autoregulatory element (Li et al., 1999b). The unknown factor that presumably binds the inverted repeat could act to inhibit activation from EXD–LAB complexes, in addition to promoting activation from EXD–DFD complexes.

In another example suggesting that the contributions of positions 5 and 6 are not straightforward, elements bearing a "TA" or "AT" at these locations (TGATTATTGA and AGATTTATGA) direct expression to the r4/5 hindbrain border in response to group 3 paralogs in the mouse (Manzanares et al., 2001). Thus, the "TA" dinucleotide of the first element fails to mediate DFD/fourth group responsiveness, which would have dictated an anterior border at r6/7, not r4/5 (Chan et al., 1997). Additionally, while "TT" has been shown to confer preferential binding of posterior HOX proteins in complexes with PBX (Chang et al., 1996; Phelan and Featherstone, 1997), the second element, which bears this dinucleotide, responds in vivo to the more anterior third group HOX proteins. These observations introduce a note of caution: DNA-binding preferences established in vitro may not accurately predict the specificity of HOX function in vivo.

The question arises as to whether the YPWM can confer specificity in HOX–PBX interactions. There are minor variations to the motif that could play such a role. Arguing against this notion, however, several different YPWM peptides interact with the PBX homeodomain in the same way (Sprules et al., 2000). Residues flanking the YPWM are conserved within paralog groups (Neuteboom et al.,

1995; Shanmugam et al., 1997; Sharkey et al., 1997; Morgan et al., 2000) and influence the stability of PBX–HOX complexes on co-operative binding sites that differ at position six (Shanmugam et al., 1997). These amino acids could modulate interactions with PBX by inducing conformational changes in the PBX homeodomain.

As noted above, the only points of contact noted in the crystal structures of minimal HOX and PBX proteins cooperatively bound to DNA are between the YPWM and PBX homeodomain. Moreover, the PBX–HOX complex does not have dramatic effects on the conformation of DNA in the binding site. It is therefore surprising that separation of the PBX and HOX core recognition motifs by even a single base pair is deleterious to co-operative DNA-binding (Knoepfler et al., 1996). This is all the more striking since the flexible linker between the YPWM and HOX homeodomain (Fig. 2A) can be up to 53 residues, and should be able to span a considerable separation and rotation of the individual recognition sites (Phelan et al., 1995). Interestingly, DFD, along with other HOX proteins, can act together with EXD to regulate transcription through non-abutting sites that do not support co-operative DNA-binding in vitro (Pinsonneault et al., 1997; White et al., 2000), but which may do so in vivo under the stabilizing influence of HTH. Thus, HTH allows co-operative DNA-binding by EXD and UBX to separated half sites in a *distalless* (*dll*) regulatory region (White et al., 2000; Gebelein et al., 2002). In heterotrimers, therefore, the length of the HOX linker may set the tolerable distance between EXD and HOX half-sites.

2.5. DNA-binding by PBX and MEIS

Members of the MEIS family (MEIS, PREP, HTH) have an isoleucine at position 50 that is expected to dictate the preference for the AG dinucleotide in the MEIS binding site 5′ TGACAG 3′. HOX proteins from paralog groups 9 through 13 (Fig. 1) bind DNA cooperatively with MEIS to the sequence 5′ TGACAGTTTTACGAC 3′, where the first underlined bases are the core of the MEIS binding site, and the second set that of the HOX partner (Shen et al., 1997). The level of cooperativity is modest and limited to stabilization of MEIS binding, with little effect on HOX–DNA interaction (Shen et al., 1997; Shanmugam et al., 1999).

PBX/EXD and MEIS/PREP family proteins can also form homo- and heterodimers (Chang et al., 1997b; Knoepfler et al., 1997; Neuteboom and Murre, 1997; Berthelsen et al., 1998b,c; Calvo et al., 1999; Jacobs et al., 1999; Ryoo et al., 1999; Shanmugam et al., 1999; Fognani et al., 2002; Haller et al., 2002). Unlike PBX–HOX, PBX–MEIS/PREP interactions are stable in the absence of DNA-binding (Chang et al., 1997b; Calvo et al., 1999). Also unlike PBX–HOX, PBX and MEIS binding sites can be separated by up to 24 bp and can lie in direct or inverted orientations (Fig. 3B, D). The protein interfaces are formed by the PBC-A domain of PBX and the HR2 (and possibly HR1) domains of MEIS/PREP (Knoepfler et al., 1997; Berthelsen et al., 1998b; Shanmugam et al., 1999). These domains are relatively N-terminal in both proteins (Fig. 2B), which should permit the downstream homeodomains to swing away from each other analogous to the tips of a drawing compass. In addition, the regions of the proteins lying between their N-terminal

contacts and homeodomains must allow considerable rotation to accommodate both directly repeated and inverted binding sites on DNA. All of this implies a highly flexible region N-terminal to the PBX and MEIS homeodomains.

2.6. Trimeric interactions

PBX interacts with MEIS and HOX via non-overlapping domains located in its PBC-A and homeodomain, respectively (Fig. 2B). This allows the formation of PBX–HOX–MEIS heterotrimers that have been observed in vitro and implicated in enhancer function in vivo. Three types of trimer have been described, and are denoted here by means of the dimer that forms the core of the complex. In the first (Fig. 3C, D), a DNA-bound PBX–HOX heterodimer tethers a member of the MEIS/PREP/HTH family (e.g. PBX–HOX–MEIS) (Berthelsen et al., 1998c; Swift et al., 1998; Jacobs et al., 1999; Ryoo et al., 1999; Shanmugam et al., 1999; Shen et al., 1999; Ferretti et al., 2000). In the next form, a second molecule of PBX is brought into the complex by homodimeric interactions to give a PBX–HOX–PBX complex (K. Shanmugam and M. F., unpublished observations). Third, a MEIS–HOX dimer recruits PBX (MEIS–HOX–PBX) (Shanmugam et al., 1999). In the first and last of these complexes, the third partner need not bind DNA in vitro, and can be recruited by protein–protein interaction alone (Fig. 3C) (Berthelsen et al., 1998b). However, DNA-binding by MEIS/PREP family members may be critical when the PBC–HOX complex is presented with a sub-optimal site (Fig. 3D) (Jacobs et al., 1999; Ryoo et al., 1999; Ferretti et al., 2000; Gebelein et al., 2002). Even when the third partner does not directly contact DNA, it contributes to the DNA-binding stability of the complex as a whole (Shanmugam et al., 1999). The flexibility of PBX–MEIS interactions (see above) is reflected in the organization of binding sites for PBX–HOX–MEIS trimers in naturally occurring enhancers where the MEIS/PREP binding site can lie either 5′ or 3′ to the HOX–PBX site at a distance of several base pairs (Fig. 3D) (Jacobs et al., 1999; Ryoo et al., 1999; Ferretti et al., 2000; Gebelein et al., 2002). The ability to bind DNA as a trimeric complex may distinguish HOX function, since UBX, but not ANTP, forms heterotrimers with EXD and HTH on a *distalless* (*dll*) enhancer. The specificity of this interaction is dependent on a region of UBX C-terminal to the homeodomain (Gebelein et al., 2002).

3. Transcriptional regulation

3.1. Activation and repression: the role of co-factors

To determine whether HOX proteins, with or without their partners, act as repressors or activators of transcription requires access to direct targets in a given system. This has been approached in two ways: through the construction and testing of artificial HOX-responsive enhancers, and the identification of naturally occurring target elements. Both approaches confirm a role for HOX proteins in activation and repression (Krasnow et al., 1989; Johnson and Krasnow, 1990;

Pinsonneault et al., 1997; Jacobs et al., 1999; Ferretti et al., 2000; White et al., 2000). This duality is verified by genetic studies showing, for example, that UBX represses *Antp* (Peifer and Wieschaus, 1990), but activates *dpp* (Capovilla et al., 1994; Sun et al., 1995). More controversial is whether activation is the exclusive jurisdiction of PBC–HOX heterodimers.

McGinnis and colleagues have proposed an elegant and intriguing model whereby HOX proteins are converted from repressors (or neutral regulators) to activators by interaction with PBC members (Pinsonneault et al., 1997). The salient points of this EXD-switch model are as follows. First, EXD (or PBX) is strictly required for transcriptional activation, but not repression, by HOX proteins. Second, without denying the validity or biological importance of co-operative binding by EXD–HOX heterodimers to specialized compound sites, the authors propose that such cooperativity is neither required nor the norm. Rather, non-cooperative interaction between HOX and EXD can take place between non-adjacent sites. Third, when HOX proteins act as repressors, they do not require EXD/PBX, though they may need other co-factors. Fourth, the principal function of EXD/PBX is not to promote target site discrimination at the level of DNA-binding, but rather to stabilize a conformation of the HOX partner that favors activation.

Cogent arguments back up the model. In favor of an obligatory role for EXD in transcriptional activation by HOX proteins, the majority of known HOX-responsive elements that mediate transcriptional activation require the function of EXD or PBX proteins (Chan et al., 1994; Rauskolb and Wieschaus, 1994; Pöpperl et al., 1995; Sun et al., 1995; Chan et al., 1996; Gould et al., 1997; Maconochie et al., 1997; Pinsonneault et al., 1997; Jacobs et al., 1999; Ryoo and Mann, 1999; Ryoo et al., 1999; Ferretti et al., 2000; Brodu et al., 2002). Even a LAB derivative rendered hyperactive by mutation of its YPWM motif is still dependent on *exd* for transactivation function, confirming the importance of EXD for positive regulation by HOX proteins, and incidentally bearing out alternative mechanisms for HOX-EXD interaction (Chan et al., 1996; Pinsonneault et al., 1997).

Insight into the dispensability of co-operative DNA-binding derives in part from the study of an EXD- and DFD-dependent autoregulatory element of the fly *Dfd* gene, the 120 bp module E. Components of module E required for activity include a region of EXD binding, a recognition site for DFD, and a region of approximately 50 bp bearing an imperfect palindrome (Pinsonneault et al., 1997; Li et al., 1999b). Importantly, the DFD and EXD binding sites are not abutting and do not permit co-operative DNA-binding, although EXD does induce a mild increase in the association of DFD monomers with DNA in vitro. Despite the non-cooperative nature of these sites, EXD is required for the activity of module E, and mutations that increase or decrease the binding of EXD to module E cause proportional changes in enhancer activity in vivo (Pinsonneault et al., 1997). In a more extreme case, EXD is required for the activity of a *dpp* enhancer (Rauskolb and Wieschaus, 1994) despite the absence of EXD binding sites in a subfragment activated by UBX (Manak et al., 1995). Thus, co-operative DNA-binding and joint target site discrimination may not be prerequisites of transcriptional activation by EXD and HOX proteins (however, see following).

The authors also argue for the dispensability of EXD for transcriptional repression. The most compelling is genetic evidence showing that fly HOX proteins retain the ability to repress in the complete absence of *exd* function, that is to say, under conditions of maternal and zygotic *exd* deficiency (Peifer and Wieschaus, 1990). Moreover, UBX and ABD-A are known to repress through clusters of monomer binding sites not expected to mediate co-operative DNA-binding with EXD (Krasnow et al., 1989; Appel and Sakonju, 1993; Capovilla et al., 1994; Capovilla, 1998; Galant et al., 2002). In an insightful analysis of the zygotic *exd* phenotype, the authors show how the ability of UBX to confer the morphology of abdominal segments 2 and 4 (A2/4) on A1 could be explained by the acquisition of repressor functions by UBX following the loss of EXD, thereby making UBX mimic the normal repressive role of ABD-A in specifying A2/4 identity. That UBX indeed plays a role in the *exd* background is shown by comparison to the phenotype of *exd*/*Ubx* double mutants (Peifer and Wieschaus, 1990; Pinsonneault et al., 1997).

Fourth, providing a mechanistic basis for the conversion of HOX proteins to activators, EXD relieves an inhibitory effect of the DFD homeodomain on the activation function of the DFD N-terminus (Li et al., 1999a). This is demonstrated in two ways. First, the DFD activation domain as well as DFD–VP16 fusions are more active in the presence of EXD. The authors were careful to define and compare DFD and EXD–DFD binding sites of *equivalent affinity*, a key condition that strengthens their interpretation. Second, in experiments testing GAL4 fusion proteins on a GAL-responsive reporter, deletion of the DFD homeodomain potentiates the function of the activation domain. The DFD homeodomain is proposed to mask the DFD activation domain by direct intramolecular contact or (more likely) via an intermediary masking factor. EXD would liberate the DFD activation domain by contact to the DFD homeodomain, a proposal backed up experimentally (Li et al., 1999a). In agreement with this, mammalian HOX mutants unable to interact with PBX (but retaining full monomeric DNA-binding activity) fail to activate transcription in response to a histone deacetylase (HDAC) inhibitor (see Section 3.6) (Saleh et al., 2000b).

The EXD-switch model is further strengthened by analogy to the fly engrailed (EN) homeoprotein. EN is known to act as a potent repressor in many contexts. However, like HOX proteins, EN interacts with EXD via a tryptophan-containing motif (Peltenburg and Murre, 1996,1997), and the transcriptional activation functions of EN are dependent on EXD (Peifer and Wieschaus, 1990; Pinsonneault et al., 1997). This supplies strong ‘‘proof of principle’’ for the switch model.

Despite these persuasive arguments, some recent observations oppose a strict EXD-switch. First, HOX proteins have been shown to activate transcription through sites that do not mediate co-operative DNA-binding with PBC members. Thus, an enhancer of the fly *1.28* gene is positively regulated by four DFD binding sites that do not permit co-operative association with EXD (Pederson et al., 2000). Because the activity of this enhancer was not examined in an *exd* null background, however, it is possible that EXD exerts an effect nonetheless. It could be proposed, therefore,

that physical interaction with EXD is stabilized within the cell, and is sufficient to switch DFD from repressor to activator. This may also explain positive regulation by UBX and ABD-A through a 45 bp fragment of a *dpp* enhancer that does not bind EXD, despite the known *exd*-dependence of *dpp* expression (Rauskolb and Wieschaus, 1994; Manak et al., 1995). Such reasoning would not seem to apply to transfection results with a mammalian DFD ortholog. HOXD4 activates transcription of a reporter gene driven by HOX monomer binding sites in transfected mammalian cells. Moreover, mutation of the YPWM motif (which should abolish interaction with PBX) actually increases the transcriptional activation potential of HOXD4 (Rambaldi et al., 1994). However, PBX function may still be required for activation by the HOXD4 mutant, especially in light of the EXD-dependence of a similar mutant in flies (Chan et al., 1996). This is not the case for CR3, a HOX-responsive element of murine *Hoxb4*. CR3 binds the HOXB4 homeodomain (Gould et al., 1997), PBX–HOXD4 heterodimers (K. Shanmugam and M.F., unpublished observations), and responds to HOXB4, HOXD4, and HOXB5 in mouse embryos. It is also activated by DFD, SCR, and ANTP during fly development, but in a partially *exd*-independent fashion (Gould et al., 1997), arguing against an obligatory role for EXD in activation by HOX proteins.

Important objections to the EXD-switch model are raised by studies on a *dll* enhancer showing that HOX–EXD interactions lead to transcriptional repression. The expression of *dll* is repressed by UBX and ABD-A acting through a variant EXD–HOX recognition sequence bearing a one-base-pair spacer between half-sites (TGATTTAAT) (Vachon et al., 1992; White et al., 2000). This sequence is not cooperatively bound by EXD and UBX in vitro (White et al., 2000). Three copies of the element are able to repress a heterologous enhancer in *cis* in a manner dependent both on EXD and HOX bindings sites and on *exd* function (White et al., 2000). This provides a clear exception to the EXD-independence of HOX-mediated repression. Deletion of the intervening base pair to yield a more typical EXD–HOX binding site (TGATTAAT) yields an element that can now be activated by DFD and SCR, but which continues to mediate repression by UBX and ABD-A (White et al., 2000). Therefore, the ability to repress is not a strict function of half-site spacing. A subsequent investigation used a minimal *dll* enhancer containing additional flanking sequences that included an HTH binding site 7 bp from that of EXD. Unlike the first study, this expanded region supports co-operative DNA binding by an EXD–BX–HTH trimer that is dependent on sites for each of the three proteins. These same sites are also required for repression in vivo, confirming the biological relevance of trimer function for this activity (Gebelein et al., 2002). While the previous study observed repression despite the absence of the natural HTH recognition sequence (White et al., 2000), the use of three tandem copies of the EXD–HOX element may have fortuitously allowed binding of HTH to one of the EXD half-sites. Together, these findings implicate both EXD and HTH in repression by HOX proteins.

In another example, a HOX–EXD binding site in an enhancer of the fly *forkhead* (*fkh*) gene mediates activation by complexes of EXD with SCR, ANTP or UBX, but

repression by EXD–ABD-A (Ryoo and Mann, 1999). This repression is dependent on ABD-A since it is lost in *abd-A*/*Abd-B* double mutants. Moreover, the apparent repression is not just a failure to activate due to the decreased levels of EXD observed in abdominal segments, because activation of the enhancer within the *abd-A* expression domain is not rescued by increased levels of EXD and HTH. These findings corroborate a repression function of EXD–ABD-A, but other explanations are possible. Importantly, the authors did not directly demonstrate repression, but the lack of activation. Thus, EXD–ABD-A may not repress the *fkh* enhancer but simply fail to activate it. Since ABD-A normally represses *Ubx* expression in abdominal segments, *fkh* activation in *abd-A*/*Abd-B* double mutants could be due to EXD–UBX complexes formed following derepression of *Ubx*. Nonetheless, indications remain that EXD and HOX proteins in flies can act together to repress transcription.

In a mammalian system, transcriptional repression has also been ascribed to PBC–HOX heterodimers. Multimers of a PBX–HOX co-operative binding site (TGATTGAT) decrease reporter gene activity in transfected cells by comparison to an otherwise identical reporter bearing HOX monomer binding sites (Saleh et al., 2000b). However, at least some of this repression could be due to PBX homodimers and PBX–MEIS or PBX–PREP heterodimers that are likely to bind multimerized PBC–HOX sites. Two instances of EXD-dependent but HOX-independent repression could be mediated by comparable EXD homodimers or EXD–HTH heterodimers (Rauskolb and Wieschaus, 1994; Pinsonneault et al., 1997).

Various indirect arguments also suggest that the EXD switch model may require modification. First, while a number of HOX proteins have been shown to harbor activation domains (Samson et al., 1989; Rambaldi et al., 1994; Zappavigna et al., 1994; Viganò et al., 1998; Chariot et al., 1999; Li et al., 1999a; Saleh et al., 2000b; Tan et al., 2002), there are very few reports describing HOX repression domains (Schnabel and Abate-Shen, 1996; Galant and Carroll, 2002; Ronshaugen et al., 2002). This implies that co-repressor recruitment is not a widespread HOX function. However, repression could still be carried out by alternative mechanisms such as competition for activator binding sites, occlusion of activators through chromatin reorganization, or the generation of repression domains in arrays of multiply-bound HOX proteins. These possibilities are borne out by the ability of HOX proteins to repress through natural regulatory elements bearing clusters of HOX monomer binding sites (Krasnow et al., 1989; Appel and Sakonju, 1993; Capovilla, 1998; Galant et al., 2002). HOX monomers binding over several hundred base pairs could interact with each other to restructure chromatin, thereby rendering activator binding sites inaccessible (in a manner distinct from effects mediated by HDACs and SWI-SNF-like remodeling complexes) (Biggin and McGinnis, 1997).

Second, PBX is known to act as a transcriptional repressor, and to recruit co-repressor complexes bearing HDAC activity (Asahara et al., 1999; Saleh et al., 2000b). It follows that PBX homodimers may be expected to act as strict repressors, but co-repressor recruitment by PBX complexed with MEIS/PREP and/or HOX could also result in net repression. For the PBX–HOX complex to act invariably as a net activator of transcription, the activation function supplied by the HOX partner

(and/or other factors such as MEIS) would have to predominate over repression mediated by PBX. By analogy to the proposed unmasking of the HOX activation domain, this could be achieved by masking of the PBX repression domains upon interaction with HOX. Non-exclusively, the PBX–HOX complex may respond to cell signaling cues. For example, PKA dramatically increases transcriptional activation through PBX–HOX binding sites in transfected mammalian cells (Saleh et al., 2000b).

Interestingly, the limited HOX representatives in hydra do not display a YPWM motif (required for most interactions with PBC family proteins), nor has a *Pbx* ortholog been detected in their genomes (Galliot, 2000; Gauchat et al., 2000). This supports the notion that HOX proteins in all species must display PBC-independent functions, since it is unlikely that hydra evolved such features in isolation. However, if PBC members are required to switch HOX proteins from repressors to activators, then HOX proteins would act strictly to repress gene expression in hydra. This seems unlikely, and is refuted by studies on the *Hoxb4* CR3 noted above.

In summary, the evidence favors a tendency for HOX monomers to function as transcriptional repressors, and PBC–HOX dimers as transcriptional activators. However, it is clear that variables such as sequence context, co-factor availability, and cell signaling play determinative roles in establishing the polarity of regulation by HOX proteins.

3.2. Activation domains and co-activator recruitment

DNA-binding transcriptional regulators of the "enhancer factor" class possess activation and/or repression domains that recruit co-activators or co-repressors to target promoters (Triezenberg, 1995; Featherstone, 2002). To increase our understanding of gene regulation by HOX proteins, a number of groups have mapped activation domains in these products (Krasnow et al., 1989; Samson et al., 1989; Ali and Bienz, 1991; Rambaldi et al., 1994; Zappavigna et al., 1994; Zhao et al., 1996; Zhu and Kuziora, 1996; Di Rocco et al., 1997; Viganò et al., 1998; Chariot et al., 1999; Li et al., 1999a; Saleh et al., 2000b; Tan et al., 2002). Some shared features are apparent. All HOX proteins examined to date have activation domains located N-terminal to the homeodomain, though (rarely) additional activation functions may lie within and C-terminal to the homeodomain. Three types of generally recognized activation domain are characterized by amino acid content-acidic, glutamine-rich, and proline-rich—though such designations have little to do with functional specificity (Carey and Smale, 1999). Most HOX activation domains are enriched in proline. None are acidic, though many could be rendered so by phosphorylation of numerous serine and threonine residues. However, it is doubtful that amino acid character per se defines the function of HOX activation domains; HOXD4 and DFD, orthologous *Hox* products from humans and flies, can both activate expression of the *Dfd* gene in fly embryos (McGinnis et al., 1990), though HOXD4 contains a proline-rich activation domain while that of DFD is histidine- and glycine-rich (Rambaldi et al., 1994; Zhu and Kuziora, 1996; Li et al., 1999a). Likewise, functional differences between HOX proteins do not generally map

to known or suspected activation domains (Gibson et al., 1990; Kuziora and McGinnis, 1991; Lin and McGinnis, 1992; Zeng et al., 1993; Zhao and Potter, 2001, 2002), and for paralogs are attributable to their distinct expression patterns rather than divergences at the protein level (Greer et al., 2000). While it might be expected that secondary structure will be more important than amino acid content, a predicted alpha helix within the UBX activation domain is not conserved among orthologs beyond flies (Tan et al., 2002). Rather, an understanding of HOX activation function will best be served through the identification of their target co-activator complexes. Early results indicate that HOX proteins, despite differences in primary and secondary structure, may recruit the same coactivators. Thus, HOXB7 and HOXD4—diverged non-paralogous proteins—both recruit the CBP co-activator to N-terminal activation domains (Chariot et al., 1999; Saleh et al., 2000b) as does the HOX-like pancreatic factor PDX (Asahara et al., 1999). In addition, the *Drosophila nejire* gene, encoding a member of the CBP/p300 family, is a modifier of *Dfd* and *Ubx* function (Florence, 1998). The strength of these associations may be as important as the identity of the recruited co-factors (see Section 3.4).

HOX partners may also contribute directly and indirectly to activation functions. A weak activation domain in the PBX1A C-terminus has not been consistently observed (Lu and Kamps, 1996a; Di Rocco et al., 1997). A more important positive role for PBC family proteins may be to induce a conformational change in the HOX partner so as to expose its activation domain (Li et al., 1999a). Transcriptional activation domains have also been mapped to the MEIS1A and MEIS1B C-termini (H. Huang and M.F., unpublished observations), providing a possible explanation for the requirement of MEIS/PREP family members for activation through endogenous enhancers (Jacobs et al., 1999; Ryoo et al., 1999; Ferretti et al., 2000). The effects on phenotype and gene expression caused by fusion to strong activation or repression domains have been interpreted to indicate normal roles for HTH and MEIS in transcriptional activation (Dibner et al., 2001; Inbal et al., 2001; Maeda et al., 2002; Zhang et al., 2002) that are complemented by (or dependent on) the HOX partner (Vlachakis et al., 2001).

3.3. Repression domains and co-repressor recruitment

Genetic and molecular evidence demonstrates that HOX proteins are transcriptional repressors. Convincing examples in *Drosophila* include the repression of *Antp* sand *dll* by UBX (Vachon et al., 1992; Appel and Sakonju, 1993), and of *dpp* by ABD-A (Capovilla, 1998). One study mapped a UBX C-terminal repression domain composed of a monotonic stretch of alanine residues (Galant and Carroll, 2002), in line with previous reports ascribing a repressive role to alanine-rich regions (Hanna-Rose and Hansen, 1996; Lu and Kamps, 1996a; Saleh et al., 2000b). However, independent studies map the UBX repression function further N-terminal (Gebelein et al., 2002; Ronshaugen et al., 2002). Repression through a *dll* enhancer requires an extended linker separating the YPWM and homeodomain that is encoded by only some *Ubx* splice forms (Gebelein et al., 2002). The linker could directly harbor

repression domain, or interact with EXD or HTH to expose such activities. Analysis of mammalian HOXA7, HOXB4, and HOXC8 proteins implicates the homeodomain, plus additional regions, in repression (Schnabel and Abate-Shen, 1996), and could be due to homeodomain-mediated inhibition of CBP HAT activity (Shen et al., 2001).

The ability of EXD to repress target gene expression in HOX-dependent and -independent manners (Pinsonneault et al., 1997; White et al., 2000) implies that PBC family members harbor repression domains. A discrete region of the PBX N-terminus, including an evolutionarily conserved stretch of alanines, specifically represses SP1-mediated activation in a DNA-binding independent fashion (Lu and Kamps, 1996a). This seems unlikely to be due to a squelching mechanism since the PBX N-terminus has no transcriptional activation function (Lu and Kamps, 1996a; Di Rocco et al., 1997). Neither does PBX appear to interact directly with SP1 or TAF110, the SP1 co-activator (Lu and Kamps, 1996a). This DNA-binding-independent repression appears to have its parallel in homeodomain-independent transformation function by the E2A-PBX1 oncoprotein (Monica et al., 1994). Similar homeodomain-independent functions have been observed for the fushi tarazu (FTZ) homeoprotein, and are explained by the recruitment of FTZ to target promoters by protein–protein interaction (Fitzpatrick et al., 1992; Copeland et al., 1996).

A mechanism for repression by the PBX N-terminus derives from its interaction with a co-repressor complex including HDAC1 and 3, N-CoR/SMRT and mSIN3B (Saleh et al., 2000b). The small interaction domain includes the conserved alanine stretch reported previously to display repressor function. PBX could thus exert its non-DNA-binding repressive effect through the stabilization of repressor complexes at target promoters. However, the majority of PBX-mediated repression likely will be achieved through DNA-binding as homodimeric and heteromeric complexes. Another study mapped an N-CoR/SMRT-interacting repression domain to the C-terminus of PBX1A, but not PBX1B, suggesting isoform-specific PBX functions could be exploited in the cell to fine-tune gene expression patterns (Asahara et al., 1999). The conservation of the N-terminal alanine stretch in fly EXD, but not worm CEH-20, may indicate that co-repressor recruitment is a widespread but not universal regulatory mechanism of PBC proteins. Alternatively, the C-terminus of CEH-20 may have conserved repressor function, or novel repression domains may have evolved elsewhere in the protein.

Although repression domains have not yet been mapped to MEIS/HTH, genetic evidence demonstrates a role for HTH in the repression (and activation) of eye-specific gene activity (Bessa et al., 2002).

3.4. The specificity of HOX function: the balance between repression and activation

The deployment of different *Hox* genes in discrete domains along the AP axis confers positional identity to the embryonic trunk. It follows, therefore, that differences in the sequences between given HOX proteins must account for this specificity of function. However, functional studies on the one hand, and

biochemical analysis on the other, have generated an apparent contradiction. For, while the majority of investigations have mapped at least some functional differences between HOX proteins to the homeodomain (Gibson et al., 1990; Kuziora and McGinnis, 1991; Lin and McGinnis, 1992; Chan and Mann, 1993; Zeng et al., 1993; Chauvet et al., 2000; Zhao and Potter, 2001; Zhao and Potter, 2002), the differences in DNA-binding specificity mediated by the homeodomain are minimal (Biggin and McGinnis, 1997). The discovery of PBC family co-factors with which HOX partners bind DNA with higher affinity and specificity was thought to resolve this issue, but significant problems remain. First, while there are apparent preferences of given PBC–HOX complexes for co-operative sites defined by the sixth position, there is a great deal of overlap (Chang et al., 1996; Knoepfler et al., 1996; Shen et al., 1996; Chan et al., 1997; Phelan and Featherstone, 1997; Ryoo and Mann, 1999). Second, some enhancers responsive to HOX and EXD do not co-operatively bind these proteins (Pinsonneault et al., 1997; Pederson et al., 2000; White et al., 2000), although cooperativity may be achieved in the presence of HTH (Gebelein et al., 2002). Third, specificity can be switched from LAB to DFD responsiveness without altering the EXD–HOX binding site (Li et al., 1999b). Fourth, if the optimal binding sites defined in vitro were important for distinguishing PBC–HOX function, then this should be reflected in naturally occurring enhancers. Quite to the contrary, endogenous PBC–HOX-responsive sites are notable for deviations from the optimum that are expected to decrease DNA-binding affinity (Pöpperl et al., 1995; Maconochie et al., 1997; Ryoo et al., 1999; Manzanares et al., 2001; Gebelein et al., 2002). For example, the site in the *Hoxb1* autoregulatory element with the strongest in vivo activity, repeat 3, has a G instead of the optimal T at the fifth position (TGATGGAT) (Pöpperl et al., 1995). This substitution destabilizes a PBX–HOXA1 heterodimer by 6-fold (Phelan and Featherstone, 1997). Such results argue that differences in DNA-binding affinity may have little to do with distinguishing the specificity of HOX function. A similar lack of correlation between in vitro DNA-binding affinity and in vivo function has been noted for repression by ABD-A monomers (Capovilla, 1998). At least part of the discrepancy is likely due to the greater influence of enhanceosome context (Carey, 1998), such that synergy between HOX proteins (with and without their partners) and adjacently bound transcription factors is much more important than DNA-binding affinity for individual elements. Illustrating this point, although UBX and ANTP bind identical sites in an enhancer of *teashirt*, in vivo each protein uses independent subsets of these sites under the influence of adjacent tissue-specific elements (McCormick et al., 1995).

So, if specificity maps to the homeodomain, but is not determined by binding affinity, what is going on? A clue comes from examination of the N-terminal arm, a key subregion in distinguishing HOX specificity. Strikingly, residue differences in the N-terminal arm that confer specificity do not participate directly in site-specific recognition (Li and McGinnis, 1999), though arguments could be made for indirect readout by the sixth residue. The implication is that these amino acids are involved in another process, and Li and McGinnis have provided convincing evidence that this is at the level of transcriptional activation (Li and McGinnis, 1999). The authors found that increasing the transactivation function of UBX by fusion to

the strong transcriptional activation domain of VP16 resulted in a protein with ANTP-like morphogenetic properties. This is despite the identical DNA-binding specificities of the UBX and ANTP homeodomains. This important result suggests that the functional differences between HOX proteins lies in their relative effects on activation vs. repression, or the balance between these opposing activities at a given target promoter and tissue, a process termed "activity regulation" (Li and McGinnis, 1999). Just as PBC members may affect activation by the HOX partner, HOX may modulate repression by PBC. The HOX homeodomain participates in the first process by inhibiting transactivation function, an effect relieved by interaction with EXD (Li et al., 1999a). Differences in the N-terminal arm would therefore be predicted to influence this inhibitory effect, for example, by setting the affinity of binding to the proposed cellular masking factor, or by choosing among such factors. Alternatively, contact between the N-terminal arm and EXD could provoke a conformational change and release of the masking factor; residue differences in the N-terminal arm could affect the strength of the interaction with EXD, and thereby the efficiency of factor release. If the first of these options is correct, then mutation of the N-terminal arm should augment transactivation by GAL4-HOX fusions. The conserved G at position 6, characteristic of the optimal PBC–LAB binding sequence, but also present in the low-affinity sites found naturally, may be important not for optimizing DNA-binding affinity, but for setting a conformation of the N-terminal arm conducive to transactivation by LAB family members.

A role for activity regulation in defining the specificity of HOX function has the advantage of explaining some otherwise confounding results. The 120 bp module E is an autoregulatory element of the *Dfd Hox* gene (Zeng et al., 1994; Gross and McGinnis, 1996; Pinsonneault et al., 1997). Essential elements within module E include non-cooperative binding sites for DFD and EXD, plus an adjacent palindrome called region 5/6. Remarkably, insertion of region 5/6, which is inactive by itself, adjacent to co-operative binding sites for EXD–LAB heterodimers converts the latter from a LAB-responsive enhancer to a DFD-responsive enhancer (Li et al., 1999b). This refutes an exclusive role for DNA-binding in distinguishing HOX function, but is consistent with the activity regulation model. Thus, the unknown factor that presumably binds region 5/6 (there are no convincing sites for HTH) could specifically interact with the EXD–DFD complex, but not EXD–LAB, to activate transcription. Stereospecific intermolecular protein contacts would also explain why the activity of module E is dependent on the normal spacing between the DFD-binding site and region 5/6 (Li et al., 1999b). Extrapolating further, HOX-responsive elements may bind many different HOX proteins in vivo, but activate through only one. This could explain how interaction of EXD with ABD-A, but not UBX, leads to activation of *rhomboid* expression, despite highly similar ABD-A and UBX DNA-binding specificities (Brodu et al., 2002).

Two studies indicate that cell signaling controls HOX-specific regulation by affecting the activation/repression balance. The influence of casein kinase II (CKII) on ANTP activity may be at the level of transcriptional activation, since CKII

phosphorylation sites map outside of the homeodomain (Jaffe et al., 1997). Additionally, protein kinase A (PKA) and the HDAC inhibitor trichostatin A (TSA) greatly increase transcriptional activation through reporters driven by PBX–HOX binding sites (Saleh et al., 2000b). Both effects may be due to a tipping of the balance in favor of HOX-associated HAT activity over that of PBX-associated HDACs (see Section 3.6).

These developments confirm aspects of a recently proposed model (Biggin and McGinnis, 1997). The authors use many of the arguments summarized above to refute a ''co-selective'' model in which PBC family members would define HOX function by conferring DNA-binding specificity. Rather, they propose a ''widespread binding'' model in which HOX proteins bind to, and compete for, multiple sites in a given promoter without the aid of co-factors. The role of co-factors, such as PBC proteins, would be to act on HOX proteins that are *already bound* to convert them from repressive or neutral states to activators, following on ideas formulated elsewhere (Pinsonneault et al., 1997; Li et al., 1999a). The authors also envisage that, in addition to numerous high-affinity binding sites for HOX monomers, a given target promoter will also carry low-affinity binding sites that are only efficiently filled by co-operation with PBC members. Such sites would be especially important for mediating activation in conditions of low HOX protein concentrations. This is substantiated by the observation noted previously that many naturally occurring PBC–HOX binding sites are sub-optimal. That HOX proteins may be recruited in multiple copies to regulate target gene transcription is suggested by the mapping of ANTP- and UBX-responsive enhancers (Appel and Sakonju, 1993; Galant et al., 2002), and by physical studies on related homeodomain proteins that demonstrate binding to long stretches of DNA in target promoters (Walter et al., 1994; Walter and Biggin, 1996; Biggin and McGinnis, 1997). However, as already mentioned, an important determinant of specific HOX function is the surrounding sequence and transcription factor environment. Thus, regardless of the number of HOX proteins brought to a given promoter/enhancer, the transcriptional outcome will be highly influenced by flanking transcription factors, cell signaling, and other events (McCormick et al., 1995). This is clearly demonstrated at the *dll* enhancer where repression is dependent on the specific organization of HOX, EXD and HTH bindings sites leading to the recruitment and function of UBX but not ANTP (White et al., 2000; Gebelein et al., 2002). When comparing the relative activation and repression potentials of different HOX proteins, therefore, it should be remembered that these are unlikely to be fixed properties, and will vary qualitatively and quantitatively depending on genetic and cellular context.

3.5. The role of the third partner

As summarized earlier, DNA-bound PBC–HOX complexes can form hetero-trimers with MEIS/PREP/HTH. While not absolutely required for trimer formation, the third partner is critical for enhancer function. Thus, the activity of the r4 enhancer of the murine *Hoxb2* gene is dependent on HOX, PBX and MEIS/PREP binding sites (Jacobs et al., 1999; Ferretti et al., 2000). Likewise, an autoregulatory

element of the fly *lab* gene depends on binding sites for HOX, EXD and HTH (Ryoo and Mann, 1999). But, if PBX or EXD co-factors allow co-operative DNA-binding with HOX proteins, why further recruit MEIS/PREP/HTH co-factors? One possibility is to increase DNA-binding stability, perhaps all the more important on sub-optimal sites, since mutation of the recognition sequence for MEIS/PREP/HTH disrupts heterotrimeric binding to low-affinity PBC–HOX sites bearing a G at the fifth position (Jacobs et al., 1999; Ryoo et al., 1999; Ferretti et al., 2000) or in which EXD and HOX sites are separated by one base pair (Gebelein et al., 2002). However, DNA-binding defective MEIS proteins can still co-operate with HOX and PBX partners to activate hindbrain-specific gene expression (Vlachakis et al., 2001), and may be due to stabilizing effects on PBX–HOX (Shanmugam et al., 1999). Another explanation is that the third partner brings in activation and/or repression functions of its own that complement those of PBX and HOX. Thus, an activation domain mapping to the MEIS1A and 1B C-termini (H. Huang and M.F., unpublished results) could synergize with that of HOX proteins by creating a joint protein interface for the recruitment of transcriptional co-activators or components of the basal transcriptional machinery. Reciprocally, a PBX–HOX–PBX trimer would double the dose of PBX repression domains in the complex with possibly greater than additive effects. Mutation or deletion of the homeodomain in the third partner increases the extent and stability of trimer formation (Berthelsen et al., 1998b; Shanmugam et al., 1999), and could result from a change in conformation that frees the N-terminus for intermolecular interaction. This conformational change may normally be provoked by binding of the homeodomain to DNA. All such interactions would be potential targets for regulation by additional transcription factors and co-regulators, and by signal transduction cascades.

3.6. Chromatin remodeling and HOX function

Work of the last several years has brought chromatin to the forefront of our thinking on the control of gene expression. It is now clear that much of the function of eukaryotic transcriptional activators is to counter the repressive effects of chromatin organization. On the other hand, transcriptional repressors work in part by provoking and/or maintaining inhibitory chromatin structures. Central to these events are the activities of two classes of transcriptional co-factors: chromatin remodelers and histone modifying enzymes. Components of the yeast SWI/SNF complex are the prototypical (though perhaps not the most important) chromatin remodelers. In general, these ATP-dependent enzymes are able to alter the path of DNA around nucleosomes, expose binding sites in nucleosomal DNA, move nucleosomes from one site to another, and catalyze the exchange of nucleosomes between templates or histone pools (Näär et al., 2001; Featherstone, 2002). The N-termini of histones H3 and H4 are the targets of a number of histone modifying enzymes including kinases, histone acetyltransferases (HATs), histone deacetylases (HDACs), and histone methyltransferases. In brief, the action of HATs neutralizes the positive charge in histone N-termini leading to decreased interaction between

the nucleosome and DNA, and/or between adjacent nucleosomes. In conjunction with the activities of chromatin remodeling enzymes, this serves to "open" chromatin and render it more accessible to transcriptional regulators and components of the basal transcriptional machinery. The opposing activities of HDACs remove acetyl groups from the lysines in histone N-termini and help this process to work in reverse to repress transcription.

Leaving aside some chicken-and-egg conundrums, the role of many activators and repressors is to recruit HATs and HDACs, respectively, to target enhancers and promoters. HOX proteins and their partners have turned out to be no exception. The related CBP and p300 proteins are broadly employed co-activators with HAT activity (Vo and Goodman, 2001). Two HOX proteins, HOXB7 and HOXD4, have been shown to recruit CBP via their N-terminal activation domains, and sequestration of CBP by the adenoviral E1A protein decreases transcriptional activation by PBX–HOX (Chariot et al., 1999; Saleh et al., 2000b). By contrast, PBX contacts HDAC-containing co-repressor complexes through both N- and C-terminal domains (Asahara et al., 1999; Saleh et al., 2000b). This suggests that the PBX–HOX heterodimer may simultaneously recruit HDAC and HAT complexes with the overall output determined by the balance of these activities, supporting aspects of the activity regulation model discussed above (Li and McGinnis, 1999; Li et al., 1999a). Two findings lend credence to this scenario. First, treatment with the HDAC inhibitor TSA greatly increases transcriptional activation by HOX proteins and PBX–HOX complexes. This could be due to the inhibition of HDACs either recruited by the PBX partner or, non-exclusively, associated with the transcriptional template in a non-specific manner. Under both explanations, HDAC inhibition would swing the balance in favor of HAT activity leading to hyperacetylated chromatin and gene activation at the target locus.

Second, PKA likewise enhances transcriptional activation by PBX–HOX. While this could be accomplished through a variety of means, one possibility would unify this process with that mediated by TSA. PKA has been shown to lead to the phosphorylation of Ser10 in the histone H3 N-terminus (Salvador et al., 2001), and Ser10 phosphorylation has been strongly implicated in gene activation through its ability to promote stable HAT recruitment and histone acetylation (Lo et al., 2000; Lo et al., 2001; Salvador et al., 2001; Berger, 2002; Featherstone, 2002). In this way, PKA, like TSA, could ultimately promote histone hyperacetylation and transcriptional activation through PBX–HOX complexes. More complicated mechanisms are possible. For example, the PBX–HOX complex may also recruit ATP-dependent chromatin remodelers whose activities are complemented by the action of TSA/PKA. At naturally occurring enhancers and enhanceosomes, the action of TSA may be mimicked by adjacently bound transcriptional regulators which could recruit ATP-dependent chromatin remodelers and additional HAT or HDAC co-regulators, or which could stabilize the binding of those already enlisted by PBX–HOX. Such synergy would explain the association of HOX-responsive elements with tissue-specific enhancers (McCormick et al., 1995). While a role for PKA in the control of embryonic HOX function is neither confirmed nor refuted by genetic studies, PKA has been shown to influence the activity of

PBX-containing complexes at a natural enhancer (Kagawa et al., 1994; Ogo et al., 1995; Bischof et al., 1998a, 1998b).

Genetic studies in *C. elegans* corroborate the action of HOX proteins in the control of chromatin structure. *egl-27* is a worm gene homologous to *mta1* encoding a component of the NURD chromatin remodeling complex (Herman et al., 1999). Worm HOX proteins LIN-39 and MAB-5 interact to negate each others function in P(7–8).p cells of males. The morphogenetic activities of LIN-39 and MAB-5 are restored in *egl-27* loss-of-function mutants (Ch'ng and Kenyon, 1999), consistent with a model in which LIN-39 and MAB-5 cooperatively recruit a NURD-like complex to repress target genes that they would normally activate in isolation.

3.7. Transcriptional control through subcellular localization

A fundamental and effective way to control the activity of a transcriptional regulator is to restrict its access to the nucleus. While HOX proteins are generally constitutively nuclear, there are limited exceptions. The fly LAB protein is initially cytoplasmic in the midgut epithelium but localizes to the nucleus in response to signals from the adjacent visceral mesoderm (Immerglück et al., 1990). HOXB6 has been shown to be cytoplasmic in human fetal epidermis, though this is likely an isoform lacking the homeodomain (Kömüves et al., 2000), a region expected to harbor nuclear localization signals (Saleh et al., 2000a and references therein). Other work has detected concentrations of HOX proteins in the nucleolus (Magli et al., 1991).

A more important control of HOX function is achieved indirectly through the subcellular distribution of PBC, MEIS, and PREP proteins. EXD is strictly cytoplasmic in the *Drosophila* embryo (Mann and Abu-Shaar, 1996) until cell cycle 14 (stage 7) when cells within mitotic domains start to reveal nuclear staining (Aspland and White, 1997). Nuclear localization of EXD is dependent on HTH (Rieckhof et al., 1997; Kurant et al., 1998; Pai et al., 1998; Jaw et al., 2000). Thus, in the proximal limb primordia, EXD is co-expressed with HTH and both proteins are nuclear; more distally, however, *hth* is repressed and, as a consequence, EXD is cytoplasmic (Aspland and White, 1997; Gonzalez-Crespo et al., 1998). The importance of these interactions is underscored by their conservation in vertebrates; the subcellular localization of PBX is tightly controlled, with PBX1 excluded from the nucleus in the distal limb bud, gut epithelium and other tissues where *Meis* genes are not expressed (Cecconi et al., 1997; Oulad-Abdelghani et al., 1997; Gonzalez-Crespo et al., 1998; Capdevila et al., 1999; Mercader et al., 1999, 2000; Saleh et al., 2000a; Huang et al., 2002). Moreover, murine *Meis1* rescues the fly *hth* mutant phenotype by restoring EXD nuclear localization (Rieckhof et al., 1997), and both MEIS and PREP proteins direct the nuclear localization of PBX and EXD in cultured cells (Abu-Shaar et al., 1999; Berthelsen et al., 1999; Capdevila et al., 1999; Saleh et al., 2000a) and the fly embryo (Rieckhof et al., 1997; Jaw et al., 2000). More recent data show that the nuclear localization of MEIS and PREP proteins is reciprocally dependent on PBX (Berthelsen et al., 1999; Vlachakis et al., 2001; Huang et al., 2002). In addition to these effects on subcellular localization, EXD

and HTH also promote each others accumulation in the cell (Kurant et al., 1998; Abu-Shaar et al., 1999; Jaw et al., 2000).

While mutual interactions between PBC and MEIS/PREP family members are often key to their nuclear localization, this cannot be the whole story. First, at some sites, EXD is nuclear in the apparent absence of *hth* expression (Rieckhof et al., 1997). Additionally, PREP2, and likely PREP1, are expressed in the distal limb bud but do not direct the nuclear localization of PBX1 (Haller et al., 2002). These results are at odds with the demonstrated ability of human PREP1 (Berthelsen et al., 1999) and human and mouse PREP2 (Fognani et al., 2002) to direct PBX to the nucleus in cultured cells. Mouse PREP2 is likewise cytoplasmic in the distal limb bud despite the presence of PBX proteins which are able to localize PREP1 and PREP2 to the nucleus in cultured cells (Berthelsen et al., 1999; Fognani et al., 2002; Haller et al., 2002). The influence of cell signaling is likely to explain some of these discrepancies; the Wnt, DPP/BMP, FGF and hedgehog signaling pathways, in addition to retinoic acid, all influence the nuclear localization of PBC proteins (Mann and Abu-Shaar, 1996; Abu-Shaar and Mann, 1998; Gonzalez-Crespo et al., 1998; Capdevila et al., 1999; Mercader et al., 2000). Some of these effects are mediated through the control of *hth* and *Meis* expression, but could also impinge on the activity of the nuclear export and import machinery, or cytoplasmic retention factors like non-muscle myosin (see following).

The subcellular localization of PBC proteins is controlled at the level of nuclear export, nuclear import, and cytoplasmic retention (Fig. 4). Treatment of insect S2 cells with leptomycin B (LMB), an inhibitor of CRM1-mediated nuclear export, results in the relocation of endogenous EXD and transfected PBX to the nucleus, demonstrating the importance of this process in PBC intracellular distribution (Abu-Shaar et al., 1999; Berthelsen et al., 1999). Deletion analysis places a potential nuclear export sequence (NES) N-terminal to the PBX/EXD homeodomain (Abu-Shaar et al., 1999; Berthelsen et al., 1999). A discrepancy as to its exact position (Fig. 2B) would be resolved by direct tests of NES function. Conversely, intramolecular contacts to the PBX N-terminus mask two nuclear localization signals (NLS) in the PBX homeodomain (Fig. 2B), blocking both DNA-binding and nuclear accumulation (Calvo et al., 1999; Saleh et al., 2000a). A mutation in the homeodomain that disrupts interaction with the N-terminus (E28R, Fig. 2B) (Calvo et al., 1999) induces a conformational change in PBX and constitutive nuclear localization even in the absence of HTH/MEIS (Saleh et al., 2000a). These results suggest a model whereby PBC proteins adopt a default conformation in which the N-terminus masks the NLS in the homeodomain (Fig. 4). Interaction of the N-terminus with MEIS/PREP/HTH would expose the NLS, resulting in translocation to the nucleus. In the absence of MEIS, the NES is accessible and PBX/EXD is exported to the cytoplasm.

Although this might seem more than sufficient to control the subcellular distribution of PBC proteins, an additional mechanism comes into play at the level of cytoplasmic retention (Fig. 4). The action of a cytoplasmic retention factor was inferred from the observation that a fraction of PBC protein is resistant to LMB-induced nuclear accumulation (Abu-Shaar et al., 1999; Saleh et al.,

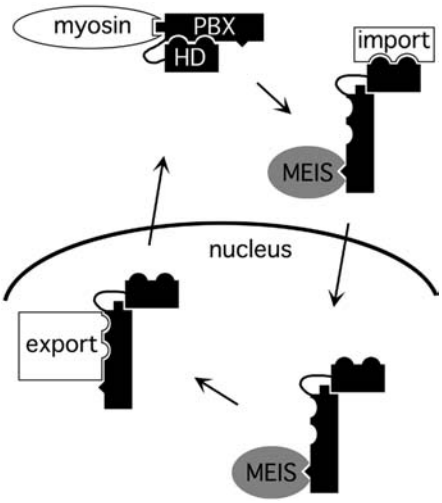

Fig. 4. A model for the control of PBX subcellular localization. Non-muscle myosin II heavy chain B (myosin) stabilizes a conformation of PBX that masks the two NLS in the homeodomain (HD). Interaction of MEIS/PREP (MEIS) with the PBX N-terminus (PBX) induces a conformational change that exposes the NLS, allowing contact with the nuclear import machinery (import). Dissociation of MEIS from PBX within the nucleus unmasks one or more NES and leads to interaction with the CRM1-dependent nuclear export apparatus.

2000a). Following a yeast two hybrid screen for novel proteins interacting with the PBX N-terminus, a fragment of non-muscle myosin II heavy chain B (NMHCB) was found to promote the efficient cytoplasmic localization of PBX, even out-competing the opposing action of MEIS. Importantly, NMHCB and PBX co-precipitate from mammalian cell extracts, and extensively co-localize in the cytoplasm of distal limb bud cells in the mouse embryo. This mechanism is evolutionarily conserved, since EXD co-localizes with insect non-muscle myosin (ZIPPER), and *zipper* mutants display aberrant EXD nuclear localization. The normal location of NMHCB in the cytoplasm, and the ability of a NMHCB fragment, which should be unable to participate in acto-myosin assemblies, to promote the cytoplasmic accumulation of PBX, suggests that it may stabilize masking of the NLS (Huang et al., 2002).

Although their function is no doubt affected by the nuclear availability of EXD, HOX proteins in turn control the levels of *hth* and *exd* expression. Thus, *exd* transcripts are reduced in the abdomen of the fly embryo through the action of BX-C *Hox* genes (Rauskolb et al., 1993), and *Scr*, *Antp*, *Ubx* and *abd-A* all repress *hth* transcription (Casares and Mann, 1998; Kurant et al., 1998; Yao et al., 1999). These effects on *hth* expression may account for the ability of BX-C *Hox* genes to inhibit the nuclear localization of EXD (Azpiazu and Morata, 1998). The multiplicity of controls at the levels of transcription, subcellular localization, and protein turnover suggests that the functions of EXD and HTH, both with and without HOX partners, must be carefully titrated to direct developmental programs appropriately.

This may be necessary to set the correct ratios of various complexes—PBC–PBC, PBC–MEIS/PREP, PBC–HOX–MEIS/PREP—with potentially opposing activities. This presumed importance of the levels of EXD and HTH stands in contrast to data suggesting that HOX proteins exert similar effects over a range of concentrations (Castelli-Gair and Akam, 1995).

3.8. The role of signaling pathways

The prediction that HOX proteins would interpret cell signaling cues (Davidson, 1991), has been amply validated (Florence, 1998; Mann and Affolter, 1998). It is telling that almost half the genes uncovered in screens for *Dfd* modifiers function in cell signaling, including components of the Notch and hedgehog pathways (Florence, 1998). Cell signaling exerts both direct and indirect effects on transcriptional regulation by HOX proteins. Some of the effects of PKA, CKII, Wnt, DPP, and hedgehog have already been discussed in context. In addition, phosphorylation by CKII attenuates the ability of ANTP to bind DNA cooperatively with EXD, providing a means by which HOX function could be tied to the cell cycle (Jaffe et al., 1997). At another level, an enhancer of the fly *lab* gene integrates—through distinct neighboring sites—DPP signaling with the activity of an EXD–LAB–HTH complex to establish region-specific expression in gut endoderm (Grieder et al., 1997; Marty et al., 2001). Synergy in such cases should employ mechanisms elaborated for enhanceosome function, including DNA bending, co-operative DNA-binding, the formation of a stereospecific multi-component activator-complex architecture, cooperative coactivator recruitment, and nucleosome displacement (Carey, 1998; Featherstone, 2002; Mann and Carroll, 2002). Genetic studies in the nematode show that *Hox* genes both respond to and interpret RAS signaling events (Clandinin et al., 1997; Eisenmann et al., 1998; Maloof and Kenyon, 1998). Given the involvement of RAS in the activities of numerous growth factors and morphogens, HOX proteins should heavily influence the competence of cells responding to *ras*-dependent inducers. As noted above, phosphorylation is likely to affect numerous HOX functions such as subcellular localization, heteromeric interactions, DNA-binding affinity, the strength of transcriptional activation and repression domains, co-regulator recruitment, and protein turnover.

3.9. The E2A-PBX1 oncoprotein

Human *PBX1* was first identified by analysis of the t(1;19) breakpoint present in one quarter of pediatric pre-B cell leukemias (Kamps et al., 1990; Nourse et al., 1990). The translocation results in a fusion protein whose N-terminus bears the strong transcriptional activation domain encoded by E2A. A number of studies have documented the increased transactivation potential of E2A-PBX (Van Dijk et al., 1993; LeBrun and Cleary, 1994; Lu et al., 1994; Monica et al., 1994; Phelan et al., 1995), and proven that this is essential for E2A-PBX-mediated transformation

(Monica et al., 1994; Kamps et al., 1996). Like PBX, activation by the fusion protein is augmented by PKA (Ogo et al., 1995).

In terms of co-factor interactions, two points are noteworthy. First, E2A-PBX1 lacks the N-terminal 89 residues of PBX1, impinging on the PBC-A domain. This abolishes interaction with MEIS/PREP family members (Chang et al., 1997b; Knoepfler et al., 1997) but does not impair homodimerization on abutting half-sites (TGATTGAT) (Calvo et al., 1999). By contrast, E2A-PBX1A homodimers do not form on sites separated by 18 bp under conditions allowing homodimerization by wild-type PBX1A (K. Shanmugam and M.F., unpublished observations). Second, because the homeodomain is intact, E2A-PBX continues to present a molecular target for the HOX YPWM motif and is able to bind DNA cooperatively with HOX proteins and activate transcription in a HOX-dependent manner (Phelan et al., 1995; Chang et al., 1997a; Lu and Kamps, 1997).

Since HOX proteins cooperate with PBX to transform fibroblasts in YPWM-dependent manner (Krosl et al., 1998), it seems reasonable that they would also cooperate with E2A-PBX in cancer causation; and in fact, this has been demonstrated (Thorsteinsdottir et al., 1999). Does this mean that such synergy is at the level of co-operative DNA-binding? Not necessarily, since the YPWM/ANW motif is dispensable for myeloid progenitor immortalization (Calvo et al., 2000), and HOXD13, which naturally lacks a PBX interaction motif, is leukemogenic as a translocation-induced fusion protein (Raza-Egilmez et al., 1998). A similar fusion oncoprotein derived from HOXA9 also continues to promote the proliferation of bone marrow progenitors after mutation of the ANW motif (Kroon et al., 2001). Additionally, the E2A-PBX homeodomain is dispensable for transformation of fibroblasts in vitro, or induction of lymphomas in transgenic mice (Monica et al., 1994; Kamps et al., 1996), though it is necessary to immortalize cultured myeloid progenitors (Kamps et al., 1996). Since it is required for co-operative DNA-binding with HOX partners, the dispensability of the PBX homeodomain is at odds with a HOX-dependent co-operative DNA-binding mechanism of E2A-PBX-mediated transformation. This does not prove, however, that the two classes of protein do not synergize on the same oncogenic target genes. Synergy could rely on protein–protein contacts that are independent of the EXD/PBX homeodomain, analogous to the recruitment of a homeodomainless FTZ to a target promoter by PAIRED (Copeland et al., 1996).

4. Conclusions

The relatively simple structure of the homeodomain has been a poor indicator of the complex molecular interactions governing HOX function. One of the surprises of recent years is that binding site recognition may not play a predominant role in distinguishing the function of different HOX proteins. Rather, the sequence and organization of these sites is likely to collaborate with variations in primary structure to affect protein conformation and stereospecific contacts leading to differential

interaction with other transcription factors and co-regulators. One of our challenges is to determine how this occurs at a molecular level.

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Msx genes in organogenesis and human disease

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Contents

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1. Introduction

Msx genes are a highly conserved family of homeobox genes whose members have been identified in a wide variety of metazoans (Davidson, 1995). The *Msx* gene family takes its name from the *msh* (muscle segment homeobox) gene of *Drosophila*, which functions in neurogenesis, myogenesis, and the dorsoventral patterning of the wing. A decade of investigation has revealed that *Msx* genes function in diverse animal groups in the control of cell identity, cell migration, and cell proliferation, and that they have causative roles in human genetic syndromes that affect the development of the skull, teeth, and limbs (Maas et al., 1997; Wilkie, 1997). Current research has converged on an association between *Msx* genes and the problem of how extracellular signals control the balance of proliferation and differentiation—and thus pattern. We begin our review with an overview of the evolution and function of the *Msx* gene family. We then focus the role of one of the three mammalian *Msx* genes, *Msx2*, in human genetic disease and cranial development.

2. *Msx* gene structure and evolution

Beginning with the first comparative analyses of *Msx* homeodomain sequences more than a dozen years ago, it was evident that the *Msx* homeodomain was highly conserved across large phylogenetic distances (Holland, 1991). These initial findings together with data that have accumulated in the intervening decade show that *Msx*-related genes are clearly recognizable throughout the metazoa in groups as divergent as porifera (sponges) and vertebrates (Seimiya et al., 1994). Indeed, a 70% identity in *Msx* homeodomain amino acid sequence is apparent between sponges and vertebrates, a level of similarity that is remarkable even among highly conserved homeobox genes. This high degree of conservation suggests that aspects of *Msx* gene function are fundamental and unchanging over wide phylogenetic distances.

Based on the amino acid sequences of their homeodomains, *Msx* genes are grouped in the *Nk*-like family of homeobox genes (Gauchat et al., 2000; Pollard and Holland, 2000). Other *Nk* family members include *Nk1*, *Nk3*, *Nk4*, *Lbx*, *Tlx*, *Emx*, *Nk6*, *Vax*, *Hmx*, *Dlx*, *Not*, *Barx*, and *Hex*. *Nk*-like genes are one of four families of homeobox genes that comprise the *Antennapedia* superclass

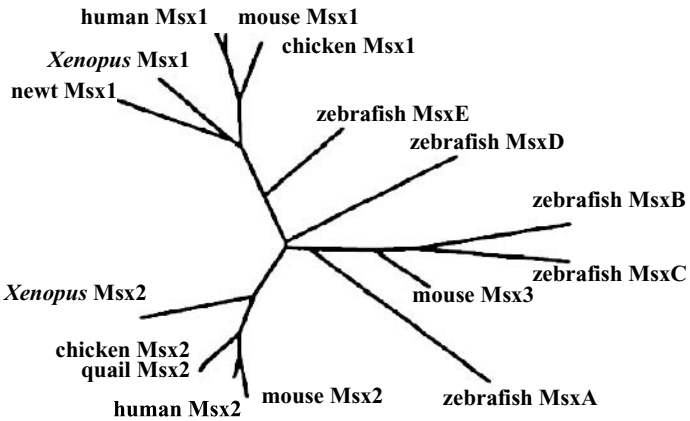

Fig. 1. Phylogeny of vertebrate *Msx* genes (after Westerfield et al., 1997). The tree is unrooted. Branch lengths are proportional to the number of substitutions.

(Pollard and Holland, 2000). The other families in this superclass are the "extended Hox" (*Hox*, *Evx*, and *Mox*), ParaHox (*Cdx*, *Xlox*, *Gsx*), and *EHGbox* (*En*, *HB9*, *Gbx*). Each family exhibits some degree of clustered organization, which is most extreme in the highly conserved clusters of Hox genes. That each family is clustered has led to the proposal that an ancestral metazoan had four clusters of *Antennapedia*-related genes, each of which arose from the duplication of an ancestral family member. These four founding family member genes are presumed to have arisen from the duplication of a single ancestral gene co-incident with the emergence of multicellular life (Gauchat et al., 2000; Pollard and Holland, 2000).

All invertebrates examined to date, including sponges, nematodes, arthropods, sea urchins, ascidians, and cephalochordates, have single *Msx* genes. Teleostean fishes, amphibians, birds, and mammals each have several *Msx* genes (Ekker et al., 1997). Birds, mice, and humans have at least three *Msx* genes, *Msx1–3*. This suggests that *Msx* genes duplicated some time after the divergence of basal chordates from the line that led to the vertebrates. The relationships among vertebrate *Msx* genes are shown in Fig. 1. Orthologies are difficult to discern outside of the bird-mammal group, suggesting that independent duplications or gene losses probably occurred in different chordate lineages (Ekker et al., 1997).

Sequence comparisons among mammals and birds have identified conservative regions of *Msx* amino acid sequence outside the homeodomain (Fig. 2). These include N-terminal and C-terminal extensions of the homeodomain (designated the "extended homeodomain"), as well as conserved regions outside the homeodomain (Bell et al., 1993; Shang et al., 1994; Akimenko et al., 1995; Ekker et al., 1997). One such domain, LPSFVEAL, located in the N-terminus, likely functions in transcriptional repression (Smith and Jaynes, 1996).

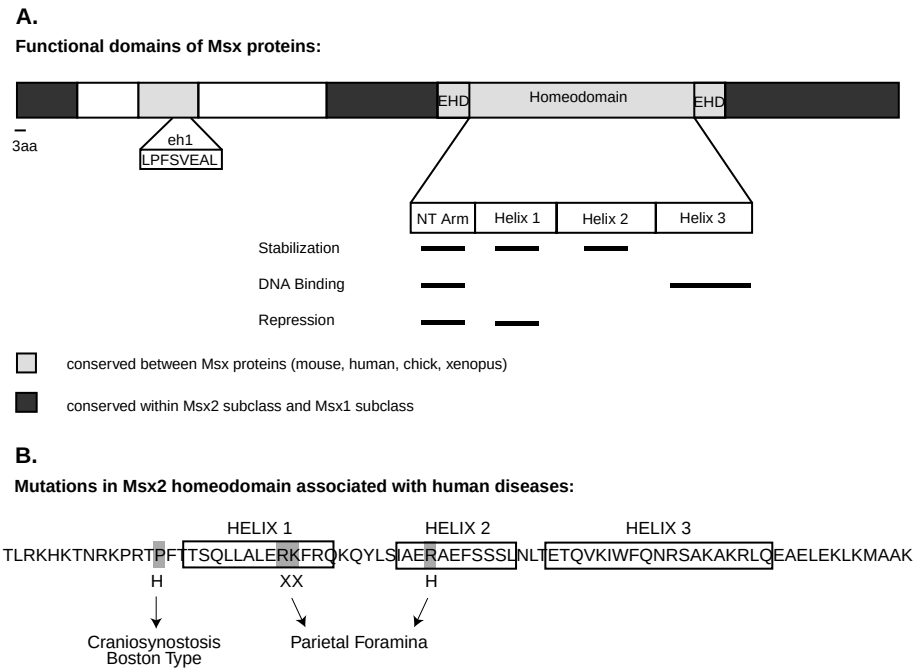

Fig. 2. A. Domain structure of *Msx* proteins (after Hu et al., 1998). Eh1 is a repressor domain shared by engrailed and a number of other homeodomain-containing proteins (Smith and Jaynes, 1996). B. Mutations in *Msx2* homeodomain that engender human diseases. Data from Jabs et al. (1993) and Wilkie et al. (2000). (See Color Insert.)

3. A comparative view of *Msx* gene function: lessons from invertebrates, fishes, and amphibians

The *Msx1* and *2* genes of mammals are expressed in a partially overlapping pattern in a broad spectrum of tissues during embryogenesis, including the neural tube, migratory neural crest cells, branchial arches, placodes, limb buds, tooth buds, skull vault, mammary gland, genital ridge, pituitary and hair follicles. *Msx3* expression is confined to the neural tube. Functional analyses have demonstrated that *Msx1* and *2* have roles in the development of the middle ear, tooth, and palate (Satokata and Maas, 1994; Foerst-Potts and Sadler, 1997). *Msx2* has roles in the development of the skull vault, hair follicle, mammary gland, and tooth (Satokata et al., 2000). *Msx* genes thus have an almost bewildering variety of activities during embryogenesis. A useful approach to untangling this multiplicity of roles in mammalian development is to compare *Msx* gene function across the metazoa.

The majority of metazoans are grouped in the bilateria, characterized by bilateral symmetry and triploblastic body plans. The bilateria are divided into two superphyla, the protostomes (arthropods, mollusks, annelids, and other phyla)

and the deuterostomes (echinoderms, hemichordates, and chordates). In this section, we review information on *Msx* gene function in a protostome (*Drosophila*) and in several invertebrate deuterostomes (echinoderms, cephalochordates and chordates). We also consider representatives of basal vertebrate classes, including zebrafish and *Xenopus*. We note parallels between the activities of *Msx* genes in these more ancient animal groups compared with the activities of such genes in mammals.

3.1. msh function in Drosophila

3.1.1. msh and the specification of neural and muscle lineages in Drosophila

Recent genetic studies have led to an increasingly detailed picture of *msh* gene function in *Drosophila*, and have thus provided useful hypotheses for approaching *Msx* gene activity in vertebrates. These studies include an analysis of an *msh* loss of function allele, as well as recent work documenting *msh* gain of function phenotypes (Tiong et al., 1995; Isshiki et al., 1997; Nose et al., 1998; Mozer, 2001; Jagla et al., 2002).

Isshiki et al. (1997) have identified a loss of function allele and investigated its effects on the development of the nervous system and the dorsal musculature. This allele was generated by imprecise excision of a P-element from the *msh* gene. In *msh* loss of function mutants, dorsal neuroblasts form but do not divide or migrate appropriately. These defects occur in two distinct neuroblast lineages. In a third lineage, marker studies provided evidence for a dorsal to ventral fate change. *msh* may thus influence not only proliferation and migration, but also, at least in subset of neuroblasts, cell fate. It remains unclear whether these defects are cell autonomous. *msh* is expressed both in neuroblasts and in adjacent ectoderm, and may function in one or both of these tissues.

Parallel findings have emerged from an analysis of the effect of this loss of function allele on muscle development (Nose et al., 1998). Each abdominal hemisegment of the *Drosophila* embryo contains a number of distinct muscle types which are specified by distinct classes of myoblasts (Bate, 1990). How such founders are segregated during mesodermal development has been a focus of research. Nose and colleagues (Nose et al., 1998) show that *msh* is expressed in subsets of muscle progenitors. In *msh* mutants, defects are evident in the number of muscle fibers as well as in the morphology of such fibers. These defects are caused by inappropriate specification of progenitor cells that give rise to different muscle types. Although progenitors formed normally, subsequent development in which the progenitors go on to form precursors of specific muscles was disrupted. *msh* is thus required for aspects of the myogenic program that result in formation of particular muscle precursors. These findings are consistent with results of Storti and colleagues (Lord et al., 1995), who showed that misexpression of *msh* causes a loss of some muscles and defects in the patterning of others.

Further evidence for the involvement of *msh* in the specification of distinct muscle precursor cells came from a genetic analysis of regulatory interactions among three

genes, ladybird, *msh* and eve, which together specify a subset of dorsal muscle precursors (Jagla et al., 2002). *Lady bird* and *msh* are members of the *Nk* family; *eve* is a member of the extended Hox family (Jagla et al., 1997; Pollard and Holland, 2000). Using a combination of gain of function and loss of function approaches, Jagla et al. (2002) showed that deregulation of each gene influences the number of cells expressing the other two genes. This results in a change in the relative number of cardiac muscle cells, as well as a change in the ratio of subsets of cells within the heart and dorsal muscle. Mutually repressive interactions between these identity genes maintain their respective domains of expression, and these interactions are essential for establishing cell identity.

Taken together, these results show that *msh* has parallel functions in cell fate specification in myogenesis and neurogenesis. In both processes, *msh* has a role in later events that result in particular neural or muscle cell types—not in the initial formation of progenitor cells. Also, expression of *msh* in both neural and muscle progenitors is preceded by expression in the adjacent ectoderm, again suggesting that *msh* participates in inductive interactions underlying the development of neural cells. It is interesting that in mammals, forced expression of *Msx1* in differentiating myoblasts can prevent, or even reverse differentiation (Odelberg et al., 2000). These findings, together with results from work in *Drosophila* (Nose et al., 1998), suggest that *Msx* genes may have conserved functions in the control of myoblast specification and differentiation.

3.1.2. Wing patterning

A role for the *msh* gene in the dorsoventral patterning of the *Drosophila* wing has been uncovered by Cohen and co-workers (Milan et al., 2001). The dorsoventral subdivision of the *Drosophila* wing is controlled by the function of the *apterous* gene in dorsal cells (Diaz-Benjumea and Cohen, 1993; Blair et al., 1994). Cells that express *apterous* form dorsal structures, while cells lacking *apterous* form ventral structures. Analysis of *msh* and apterous mutants showed that *msh* is both necessary and sufficient for dorsal fate during wing development (Milan et al., 2001). In addition, the expression of *msh* is under the control of *apterous*: *msh* expression is reduced in *apterous* mutants, and ectopic expression of *apterous* leads to ectopic activation of *msh*. That *msh* is an obligate downstream element in the *apterous* pathway is shown by the finding that expression of *msh* under the control of the Gal4 promoter restores dorsal identity in the absence of *apterous* (Milan et al., 2001).

Additional evidence of a role for *msh* in wing development in *Drosophila* came from the identification of the *Dorsal wing* (*Dlw*) locus (Tiong et al., 1995) as a gain of function allele of *msh*. Caused by transposon insertions in the upstream region of *msh*, *Dlw* results in apterous-independent expression of *msh* (Milan et al., 2001) and abnormalities in dorsoventral wing patterning. Curiously, a separate gain of function mutation in *msh*, *Drop*, was found to produce defects in eye development (Mozer, 2001). This phenotype is caused by retroposon driven ectopic expression of *msh* in the eye, and is apparently unrelated to the normal function of *msh*.

The intriguing possibility that at least some aspects of the function of *Msx* genes in the dorsoventral patterning of appendages might be conserved between *Drosophila* and vertebrates is suggested by the finding that *Msx1* is mutated in individuals affected with Witkop syndrome, also known as "tooth and nail syndrome" (Jumlongras et al., 2001). This rare autosomal dominant disorder is characterized by nail dysplasia and several congenitally missing teeth (Witkop, 1965). The mutation results in a stop codon at amino acid 37 of the homeodomain and thus a truncated protein that lacks a portion of the homeodomain as well as the entire C-terminal region. This mutation probably leads to loss of function of the mutant allele. The nail dysplasia phenotype is essentially a defect in dorsal identity of cells of the distal limb; it may thus be analogous to the loss of dorsal identity that occurs in *msh* and apterous mutants in flies (Milan et al., 2001).

There is also reason to believe that upstream elements in the appendage patterning pathway might be conserved. Apterous homologues have been identified in vertebrates, and two of these, *Lmx1* and *Lhx2*, are required in vertebrate limb development. *Lmx1* is expressed in the dorsal compartment of the vertebrate limb, and can confer dorsal identity (Vogel et al., 1995). *Lhx2* induces expression of radical fringe in the apical ectodermal ridge, and thus influences limb outgrowth (Laufer et al., 1997; Rodriguez-Esteban et al., 1997; Rodriguez-Esteban et al., 1998). Finally, it is intriguing that a targeted mutation in *Lmx1b* result in defects in skull vault development, including overgrowth of calvarial bones (Chen et al., 1998). Since gain of function mutations in *Msx2* have been implicated in similar phenotypes (Jabs et al., 1993; Liu et al., 1995, 1999), it is possible that *Lmx1b* acts in opposition to *Msx2* during calvarial development. Finally, it is interesting that Lhx2 can interact physically with Msx1 and modify its DNA binding activity (Bendall et al., 1998), providing another potential mechanism by which Lim homeodomain proteins could influence *Msx* gene activity.

3.2. Sea urchins: the patterning of primary mesenchyme and the larval skeleton

Sea urchins (echinoderms) are members of the ambulacraria, the sister group to the chordates (Furlong and Holland, 2002); thus analysis of their developmental programs can provide key insights into the evolution of developmental mechanisms in chordates. A single *Msx* gene has been detected in the sea urchin (Strongylocentrotus purpuratus) genome (Tan et al., 1998). Designated *SpMsx,* this gene is expressed broadly in blastula stage embryos. Subsequently, its expression is restricted to the vegetal plate and oral ectoderm. The vegetal plate includes precursors of the primary mesenchyme, which give rise to the larval skeleton (Ettensohn, 1992; Brandhorst and Klein, 2002). In later stages, *SpMsx* is expressed in the gut, oral ectoderm and larval arms. Tan and colleagues have shown that overexpressing *SpMsx* in sea urchin embryos causes defects in the patterning of the primary mesenchyme and larval skeleton. They showed that in wild type embryos, primary mesenchyme cells are distributed in a circumpolar ring, whereas in embryos derived from *SpMsx* mRNA injected eggs, primary mesenchyme cells are irregularly arranged. Moreover, blastomere transplantation studies showed that this irregular

arrangement is dependent on overexpression of *SpMsx* in the ectoderm. Although loss of function data are not available, these results suggest that *SpMsx* may have a role in establishing the arrangement of primary mesenchyme. Whether such a non-autonomous role in mesenchyme patterning is conserved is not clear, though it is intriguing that just such a role has been suggested *Drosophila msh* in the patterning of muscle and neural precursors (Milan et al., 2001).

3.3. Ascidians and cephalochordates

Like sea urchins, ascidians and cephalochordates probably have single *Msx* genes (Ma et al., 1996; Sharman et al., 1999). Thus the multiple *Msx* genes present in vertebrate genomes probably arose after the divergence of the cephalochordates from the vertebrate lineage. The close affinity of ascidians and cephalochordates to vertebrates makes these groups especially useful in uncovering ancestral and derived functions of vertebrate *Msx* genes. Expression studies have shown that an ascidian *Msx* gene is first expressed prior to gastrulation in the ectoderm (neural plate) and mesoderm (presumptive notochord and muscle)(Ma et al., 1996). *Msx* transcripts decline in the mesoderm but persist in the neural plate during neuralation. Transcripts of a cephalochordate (amphioxus) *Msx* gene are first detectable in the neural plate during gastrulation (Sharman et al., 1999). Subsequently they are found in dorsal cells of the neural tube and, transiently, in dorsal cells within somites. These dorsal cells are reminiscent of migratory neural crest of vertebrates (Christiansen et al., 2000). The expression patterns of the ascidian and amphioxus *Msx* genes are at least broadly similar to those of vertebrate *Msx* genes. Moreover, these patterns, particularly that of the amphioxus *Msx* gene, suggest that *Msx* gene expression may mark cells that are the evolutionary precursors of the neural crest.

3.4. Msx gene function in fishes and amphibians: roles in the regeneration of appendages, Bmp signaling, and the activity of the head organizer

The zebrafish has at least five *Msx* genes, designated *Msx*a–e (Ekker et al., 1997). *Xenopus* and the urodele amphibian, *Ambystoma*, are each known to have at least two (Ekker et al., 1991; Su et al., 1991; Carlson et al., 1998; Koshiba et al., 1998). The zebrafish genes are more closely related to each other than to *Msx1*, *2* or *3* of the bird-mammal group. Similarly, amphibian *Msx1* and *Msx2* are distantly related to their cognate genes in birds and mammals, making it difficult to discern whether they are, in fact, orthologues of *Msx1* and *Msx2* in the bird-mammal group (Ekker et al., 1997). Each of the five zebrafish *Msx* genes, *Msx*a–e, exhibits a distinctive pattern of expression during embryogenesis (Akimenko et al., 1995), suggesting distinct functions. This is also the case for amphibian *Msx1* and *Msx2*, which are expressed in distinct though partially overlapping patterns (Koshiba et al., 1998).

Expression studies in both groups suggest roles for *Msx* genes in limb regeneration (Akimento et al., 1995; Carlson et al., 1998; Koshiba et al., 1998). In zebrafish, the fin regeneration blastema is organized in two functionally distinct domains during regenerative outgrown: Distal blastemal cells, which do not proliferate, express

Msxb; proximal cells which do proliferate, do not express *Msxb* (Nechiporuk et al., 2002). *Msxb* expression thus appears to correlate with a slow cell during blastema formation. Nechiporuk and Keating (2002) suggest that upregulation of *Msxb* may be necessary to slow the cell cycle during blastema formation and to inhibit proliferation in the distal-most blastema during regenerative outgrowth.

As is the case the developing limbs of birds and mammals, the *Msx2* gene of the axolotl (Ambystoma mexicana) is expressed in the apical epidermis and mesenchyme (Carlson et al., 1998). *Msx1* is expressed more broadly in the mesenchyme (Koshiba et al., 1998). In later developmental stages, both *Msx1* and *Msx2* are downregulated, and, intriguingly, are re-expressed after limb amputation (Koshiba et al., 1998). Whether expression of either *Msx1* or *Msx2* is correlated with the slowing of the cell cycle during blastema formation—as is the case for zebrafish *Msxb*—remains to be determined.

Overexpression studies first conducted in the Solursh laboratory suggested that in *Xenopus Msx* genes may function in axial patterning (Chen and Solursh, 1995; Maeda et al., 1997; Suzuki et al., 1997; Feledy et al., 1999; Gong and Kiba, 1999). Subsequent work established that *Msx1* is a Bmp2/4 effector that can serve as a proxy for BMP2/4 when misexpressed in embryos (Suzuki et al., 1997; Yamamoto et al., 2000). Overexpression of *Msx1* in neural ectoderm results in the supression of neural fates and the promotion of non-neural ectodermal fates. Reciprocally, expression of a dominant negative form of *XMsx1* suppresses Bmp signaling in non-neural ectoderm and results in such cells adopting neural fates (Suzuki et al., 1997; Yamamoto et al., 2000).

More recently, Yamamoto et al. (2002) have addressed the potential role of *XMsx1* in head development. *XMsx1* is expressed in head ectoderm and ventral mesoderm. *XMsx1* is required downstream of Bmp for ventralization of mesoderm and ectoderm (Yamamoto et al., 2000). Dorsal overexpression of *XMsx1* results in headless embryos, similar to the phenotype that occurs when Bmp family members are overexpressed. The authors used an inhibitory form of *XMsx1* to test the hypothesis that it has a role in head formation. Consistent with this view, they found that this inhibitory form indeed did cause ectopic head formation. Inhibition of head formation was correlated with reduced expression of anterior endomesodermal markers *Xotx2*, *Xhox*, and *Xdkk1*, each of which is known to be involved in head organizer activity.

Expression of an inhibitory form of *Msx1* in *Xenopus* also activates nodal signaling (Yamamoto et al., 2002). Conversely, wild type *XMsx1* inhibits activation of an activin responsive reporter gene in dorsal blastomeres, implying that the function of wild type *XMsx1* is to inhibit nodal signaling. On a molecular level, *XMsx1* can exclude the Fast transcription factor from a complex with smads 2 and 4, resulting in an inhibition of nodal dependent transcriptional activation (Yamamoto et al., 2002). Whether *Msx1* plays a similar role in nodal signaling in mammals remains to be investigated.

These results raise the question of whether mammalian *Msx* genes may have a similar activity in the development of the head. Although in the mouse *Msx1*, *2* double mutants have severe, multiorgan defects, including exencephaly, they do not

exhibit a deficiency in head formation (Satokata et al., 2000). It remains possible, however, that *Msx1* and *Msx2* act in a functionally redundant manner with the third murine *Msx* gene, *Msx3*, in head formation.

In summary, from work in several invertebrate phyla, as well as in fish and amphibians, *Msx* genes function in cell specification and migration. Genetic studies in *Drosophila* reveal *msh* to be an identity gene, with roles in neurogenesis, myogenesis, and wing patterning. At least some aspects of these functions are likely conserved in vertebrates. For each of these functions, *msh* does not initiate the developmental process in which it participates, but rather acts at a later stage to ''fine tune'' it. This is a theme that is repeated with few exceptions over all the animal groups in which *Msx* gene function has been examined. Intriguingly, in *Xenopus*, *Msx* genes appear to have a role in the head organizer, although such a role has not been documented in other vertebrates. Since the fly *msh* gene is apparently not Bmp responsive while *Msx* genes of vertebrates do respond to BMPs, it appears that the ability of *Msx* genes to serve as effectors of the Bmp pathway is an innovation that occurred subsequent to the divergence of protostomes and deuterostomes, and possibly within the chordate lineage.

4. *Msx* genes in human disease and mammalian development

Clues from human genetics, together with the analysis of targeted mouse mutants, have provided a basic picture of the activities of *Msx* genes in mammals. In humans, loss of function mutations in *MSX1* cause selective agenesis of teeth (Vastardis et al., 1996; Hu et al., 1998; Cohen, 2000), as well as Witkop syndrome, characterized by defects in the development of teeth and nails (Jumlongras et al., 2001). *MSX2* has been linked causally with disorders that affect the development of the skull vault (Wilkie, 1997; Cohen, 2000). Targeted inactivation of *Msx1* in the mouse leads to cleft palate and a failure of tooth development (Satokata and Maas, 1994; Maas and Bei, 1997). *Msx1* mutant mice also exhibit abnormalities of the nasal, frontal and parietal bones, and of the malleus in the middle ear. Inactivation of *Msx2* causes defects in the skull vault that closely parallel defects seen in humans (Satokata et al., 2000). In addition *Msx2* mutant mice exhibit defects in the development of the tooth, hair follicle, and mammary gland. We do not attempt here to describe in detail all of the phenotypes associated with *Msx1* and *Msx2*. We focus, rather, on the role of *Msx2* in the development of the skull vault, both because it has been a focus of research on *Msx* genes, and because the principles that have emerged from this work serve to illustrate the activities of both *Msx1* and *Msx2* in other developmental processes.

4.1. Msx2 in the development of the mammalian skull

4.1.1. Developmental biology of the skull vault

The vertebrate skull consists of the viscerocranium, comprising the jaws and other derivitives of the branchial arches, and the neurocranium, comprising the skull vault

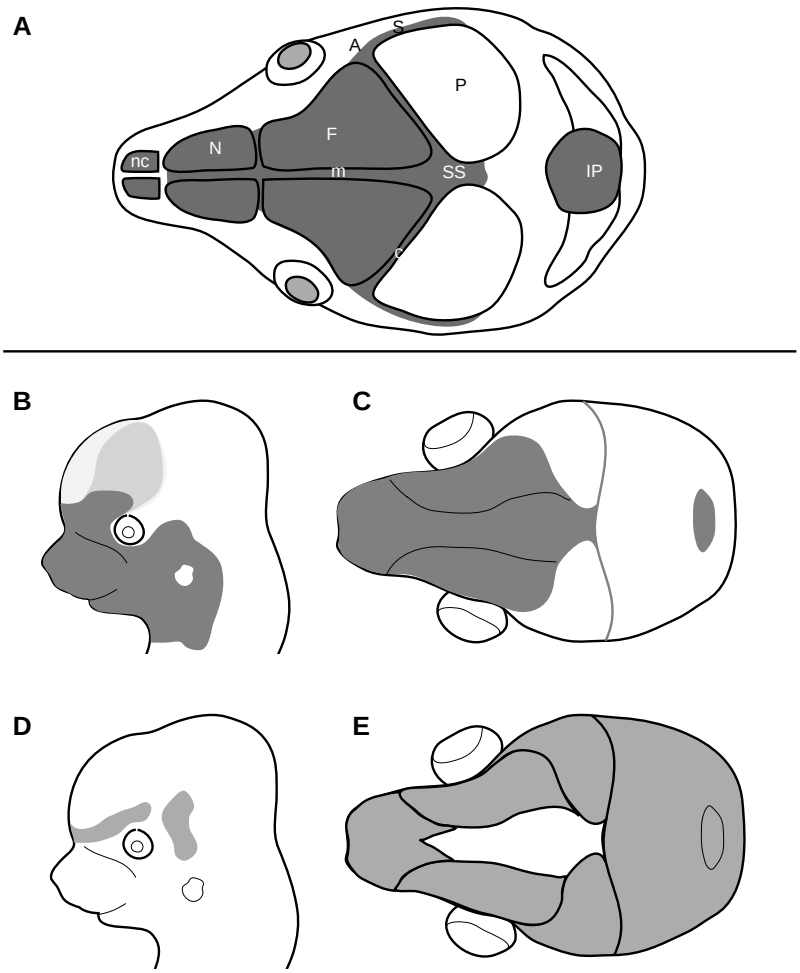

Fig. 3. Development of murine skull vault. (A) Schematic diagram of adult skull showing neural crest contribution in blue (after Jiang et al., 2002). (B, C) Distribution of neural crest cells at E12.5 (lateral view: B) and E16.5 (dorsal view: C). (D, E) Distribution of differentiated osteoblasts at E12.5 (D) and E16.5 (E). Abbreviations: a, alisphenoid; c, coronal suture; F, frontal; m, metopic (frontal) suture; N, nasal; nc, nasal cartilage; P, parietal; IP, interparietal; S, squamosal; SS, sagittal suture. (See Color Insert.)

and base. The skull vault consists of the paired frontal and parietal bones, and, in mammals, the interparietal bone (Fig. 3). The development of the skull vault commences with the migration of presumptive skeletogenic mesenchyme cells to the sites of the calvarial anlagen between the developing brain and epidermis (Fig. 3). Although the developmental origins of the mesenchymal cell populations that contribute the skull vault have been controversial (Noden, 1975, 1978; Couly et al., 1993; Morriss-Kay, 2001), it now seems probable that, at least in the mouse,

the frontal bones and a portion of the interparietal bone are of neural crest origin while the parietal bones are of mesodermal origin (Jiang et al., 2002). An inductive signal from the underlying neural tissue appears to have a role in the specification of the mesenchyme (Schowing, 1968), and a later signal from the epidermis is thought to be required for its differentiation (Tyler, 1983).

In the final phase of development, the bones grow at the sutural margins in concert with the growth of the brain (Fig. 3). This growth depends on the tight regulation of the proliferation and differentiation of osteogenic precursor cells in the sutural spaces, and is probably modulated by signals from the underlying dura mater (Opperman et al., 1995, 1998).

Msx2 is expressed in both the mesodermal and neural crest-derived mesenchymal cell populations that give rise to the skull vault (Jabs et al., 1993). It is also expressed in the dura, a membrane that lies between the brain and the skull vault. At later stages *Msx2* is expressed in osteogenic cells within the suture (Liu et al., 1999; Rice et al., 2000).

4.1.2. Msx2 in human genetic syndromes

That *Msx2* may have a functional association with disorders of skull vault development was heralded by work done by Muller, Warman, and colleagues (Muller et al., 1993; Warman et al., 1993), who mapped craniosynostosis Boston type to 5qter, close to the location of *MSX2* (5q34). Craniosynostosis is the premature fusion of calvarial bones at the sutures. Its overall incidence is approximately 1/3000 live births. Craniosynostosis, Boston type is inherited as an autosomal dominant and is so far known only from a single large kindred. The genetic causes of syndromic craniosynostosis include gain of function mutations in FGF receptors 2 and 3 (Wilkie, 1997), and loss of function mutations in the *Twist* gene cause Saethre-Chotzen syndrome (el Ghouzzi et al., 1997; Howard et al., 1997).

Jabs, Maxson, and colleagues (Jabs et al., 1993) subsequently demonstrated tight linkage between the disorder and the *MSX2* gene. Sequence analysis identified a single base change in affected individuals. This change resulted in the substitution of a proline for a histidine in position 146, or position 7 of the homeodomain (Fig. 2). This amino acid position is in the N-terminal arm of the homeodomain, two residues away from an arginine 5, which makes contact with DNA.

The location of this mutation suggested that it might affect the DNA binding properties of the MSX2 protein. Quantitative gel shift analysis performed with bacterially expressed mutant and wild type murine *Msx*2 showed that the mutant form bound a consensus *Msx2* target site several fold more tightly than did its wild type counterpart (Ma et al., 1996). This difference in affinity was due almost entirely to a change in the off-rate of the Msx2 protein. A binding site selection study showed that the mutation did not influence the set of target sequences to which Msx2 could bind. Thus the mutation affected only the DNA binding affinity of Msx2, not the specificity. Such an effect on binding affinity was consistent with the autosomal dominant mode of inheritance, and with the hypothesis that the mutation acted via a dominant gain function mechanism.

Transgenic mice in which either mutant or wild type forms of *Msx2* were expressed in developing sutures and calvarial bones provided further support for the dominant gain of function hypothesis. In one study, the broadly expressed CMV promoter was used to drive *Msx2* expression (Liu et al., 1995). This resulted in enhanced growth of calvarial bones, as well as defects in the development of the skin and eye. Subsequently the *Msx2* promoter was used to specifically overexpress mutant and wild type forms of *Msx2* in sutures and other sites of *Msx2* expression. Liu et al. (1994) showed that approximately 6 kb of 5; flanking sequence was sufficient to recapitulate the expression of the endogenous *Msx2* gene (Fig. 4). When this promoter sequence was used to drive expression of *Msx2*, resultant transgenic lines exhibited overgrowth of parietal bones. Mutant and wild type forms of *Msx2* exhibited similar phenotypes, consistent with the hypothesis that the pro to his substitution is an activating mutation (Liu et al., 1999). It should be pointed out, however, that rigorous demonstration that this is the case would require knocking the mutation into the endogenous *Msx2* locus, which has not yet been reported.

To date, no other families with gain of function mutations in *MSX2* have been identified. One explanation for this is that processes required for the viability of the embryo may be sensitive to enhanced *MSX2* gene function. Overexpression of *Msx2* causes severe neural tube defects in the mouse (Winograd et al., 1997; Liu, Y.-H., and Maxson, R.E., unpublished observations). Thus it is possible that in humans, even moderate increases in levels of *MSX2* activity cause lethality. If so, then mutations that both activate *MSX2* and are also viable would be expected to be rare.

A second disorder that has been associated with *MSX2* is familial parietal foramina. Individuals affected with this disorder exhibit persistent foramina—ossification defects in the bones of the skull vault (Cohen, 2000). These defects typically occur in the parietal bones, though they can also occur elsewhere in the skull vault. Wilkie and colleagues identified mutations in *MSX2* in several families that exhibit parietal foramina (Wilkie et al., 2000) (Fig. 2). These mutations were located in helices 1 and 2 of the homeodomain, in a region not involved directly in DNA contact, but with a role in the overall three dimensional structure and stability of the homeodomain. Analysis of the DNA binding properties of murine Msx2 showed that the mutant forms of the protein bound DNA with a much reduced affinity relative to a wild type Msx2 protein, consistent with the hypothesis that the cause of familial parietal foramina is haploinsufficiency of *MSX2*.

If this hypothesis were true, then targeted inactivation of *Msx2* in mice should produce a similar phenotype. Maas and colleagues showed that indeed *Msx2*−/− animals exhibited an ossification defect in the posterior frontal bone (Satokata et al., 2000). This defect was also present in heterozygotes with reduced severity. Intriguingly, such mice also had defects in the development of long bones, due to an effect on PTH-PTHrP signaling.

Although this result strongly supports the hypothesis that reduced *Msx2* function is responsible for familial parietal foramina in humans, one inconsistency is that in humans the defect is in the parietal bone, whereas in mice it is in the frontal bone

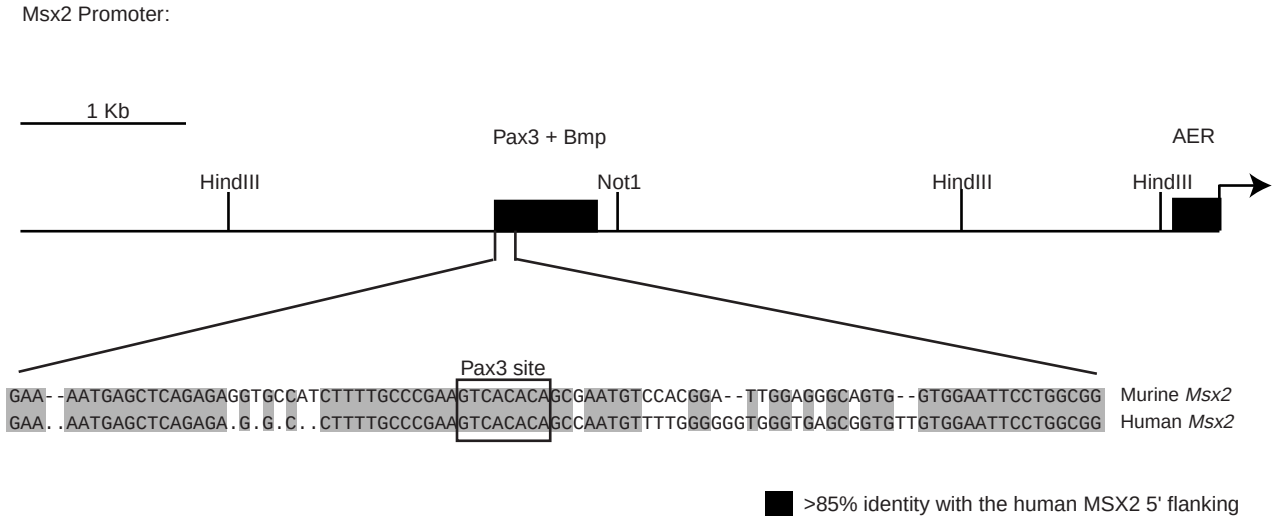

Fig. 4. Structure of murine *Msx2* promoter (after Kwang et al., 2002). Human sequences that match the Pax3 and Bmp responsive region are located approximately 90 kb upstream of the human *MSX2* transcription start site.

(Satokata et al., 2000; Wilkie et al., 2000). Analysis of *Msx1–2* double mutant mice has provided an explanation for this inconsistency. As the dosage of either *Msx1* or *Msx2* is reduced, the frontal bone phenotype becomes more severe (Satokata et al., 2000; Ishii, M., and Maxson, R., unpublished observations). Intriguingly, in double homozygote-heterozygote combinations, a foramen is also evident in the parietal bones, mimicking the situation in humans (Ishii, M., and Maxson, R., unpublished observations). These findings suggest that mice and humans differ with respect to the dosage requirements for *Msx* gene function in different bones of the skull vault. Thus in humans the development of the parietal bone is more sensitive to *Msx* gene dosage than the development of the frontal bone. In mice, the development of the frontal bone is more sensitive.

4.1.3. Cellular mechanisms underlying craniosynostosis, Boston type and familial parietal foramina

How, at the level of cellular and developmental mechanisms, mutations in *Msx2* produce craniosynostosis and familial parietal foramina, has been a focus of research, both because of the inherent appeal of the patterning of the skull vault as a model of the patterning of mesenchymal cell populations, and because of interest in understanding the pathophysiology of parietal foramina and craniosynostosis. Analysis of skull vault development in *Msx2* overexpressing transgenic mice showed a transient increase in the number of osteoblastic cells in the osteogenic fronts of the parietal bones compared with wild type controls (Liu et al., 1999). These cells can be identified by virtue of their expression of the early-stage osteoblast marker, alkaline phosphatase (alp). *Msx2* transgenic mice also exhibited an increase in BrdU labeling of cells of the osteogenic front. Thus overexpression of *Msx2* resulted in an increase in the number of proliferative osteoblasts in ostoegenic fronts. This increase appeared to be transient, and coincided with the overgrowth of the parietal bones. Maxson and colleagues (Liu et al., 1999) proposed that overexpression of *Msx2* transiently retards the terminal differentiation of osteoblasts and causes an increase in the population of proliferative osteoblasts, resulting ultimately in overgrowth of bone.

Consistent with this model, Lichtler and co-workers (Dodig et al., 1999) showed that overexpression of *Msx2* in cultured calvarial osteoblasts resulted in an increase in proliferation and a reduction in the number of cells expressing late-stage osteoblast markers. Antisense attenuation of *Msx2* expression had the opposite effect: proliferation decreased and the number of cells expressing terminal differentation markers increased (Dodig et al., 1999). Thus, at least in osteogenic cell populations present during the appositional growth phase of calvarial development, *Msx2* seems to have a role in maintaining osteoblasts in a proliferative, less-than-fully-differentiated state. This is similar to the role documented for *Msx1* in myogenesis in which *Msx1* inhibits myoD expression in cultured myoblasts and is sufficient to drive differentiated myoblasts into a less differentiated state (Odelberg et al., 2000).

The developmental basis of the calvarial foramen phenotype has been addressed using *Msx2* knockout mice (Satokata et al., 2000). By examining a developmental

series of mutant embryos, Ishii, Maxson and colleagues found that a deficiency in frontal bone development is evident as early as E12.5 (Ishii et al., in press). Using alkaline phosphatase to mark osteogenic cells, Ishii et al. showed that at E12.5 the rudiment of the frontal bone is significantly smaller in *Msx2* mutants than in normal controls. They show further that this defect is not due to a deficiency in the migration of neural crest precursor cells, but rather is caused by a defect in the development of undifferentiated neural crest-derived cranial mesenchyme into osteogenic cells.

Msx2 may thus have different functions in early and late stages of calvarial growth. Early on, it is required for the transition from undifferentiated, neural crest-derived mesenchyme to early-stage (alp-expressing) osteoblasts (Ishii et al., in press). Later, during the appositional growth phase of calvarial development, it serves to maintain osteoblasts in a proliferative state (Dodig et al., 1999; Liu et al., 1999). The early activity of *Msx2* may be analogous to the function of the *Drosophila msh* gene in establishing the identity of subtypes of myoblasts and neuroblasts (Isshiki et al., 1997; Nose et al., 1998).

The molecular pathways through which *Msx2* elicits these effects on the development of skeletogenic mesenchyme are clearly of interest. Mutations in several different genes can cause similar phenotypic outcomes in skull vault development: parietal foramina is caused not only by loss of function mutations in *Msx2* but also in the paired homeodomain gene, *ALX4* (Wu et al., 2000; Wuyts et al., 2000; Mavrogiannis et al., 2001), and in the basic hlh gene, *TWIST* (Thompson et al., 1984). Gain of function mutations in FGF receptors 2 and 3 can cause craniosynostosis (Wilkie, 1997). Whether *ALX4*, *TWIST*, and FGF receptors function in the same developmental processes as *MSX2* remains to be determined.

4.2. Immediate upstream genes

Because of the role of *Msx* genes in human disease, there has been considerable interest in mapping upstream inputs. Such studies offer a means of identifying additional candidate disease genes, as well as the promise of a more complete understanding of the pathophysiology of disease phenotypes. Analysis of the mouse and chicken *Msx2* promoters in transgenic mice has led to the identification of two cis-regulatory modules (Fig. 4) (Liu et al., 1994; Sumoy et al., 1995; Kwang et al., 2002). One, a segment of approximately 450 bp, is located in the proximal promoter and is sufficient to drive expression of lacZ reporters in the apical ectodermal ridge of the developing limb (Liu et al., 1994; Sumoy et al., 1995). This module also expresses in the developing hair follicle (Kulessa et al., 2000). The other, 560 bp in length, has been identified in the mouse promoter, and is located approximately 3 kb upstream of the transcription start site (Kwang et al., 2002). This element is sufficient to drive expression of a lacZ reporter in a manner that closely mimics the expression of the endogenous *Msx2* gene. A hallmark of these two regulatory modules is that both are highly conserved within the bird-mammal group (Liu et al., 1994; Sumoy et al., 1995; Kwang et al., 2002). Comparisons of the mouse and human sequences reveal identities in the range of 85–90% over several hundred base pairs. Intriguingly, the

location of the upstream module is also conserved. In both human and mouse it is located 3 kb upstream of the transcription start site (Kwang et al., 2002). Thus selection has apparently maintained the nucleotide sequence as well as the location of this module.

4.2.1. Dlx5

The expression patterns of the *Dlx5* and *Dlx6* overlap with that of *Msx2* in the developing limb. Both are expressed in the apical ectodermal ridge, coincident with *Msx2* (Robledo et al., 2002). *Msx2* expression is reduced in the AER of *Dlx5*, *6* double mutant mice, suggesting that one or both of these *Dlx* genes could be an upstream regulator of *Msx2*. Consistent with this possibility, Upholt and colleagues have shown that a homeodomain element in the AER enhancer of the chicken *Msx2* gene is required for expression in the AER of transgenic mice (Pan et al., 2002). Mutations that prevent binding of Dlx5 to this site in vitro also cause *Msx2* transgene expression to be lost in the AER.

4.2.2. Pax3

An additional gene that may regulate *Msx2* directly is the paired homeodomain factor, *Pax3*. Kwang et al. (2002) showed that *Msx2* is upregulated in the dorsal neural tube of the *Splotch* mutant mouse, which bears a loss of function mutation in *Pax3*. Homozygous *Splotch* mutants die at E13.5 with defects in the cardiac outflow tract and the neural tube (Franz, 1993). The outflow tract defects are the result of a failure of cardiac neural crest cells to populate the outflow tract in normal numbers and the consequent failure of outflow tract septation. That *Msx2* has a functional role downstream of *Pax3* in cardiac neural crest development was shown by a genetic cross between an *Msx2* targeted mutant and Splotch (Kwang et al., 2002). Reduced *Msx2* gene dosage in the context of the Splotch mutant rescued the Splotch cardiac defect, showing suppression of *Msx2* activity by Pax3 is required for normal cardiac neural crest development. Kwang and colleagues also presented evidence that *Msx2* is a direct target of Pax3. They showed that Pax3 binds a conserved element within the 560 bp *Msx2* promoter fragment (see above) and that mutations that prevent Pax3 binding to this element also cause upregulation of *Pax3* transgenes in the region of the hindbrain from which the cardiac neural crest originates.

Intriguingly Abate-Shen's group has documented a physical interaction between the Pax3 protein and Msx1 (Bendall et al., 1999). This interaction counteracts the ability of Msx1 to inhibit myoD transcription and thus promotes differentiation of myoblasts. Whether Pax3 can also influence Msx2 through a direct protein–protein interaction has yet to be tested.

4.2.3. Bmp and Wnt pathways

That *Msx* expression can be stimulated by exogenously supplied Bmps was first shown by Thesleff and colleagues in a series of experiments in which Bmp beads were implanted in tissues that give rise to the tooth (Vainio et al., 1993). Subsequent experiments in a variety of laboratories showed that *Msx* genes are Bmp-responsive

(Suzuki et al., 1997; Hollnagel et al., 1999; Sirard et al., 2000; Daluiski et al., 2001; Brugger, S., and Maxson, R., unpublished observations), and that this response is unaffected by cyclohexamide (Hollnagel et al., 1999), demonstrating that *Msx* genes are immediate early targets of the Bmp pathway. Subsequent analysis in Maxson's laboratory (Brugger, S., Merrill, A., Ting, M., Warrior, R., Arora, K., and Maxson, R., submitted) has localized the region of the *Msx2* promoter that mediates the Bmp response. This region is located within a 560 bp promoter domain that is highly conserved between mice and humans.

The trans-regulatory factors that mediate the Bmp response are of obvious interest. Although smad proteins are well-known as transducers of the Bmp signal from cytoplasm to nucleus (von Bubnoff and Cho, 2001), they bind weakly to DNA and are thought to require sequence-specific transcription factors for their function. It will thus be informative to examine closely the *Msx2* Bmp responsive cis-regulatory element and cognate factors.

That *Msx2* may also be a target of the Wnt pathway is suggested by recent findings from the Nusse laboratory (Willert et al., 2002). Using microarray technology, they showed that *Msx1* and *Msx2* are among genes upregulated by treatment of embryonal carcinoma cells with active Wnt protein. Intriguingly, treatment of cells with Wnts and Bmps together lead to synergistic activation of *Msx1* and *Msx2*, providing evidence for cooperativity between the Wnt and Bmp pathways in the regulation of *Msx* genes.

4.3. Downstream genes

4.3.1. Osteocalcin

Osteocalcin, a terminal marker of osteoblast differentiation, was proposed as an *Msx2* target by virtue of a homeodomain binding site in its proximal promoter. *Msx2* was shown to bind this site in vitro and to repress osteocalcin (*OC*) promoter activity in cultured osteoblasts (Hoffmann et al., 1994; Towler et al., 1994). Later work demonstrated, however, that this binding was not required for *Msx2*-mediated repression of *OC* (Newberry et al., 1997). Although there are as yet no genetic data showing that *OC* expression is altered in *Msx* mutant mice, the *OC* promoter has nevertheless provided a useful model for understanding how *Msx2* can influence transcription. Work from the Stein, Lian, and Towler groups has led to a detailed understanding of the transcriptional regulation of OC in osteoblasts (Lian et al., 1998). The complexities of this set of processes are beyond the scope of this review. Of direct relevance, however, are findings that the *Msx2*-mediated downregulation of the *OC* promoter can be suppressed by an interaction with Dlx5 (Newberry et al., 1998). The expression of Dlx5 coincides with that of *osteocalcin* during osteoblast differentiation (Ryoo et al., 1997). Abate-Shen and colleagues showed earlier that Dlx family members can form heterodimers with Msx proteins, and thus antagonize the ability of Msx1 to inhibit myoD expression during myoblast differentiation (Zhang et al., 1997). This interaction depends on the N-terminal region of the extended homeodomain.

4.3.2. Amelogenin

The *amelogenin* gene has also emerged as a possible *Msx2* target. Zhou and Snead (2000) have shown that the activity of amelogenin promoter–reporter constructs can be regulated positively by CCAAT/enhancer-binding protein alpha (C/EBPalpha). Transient co-transfection experiments in LS8 cells showed that *Msx2* can antagonize this effect, and that this antagonism is the result of a direct interaction between *Msx2* C-terminal residues 183–267 and C/EBPalpha. Similar to the situation with *Msx2* and osteocalcin regulation, the Msx2 protein does not itself bind the amelogenin promoter.

4.3.3. myoD

Using somatic cell genetic and molecular approaches, Thayer, Sassoon, and colleagues (Woloshin et al., 1995) identified *myoD* as a potential *Msx* target. They showed that transfer of the human chromosome that contains the *myoD* locus (11) from primary fibroblasts to 10T1/2 cells results in the activation of myoD. Chromosome 4 prevents this activation. Making use of microcell hybrids, they localized the inhibitory region to a portion of 4p that contains *Msx1*. Finally, they demonstrated that forced expression of *Msx1* prevents *myoD* expression. Subsequent work in the Abate-Shen and Shen laboratories showed that *Msx1* can downregulate *myoD* in developing embryos (Bendall et al., 1999). Moreover, Keating and colleagues (Odelberg et al., 2000) showed that forced expression of *Msx1* in C2C12 myotubes can reduce the expression of the muscle determinants, *myoD, myogenin,* and *MRF4*, and can cause these cells to dedifferentiate.

4.3.4. Other downstream targets

Two other genes have been identified as potential targets based on findings that their expression can be modulated by changes in *Msx* gene expression. These include the cell cycle regulator, cyclinD, shown by Abate-Shen and colleagues to be downregulated in cells in which *Msx1* is overexpressed (Hu et al., 2001), and the winged helix transcription factor, *Foxn1*. Loss of *Foxn1* function in the mouse results in loss of hair, a phenotype similar to one caused by targeted inactivation of *Msx2* (Satokata et al., 2000). Chuong and colleagues have shown that *Foxn1* is downregulated in *Msx1* mutants, providing a potential explanation for the hair loss phenotype in *Msx2* mutant mice (Ma et al., 2003).

4.4. Direct interactions between Msx2 and transcriptional co-factors

It is well established that DNA sequence specific transcription factors influence transcription through interactions not only with DNA, but also with co-activator and co-repressor proteins. Given the growing number of examples in which Msx proteins have been found to influence transcription without binding DNA (Catron et al., 1995; Newberry 1997; Zhou and Snead, 2000), it has become increasingly important to identify Msx2 co-factors/interacting proteins. By means of two hybrid and far western screens, as well as candidate approaches, several such molecules have been identified.

In addition to the DNA sequence-specific transcription factors, Pax3, Lhx, and Dlx (discussed above), these include Miz1, a member of the PIAS family of transcriptional regulators (Wu et al., 1997), and Mint, a transcriptional co-repressor (Newberry et al., 1999). The PIAS proteins have been implicated in Jak-Stat signaling (Shuai, 2000). They have also been isolated as partners of nuclear receptors (Janne et al., 2000). Miz1 (also designated PIASx beta) has autonomous transcription activating activity (Wu et al., 1997), and, in co-transfection experiments, can suppress the transcription inhibitory activity of Msx2 (Wu, L., and Maxson, R., unpublished observations). The extended homeodomain of Msx2 is necessary for the interaction with Miz1, and the single zinc finger of Miz1 is required for the interaction with Msx2 (Wu, L., and Maxson, R., unpublished observations). As expected for a transcription co-factor, Miz1 is expressed broadly in murine embryos (Wu et al., 1997). Intriguingly, several PIAS family members, including Miz1, can function as E3 Sumo-1 ligases; i.e. they can promote the attachment of SUMO-1 to target proteins (Sachdev et al., 2001; Kotaja et al., 2002). Sumo-1 is known to stabilize target proteins and to influence their intranuclear localization (Jackson, 2001; Sachdev et al., 2001). Whether Msx proteins are sumoylated has yet to be established. Neither is there any information on the developmental function of the interaction between Miz1 and Msx2.

Towler and coworkers, using a far western expression screening approach to identify Msx2 partners, isolated Mint (Msx interacting nuclear target protein) (Newberry et al., 1999). Mint is related to the human SHARP protein (Shi et al., 2001), which was identified in a two hybrid screen for proteins that can interact with the nuclear receptor co-repressor SMRT, and was shown to recruit histone deacetylases. In cultured osteoblasts, Mint can suppress the FGF/forskolin-mediated activation of the osteocalcin promoter (Newberry et al., 1997). The interaction between Msx2 and Mint requires residues 55–148 of Msx2, which are important for the transcriptional repressor activity of *Msx2* (Newberry et al., 1999). Although a *Drosophila* Mint homologue, Split ends (Spen) has been shown to be required for aspects of neurogenesis (Kuang et al., 2000), there is no information on the role of Mint in vertebrate development.

5. Summary

The picture of *Msx* genes that emerges from a comparison of their activities in a variety of metazoans is one of "middle managers" of a range of developmental processes. One aspect of function that appears to be conserved between flies and vertebrates is the dorso-ventral patterning of appendages. Although roles for *Msx* genes in the specification of specific myoblast and neuroblast lineages have not documented in vertebrates—as they have in flies—vertebrate *Msx* genes do function in myoblast differentiation, and in the differentiation of subsets of neural crest-derived cells, including osteoblasts that form the craniofacial apparatus. On a molecular level, Msx proteins can form heterodimers with other Nk-class homeobox proteins, as well as with lim-homeodomain and paired-homeodomain proteins.

Msx proteins can also interact with transcriptional co-factors that function in sumoylation and in the recruitment of co-repressors. Future work on *Msx* genes promises insight into mechanisms of normal and abnormal craniofacial development and the evolution of the neural crest.

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Cdx homeodomain proteins in vertebral patterning

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1. Introduction

Cdx (*caudal*) family members encode homeodomain transcription factors. The name *caudal* derives from the essential function of the gene product in the development of the posterior *Drosophila* embryo, a role that appears generally conserved across species. In vertebrates, Cdx proteins perform essential functions in the trophoblast, intestinal development, posterior specification and vertebral patterning. While this review focuses largely on the function of murine Cdx homologues, a number of relevant findings have been established initially from other systems, and will be briefly discussed.

1.1. Identification of caudal

The first *Cdx* member cloned, the *Drosophila caudal* (*cad*) gene, was isolated in a screen for homeobox-containing genes using *ultrabithorax* homeobox sequences as a probe (Levine et al., 1985; Mlodzik and Gehring, 1987). *Cad* encodes a 427 amino acid homeodomain transcription factor, and is expressed under the control of both maternal and zygotic regulatory elements. Maternal *cad* transcripts are subject to post-transcriptional regulation by the homeodomain protein, bicoid, an essential anterior determinant which is expressed in an anterior to posterior gradient (Macdonald and Struhl, 1988). *Cad* mRNA is evenly distributed in the *Drosophila* embryo, but is translationally repressed by bicoid through a motif in the 3′ untranslated region of *cad*, resulting in a complementary posterior to anterior gradient of cad protein (Rivera-Pomar et al., 1996 Niessing et al., 1999, 2000). Later zygotic expression of *cad* is observed in the hindgut primordium and later in the hindgut, posterior midgut, Malpighian tubules and anal pads (Mlodzik and Gehring, 1987).

1.2. Cad function

Cad is required for specification of the posterior *Drosophila* embryo through regulation of gap and pair-rule genes, including *giant*, *knirps*, *spalt*, and *fushi tarazu* (Macdonald and Struhl, 1986; Mlodzik and Gehring, 1987; Mlodzik et al., 1990). Cad is also essential for gastrulation processes and formation of the hindgut, where it has been proposed to function in conjunction with other factors, including forkhead, brachyenteron and wingless (Wu and Lengyel 1998). It is interesting to note that homologues of these gene products have been implicated in gastrulation and development of the posterior embryo in diverse species, suggesting an

evolutionarily conserved role for this gene "cassette" (reviewed in Lengyel and Iwaki, 2002).

1.3. Cad, an ancestral Hox gene?

As anticipated from the means of isolation, the *cad* homeobox shows a high degree of homology to the *Hox* homeobox family, being most related to *Antennapedia* (*Ant*) homeobox sequences (62% homology). *Hox* genes are clustered in two complexes in *Drosophila*, the *BX-C* and the *ANT-C* (reviewed in Lewis 1978; Popadic et al., 1998; Mann and Morata 2000). However, *cad* is not linked to either of these loci and is therefore not classified as a *Hox* gene per se.

The *Drosophila Hox* gene products function as selector genes to specify segment identity along the antero-posterior (A-P) axis (reviewed in Bienz 1994; Mann, 1995; Dennell et al., 1996; Mann and Morata, 2000). Notably, however, Hox function is not required in the posterior- or anterior-most segments, and expression of *Abdominal-B* (*Adb*), the posterior-most *Hox* gene expressed in *Drosophila,* is delimited to the penultimate posterior abdominal segment, A9, from which male genitalia are derived. Interestingly, *cad* is expressed in the last abdominal segment (A10), and is restricted to cells that form the analia in adults (Calleja et al., 1996). In the absence of cad, this posterior-most segment expresses *Adb* and is transformed into male genitalia in male mutants. Thus, loss of cad results in transformation of A10 into an A9 identity (Moreno and Morata, 1999). Such an alteration in segment identity, or homeotic transformation, is a typical outcome of *Hox* loss-of-function (reviewed in Lewis 1994; Mcginnis 1994). Conversely, mis-expression of *cad* in certain domains leads to the formation of ectopic analia, another outcome characteristic of *Hox* gain-of-function studies. These data demonstrate that, although *cad* is not linked to the *Hom-C* loci, it behaves functionally in a manner similar to *Hox* gene products to specify the posterior-most segment in *Drosophila*.

1.4. The ParaHox genes

The lack of physical linkage of *cad*, or its homologues, to a *Hox* locus in any organism, together with its function as a *Hox*-like gene product, suggests that *cad* and *Hox* genes may have diverged from a related ancestral *Hox* cluster. In this regard, a "*ParaHox*" gene cluster, initially identified in the cephalochordate *amphioxus*, is suggestive of such an origin (Brooke et al., 1998). This cluster consists of the homeobox genes *AmphiCdx, AmphiXlox* (*pdx-1* in mammals) and *AmphiGsx* (*Gsh1* and *Gsh2* in mouse). Moreover, in a manner analogous to *Hox* genes, members of the *ParaHox* cluster exhibit co-linear expression during embryogenesis related to their physical location in the locus; *AmphiGsx* expression is observed in an anterior region, with *AmphiXlox* and *AmphiCdx* transcripts observed in progressively more posterior domains of the developing embryo (Brooke et al., 1998).

In mammals, the *ParaHox* cluster is conserved, and, as with the *Hox* genes, appears to have duplicated to give rise to four related loci (Pollard and Holland, 2000). This

observation is consistent with the proposal that the *ParaHox* cluster arose from a gene cluster (*ProtoHox*) that is also ancestral to *Hox*/*Hom-C* (Brooke et al., 1998; Ferrier and Holland, 2001). Such a common origin may underlie the apparent comparable function between cad and *Hox* gene products. In this regard, it has been proposed that *cad* (or its homologues) is the *ParaHox* paralogue of *AbdB* (Brooke et al., 1998) or *Evx1*/*Evx2*, homeobox genes which are linked to *Hoxa* and *Hoxd* clusters in vertebrates (Bastian and Gruss, 1990; D'Esposito et al., 1991; Moreno and Morata, 1999). However, sequence comparison of homeodomains, and the presence of a conserved hexapeptide motif, suggest that in the mouse, the *Cdx1* and *Cdx2* genes are more related to *Hox* paralog groups 8 and 9 (van Den et al., 2002).

2. Caudal homologues

Following isolation of *cad*, homologues were subsequently identified in a variety of species, including amphioxus (*AmphiCdx*; Brooke et al., 1998), *C. elegans* (*pal-1*; Burglin et al., 1989; Waring and Kenyon, 1991), the beetle Tribolium castaneum (*Tc-cad;* Schulz et al., 1998), silk moth (*B mori caudal*; Xu et al., 1994), *Xenopus* (*Xcad1*, *Xcad2*, and *Xcad3*; Blumberg et al., 1991; Northrop and Kimelman, 1994), zebrafish (*zf-cad1*; Joly et al., 1992), carp (*Carp-Cdx1*; Stroband et al., 1995), chick (*Cdx-A*, *Cdx-B*, and *Cdx-C*; Frumkin et al., 1991; Morales et al., 1996; Marom et al., 1997), mouse (*Cdx1*, *Cdx2*, and *Cdx4*; Duprey et al., 1988; James and Kazenwadel, 1991; Gamer and Wright, 1993), hamster (*Cdx2*/*3*; German et al., 1992) and human (*Cdx1*, *Cdx2*, and *Cdx4*; Bonner et al., 1995; Horn and Ashworth, 1995; Drummond et al., 1997).

Although details are beyond the scope of this review, in all species investigated, cad homologues appear to play a role in one or more processes related to the specification of the posterior embryo, vertebral patterning, trophoblast function and intestinal development. For example, the *C. elegans Cdx* homologue, *pal-1* is critical to axis establishment and patterning of the posterior embryo during gastrulation (Hunter et al., 1999; Edgar et al., 2001), and similar roles have been established for *Xenopus*, chick, and mouse homologues (Chawengsaksophak et al., 1997; Epstein et al., 1997; Isaacs et al., 1998; Subramanian et al., 1998; Isaacs et al., 1999; Ehrman and Yutzey, 2001).

2.1. Expression of Cdx genes in vertebrates

Analysis of *Cdx* expression and function in vertebrate models has relied largely on the mouse, chick, and the frog *Xenopus*. While the following description is based on work from the mouse, similar patterns of expression have also been described in other models, and may possibly apply to vertebrates in general.

Cdx1 maps to chromosome 18 in the mouse (Duprey et al., 1988). *Cdx1* transcripts are first detected during early gastrulation in the yolk sac (McGrath and Palis, 1997). At embryonic day (E)7.5 expression is subsequently observed in

the ectoderm and nascent mesoderm in a broad region of the primitive streak (Meyer and Gruss, 1993). Shortly thereafter, at E7.75, *Cdx1* transcripts can be detected anterior to the node in the neural plate and paraxial mesoderm. The anterior boundary of expression at this stage is at the level of the preotic sulcus in the presumptive hindbrain in neural ectoderm, with a slightly more posterior boundary in the mesoderm. As development proceeds, the anterior boundary of *Cdx1* expression in the neural tube progressively recedes to more posterior domains, and at later stages is positioned at the level of the presumptive spinal cord. Within the neural ectoderm, *Cdx1* becomes localized to the dorsal region of the neural folds, and in some populations of newly migrating neural crest cells. *Cdx1* is also detected in the nascent paraxial mesoderm and, transiently, in the somites.

As the somites differentiate (E9.0–9.5), *Cdx1* becomes restricted to a dorsal region corresponding to the presumptive dermamyotome. This domain of expression is most evident in the first 16 to 17 somites at E10.5. The more posterior somites exhibit significantly reduced, or undetectable levels, with the exception of somites 24 to 30 which lie at the level of the hindlimb bud and which exhibit higher levels of *Cdx1*. Expression continues in the posterior embryo in the tail bud as well as the mesenchyme of the developing forelimb bud at E9.5, and at relatively lower levels at E10.5 in the hindlimb bud. *Cdx1* is also detected in the nephrogenic cord and later in the mesonephric ducts. Expression in the embryo gradually recedes until E12, when transcripts are no longer detected. However, *Cdx1* is subsequently transcribed in the hindgut endoderm starting at E14 with an anterior limit corresponding to the presumptive duodenum, and extending caudally through the colon with a posterior-high domain of expression reminiscent of its distribution along the embryonic A–P axis at earlier stages (Duprey et al., 1988; James et al., 1994). This expression is maintained in the intestinal tract throughout adulthood with both *Cdx1* transcripts and protein localized in the crypt cells (James and Kazenwadel 1991; Subramanian et al., 1998).

Cdx2, which maps to murine chromosome 5 (Chawengsaksophak and Beck, 1996), has an early onset of expression in the extraembryonic trophectoderm lineage at E3.5, with expression continuing in the placenta through at least E12.5 (Beck et al., 1995). The onset of embryonic expression of *Cdx2* succeeds that of *Cdx1*, being first observed at E8.5 in the posterior region of the embryo in all tissues of the primitive streak remnant as well as the base of the allantois. At this stage, *Cdx2* is also observed in the posterior neural plate and notochord, hindgut endoderm and in unsegmented paraxial mesoderm. At later stages, *Cdx2* expression continues in the tail bud and by E12.5 is localized to the posterior-most embryo and the gut endoderm with a sharp anterior boundary slightly rostral to the junction of the foregut and midgut. In the gut endoderm, expression gradually declines in the caudal direction until the level of the rectum, where it can no longer be detected. As with *Cdx1*, *Cdx2* expression is maintained in the endoderm of the intestine and colon in the adult, with transcripts observed in the crypt cells (Freund et al., 1992; Duluc et al., 1997). However, unlike Cdx1, which is confined to the crypt cells, Cdx2 protein is also detected in the differentiated progeny that

derive from these cells. It is also notable that, in the large intestine, Cdx2 is more abundant in the rostral colon, while Cdx1 is higher in the caudal colon. In the adult, the only other tissue found to express appreciable levels of *Cdx2* is the pancreas, where is has been proposed to have a role in regulating the expression of glucagon and insulin (German et al., 1992; Jin and Drucker, 1996).

The pattern of expression of *Cdx4* is more restricted relative to that of *Cdx1* and *Cdx2* (Gamer and Wright, 1993). *Cdx4* maps to the X inactivation center of the murine and human X chromosome (Horn and Ashworth, 1995). Low levels of expression are first detected at E7.5 in the allantois and the posterior tip of the primitive streak. By early neurulation stages, *Cdx4* transcripts are observed in all tissues of the primitive streak region with levels increasing toward the caudal embryo. Expression in the paraxial mesoderm reaches a rostral limit posterior to the last formed somite, while the limit of expression in the neurectoderm is slightly more anterior. However, a sharp boundary is not apparent for either neurectoderm or mesoderm. *Cdx4* is also detected in the posterior lateral plate mesoderm, intermediate mesoderm, and the hindgut endoderm from E8.5 to E9.5. After E10.5 *Cdx4* is no longer detected, although data from the adult are limited.

In the chick, a comparative analysis of expression for all three *Cdx* homologues shows strong similarities to observations made in the mouse (Marom et al., 1997). At full streak (Hamburger Hamilton stage 4), *Cdx-A* is expressed in the mid section of the primitive streak, with *Cdx-C* and *Cdx-B* expressed in progressively more posterior domains. At early somitogenesis (stage 7) transcripts for all three members are distributed throughout the primitive streak region, with Cdx-A exhibiting a more rostral limit of expression slightly anterior to Henson's node. During streak regression to tailbud stages, there is a sequential extinction of expression, with loss first of *Cdx-A* at stage 10, *Cdx-C* by stage 14, and *Cdx-B* at stage 18.

In summary, the above data suggest that both murine and chick *Cdx* genes are expressed in nested domains along the developing A–P axis from mid-gastrulation to tail bud stages, as summarized for the mouse in Fig. 1. These dynamic patterns of expression have been proposed to suggest that relative combinations of Cdx levels in the primitive streak may be an important determinant of their function (Marom et al., 1997; Charité et al., 1998). As discussed below, recent data suggest that such a model is applicable as regards a role for Cdx proteins in axial patterning and posterior specification.

2.2. Cdx function during vertebrate development

Translational repression of *cad* in the anterior region of the *Drosophila* embryo is affected by the *bicoid* gene product (Rivera-Pomar and Jackle, 1996; Rivera-Pomar et al., 1996), and this repression is necessary for proper development of anterior structures. Ubiquitous expression of *cad* early in embryogenesis leads to defects in head formation and segmentation (Mlodzik et al., 1990). Conversely, loss of

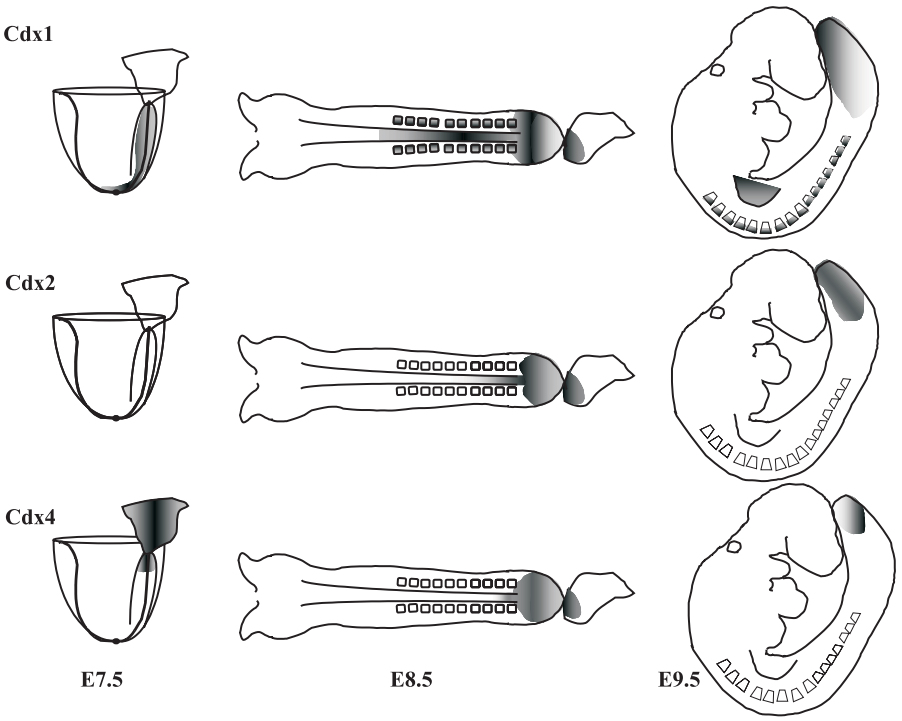

Fig. 1. Schematic representation of expression of *Cdx* members in E7.5–E9.5 mouse embryos. Relative level of expression is denoted by shading for each gene. Note that extraembryonic patterns of expression are not indicated.

maternal *cad* expression results in the deletion and malformation of posterior segments (Macdonald and Struhl, 1986), whereas loss of zygotic and maternal *cad* expression causes an anterior transformation of the tenth abdominal segment (Moreno and Morata, 1999). Moreover, expression of *cad* in the head and wing discs of the *Drosophila* larvae results in the formation of ectopic analia structures (Moreno and Morata, 1999). Taken together, these data suggest that cad has two separate functions, the first is to ensure proper A–P patterning and segmentation of the embryo and the second is to direct the formation of the posterior-most structures.

The A–P patterning function of cad in *Drosophila* appears to have been conserved in at least two of the vertebrate *Cdx* gene products. Mice bearing null mutations in *Cdx1* and *Cdx2* have been generated by homologous recombination (Subramanian et al., 1995; Chawengsaksophak et al., 1997). *Cdx1* homozygous null mice survive until adulthood and are fertile. Externally, these mice appear normal, however their vertebrae have undergone a series of homeotic transformations, most of which are anterior in nature (Fig. 2; see below). Certain of these defects are also observed at lower frequency in heterozygous offspring, consistent with the concept that the level

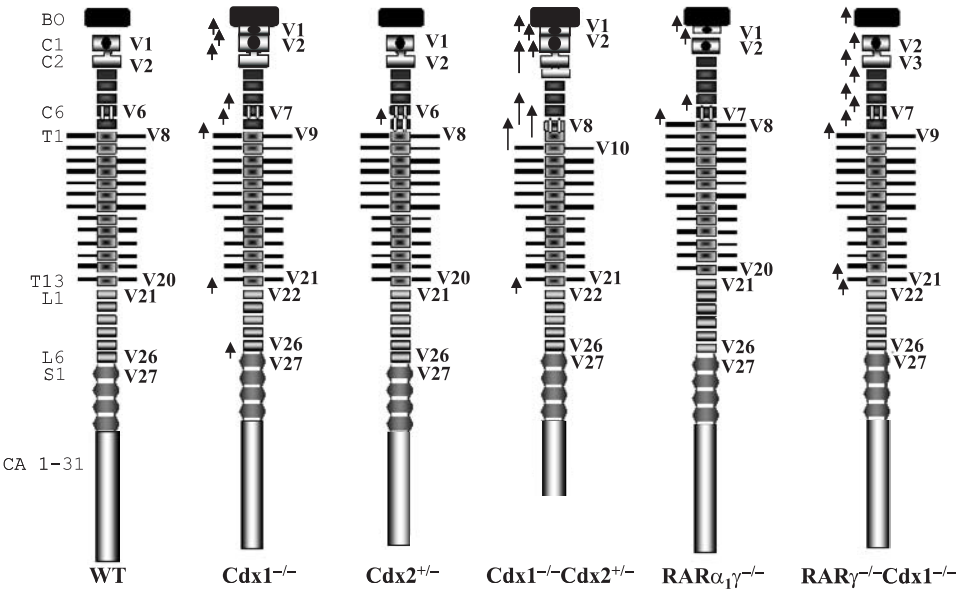

Fig. 2. Schematic representation of vertebral defects in *Cdx* and *RARγ-Cdx1* mutant offspring. Wild type, $Cdx1^{-/-}$, $Cdx2^{+/-}$, $Cdx1^{-/-}Cdx2^{+/-}$, $RAR\alpha\gamma^{-/-}$, and $RAR\gamma^{-/-}Cdx1^{-/-}$ skeletal patterns are shown. Designation of vertebrae is indicated to the left of the each mutant (BO, basioccipital; C, cervical; T, thoracic; L, lumbar; S, sacral; CA, caudal) Vertebral numbering, with C1 denoted as the first element, is indicated to the right. Arrows indicate vertebral transformations, with the length of the arrow denoting transformation extending over one (short arrow) or two (long arrow) vertebrae.

of Cdx protein is an important determinant of their function. These vertebral transformations are most highly penetrant in the cervical region, although transformations are also observed in the anterior thoracic region. Despite the expression of *Cdx1* in other tissues, including the limb buds, dermamyotome, intestine, and mesonephros, no abnormalities have been reported in these tissues in the cognate null offspring.

Cdx2 homozygous null embryos fail to implant, and do not survive past E3.5 (Chawengsaksophak et al., 1997). This peri-implantation lethality is most likely due to a defect in the trophectoderm, where *Cdx2* is first expressed, although the particular nature of this deficiency has not been analyzed in detail. Although *Cdx2* heterozygotes are viable at weaning, they are underrepresented relative to wild type littermates, suggesting an additional window of lethality which has yet to be characterized.

Like *Cdx1* null mice, *Cdx2* heterozygotes exhibit homeotic transformations of the vertebral column which affect the posterior cervical and anterior thoracic vertebrae (Chawengsaksophak et al., 1997; Fig. 2). These defects overlap with the posterior region of the vertebral column impacted in *Cdx1* homozygous null mutants, but extend to more caudal levels. This may reflect relative patterns of expression

during development, with *Cdx1* expressed more anteriorly, and with an earlier onset, than *Cdx2*.

In addition to vertebral transformations, *Cdx2* heterozygous offspring also exhibit a caudal truncation defect manifested by a shortened tail, a defect not observed in *Cdx1* null mutants. While the basis for this malformation is presently unknown, it would appear to differ from the events underlying the vertebral homeosis.

Cdx2 heterozygous offspring also exhibit polyp-like lesions which arise primarily in the proximal colon, a region of the intestinal tract which exhibits the highest level of the transcription factor (Chawengsaksophak et al., 1997; Beck et al., 1999) These lesions consist of stratified squamous epithelium typical of esophagus and forestomach. Epithelium displaying characteristics of intestinal tract tissues progressively caudal to the forestomach, including cardiac, pyloric and small intestine epithelium, is found interspersed between these lesions and areas of normal colon. It is noteworthy that the ectopic stratified squamous epithelium does not express Cdx2, but the adjacent intervening tissue expresses Cdx2 in a manner which progressively increases towards the region of normal colon peripheral to the lesions. This situation reflects the graded A–P pattern of expression seen along the digestive tract, with Cdx2 absent from esophagus to pylorus, expressed at low levels in the small intestine, and at maximal levels in the proximal colon (Freund et al., 1992; Duluc et al., 1997; Freund et al., 1998; Beck et al., 2000). The extinction of expression in the lesions is not due to loss of heterozygosity, but may reflect epigenetic events leading to failure of an autoregulatory loop, as discussed below.

Taken together, the above observations led to the supposition that *Cdx2* encodes information necessary to caudalize gut endoderm to a proximal colon identity. In the absence of this information, cells differentiate to a rostral (esophageal) fate. The development of tissues of progressively more posterior identity surrounding the periphery of the lesions has been proposed to represent intercalary regeneration between rostral-most (Cdx null) and normal proximal colon fates (Beck et al., 1999).

A *Cdx4* null mouse has not yet been described. As *Cdx4* localizes to the X chromosome, and is normally X-inactivated in the mouse (Horn and Ashworth, 1995), this chromosomal linkage may prove problematic for germ-line transmission. While a definitive role for Cdx4 in mouse development awaits description of the null mutant phenotype, several lines of evidence suggest that it plays a role in A–P patterning and/or posterior specification. This is supported by the demonstration that altered Xcad3 function, the *Xenopus* homologue of *Cdx4*, leads to profound effects in the developing frog. Ectopic expression of an active form of *Xcad3* inhibits anterior development, while expression of a dominant-negative results in the loss of trunk and tail structures (Pownall et al., 1996; Isaacs et al., 1998). Moreover, in the mouse, anteriorization of *Cdx4* expression results in the rostral expansion of the limit of a putative Cdx target gene, *Hoxb8*, suggesting a direct link between Cdx4, *Hox* gene expression and A–P patterning (Charité et al., 1998).

2.3. Functional specificity or redundancy among Cdx members?

From the above data, it is unclear if the Cdx proteins exhibit unique or redundant functions. Although potential overlap with Cdx4 cannot be excluded at present, certain observations from mouse loss-of-function models suggest that some aspects of Cdx1 and Cdx2 function are specific. For example, the vertebral homeosis in *Cdx1* null and *Cdx2* heterozygous offspring are largely distinct, although there is some overlap in the posterior cervical/anterior thoracic region. A reduction of *Cdx2*, but not *Cdx1*, leads to lesions in the colon, although both genes are co-expressed in crypt cells in this tissue; posterior truncation of the axis is likewise specific to a reduction in Cdx2. Additional observations also support a specific role for Cdx members. For example, a number of intestinal or pancreatic target genes have been described (Table 1) which respond well to Cdx2 but poorly to Cdx1 (Mitchelmore et al., 2000; Sakaguchi et al., 2002; reviewed in Freund et al., 1998). The effect of *Cdx1* and *Cdx2* overexpression on the growth, differentiation or tumorigenic phenotype of some intestinal cell lines has likewise been reported to be divergent (Lorentz et al., 1997; Mallo et al., 1998).

In contrast to specificity, several studies suggest that Cdx members may functionally overlap. For example, the phenotypes evoked by inhibition of Xcad3 and Xcad2 function in the frog are much stronger than the effects of disruption of any single murine Cdx gene. A potential explanation for this observation is that these dominant-negative strategies affect all Xcad target genes, whereas gene knockout in the mouse impacts on only a subset of targets depending on the pattern of expression of the disrupted gene and the relative abundance of other, compensatory, Cdx members. Gain-of-function for Xcad2 and Xcad3 in *Xenopus* and Cdx-B in the chick also results in some related phenotypes, again suggesting that they converge on a similar cohort of target genes (Epstein et al., 1997; Isaacs et al., 1998; Ehrman and Yutzey 2001).

The issue of functional specificity in the mouse has recently been directly assessed by analysis of an allelic series of *Cdx1–Cdx2* compound mutants (van Den et al., 2002). Both *Cdx1* null and $Cdx1^{+/-}Cdx2^{+/-}$ offspring were found to exhibit nearly identical defects of the anterior vertebral column, while $Cdx1^{-/-}Cdx2^{+/-}$ offspring exhibit more pronounced vertebral homeosis than is seen in either respective single mutant background (Fig. 2). Finally, the severity of the caudal truncation seen in Cdx2 heterozygotes is markedly increased by the subsequent loss of *Cdx1*. In this regard, it is noteworthy that Cdx2 heterozygotes do not exhibit anterior cervical homeotic transformations, and that Cdx1 null mutants are unaffected as regards the caudal embryo. These data therefore support a role for functional overlap between these two gene products and suggest that a better understanding of Cdx function awaits disruption of Cdx4 and generation of a comprehensive series of compound null mutants.

Intriguingly, in addition to vertebral malformations and axial truncation, *Cdx1–Cdx2* compound mutants also exhibit a low incidence of hindlimb defects, notably polydactyly. This is somewhat perplexing as *Cdx1* null mutant offspring do not exhibit limb defects, and only transcripts for *Cdx1* and not *Cdx2*, are observed in the

Table 1
Cdx1/*cad* target genes

Gene	Cdx response element
Drosophila *Fushi tarazu* (Dearolf et al., 1989)	5′ TTTTAGG GAAC CATAAA … TTTTATG TCTTTATG 3′
Mouse *hoxb8* (Charité et al., 1998)	5′ CAATAAAA … CTATAAAAGTTTATAGGGTATAAAT 3′
Mouse *hoxa7* (Subramanian et al., 1995)	5′ TTTATG 3′
Mouse *Sucrose isomaltase* (Suh et al., 1994)	5′ CAATAAA ACTTTATGA 3′
Hamster *insulin I* (German et al., 1994)	5 CTAATTAC 3′ (Flat element)
Mouse *proglucagon* (Jin and Drucker 1996)	5′ AGAAATTTATATTGTCAGCGTAATATCTG 3′
Mouse *Cdx2* (Xu et al., 1999)	5′ ACTAATAGAGTCTTGTAAACACTCGTTAATCA 3′
Pig *lactazse-phlorizin Hydrolase* (Mitchelmore et al., 2000)	5′ AATTTTATTATCA…GTTACATATTAAG… Cdx Hnf1α TAGTATTTTAC 3′ Cdx
Human *Claudin-2* (Sakaguchi et al., 2002)	5′ GTCAATATTTAAT…GTTTATGGATTTTTTTAGGT 3′ Hnf1α Cdx Cdx
Human calbindin-D9k (Barley et al., 1999)	5′ TGCCCGTAAAGACTATAAAAGT 3′
Human VDR (Yamamoto et al., 1999)	5′ ATAAAAACTTAT 3′
Human carbonic anhydrase 1 (Drummond et al., 1996)	5′ AATTTTTTACAACACCT 5′
Rabbit phospholipase A (Taylor et al., 1997a)	5′ CAAGATTTATGACAAGTA

Consensus response elements are underlined. Mutations that either abolish transcriptional response, or *Cdx*/*cad* binding are indicated in bold.

limb bud. This suggests that other domains of expression common to both genes, such as the lateral plate or intermediate mesoderm, may be indicative of a field of function for Cdx required for proper limb patterning (reviewed in Tickle and Munsterberg, 2001).

2.4. Transcriptional regulation by Cdx

Transcription factors are typically modular in nature, harboring, at a minimum, a sequence-specific DNA-recognition motif and an activation domain. In this regard, Cdx proteins possess three highly conserved motifs: (i) an N-terminal region necessary for proper subcellular trafficking (Trinh et al., 1999b); (ii) a homeodomain, a well known DNA-binding motif; and (iii) a highly conserved "hexapeptide" sequence N-terminal to the homeodomain (Fig. 3).

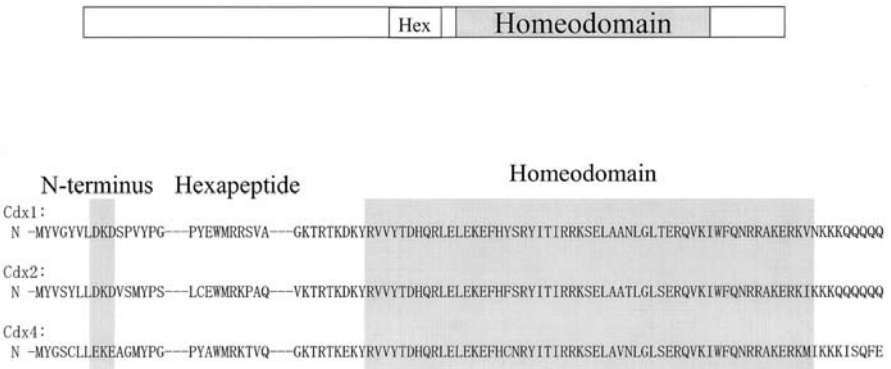

Fig. 3. Homology between murine Cdx family members. Regions of conservation between Cdx1, Cdx2, and Cdx3 proteins are indicated. Note also the "extended" homology flanking the homeobox sequences.

Hexapeptide motifs have been shown to be essential for protein–protein interactions between other transcription factors, most notably between certain Hox proteins and members of the Pbx family (Chang et al., 1995; Phelan et al., 1995; reviewed in Mann and Affolter, 1998). In this regard, however, Hox–Pbx interactions on target gene promoters involve an element consisting of two closely apposed binding sites, a 5′ Pbx binding site and a 3′ Hox binding motif (Knoepfler et al., 1996; Mann and Affolter, 1998). Such a bipartite response element has not been shown to be essential for Cdx-dependent gene activation. Moreover, protein–protein interaction assays have failed to reveal Pbx–Cdx complexes (M. Featherstone, personnel communication). Whether the Cdx hexapeptide sequence is a vestigial element retained from an ancestral *ProtoHox* gene, or whether it mediates association with Pbx (or other co-factors) under specific conditions is presently unknown.

The prototypical cad target gene is the pair-rule gene *Fushi Tarazu* (*Ftz*). The *Ftz* promoter harbors four cad response elements (CDRE), arranged as two inverted doublets of the sequence TTTATG; these elements are essential for expression from the *Ftz* promoter in transgenic flies (Dearolf et al., 1989). A consensus DNA-binding sequence for chicken Cdx-A was subsequently defined by site selection from pools of oligonucleotides of random sequence (Margalit et al., 1993). The consensus derived from this approach (A/CTTTATA/G) can convey Cdx-response to a basal promoter, and is represented in the *Ftz* CDRE. A number of *Cdx* target genes have since been identified, most of which are regulated through elements highly related to this consensus sequence (Table 1).

Although a consensus CDRE has been demonstrated, and a number of target genes have been identified, the molecular basis by which Cdx proteins function remains unclear. By analogy to other transcription factors, Cdx proteins are presumed to affect the transcription of target genes by binding to cognate response elements and recruiting regulatory co-factors (co-activators or co-repressors) to the target locus with subsequent impact on transcription. Transcriptional co-factors may

interact with constituents of the basal transcriptional machinery and/or affect chromatin remodeling. Alternatively, many co-regulators target the amino-terminal "tails" of one or more core histones of the nucleosome, presumably altering access to the underlying DNA. Typical histone modifications include phosphorylation, methylation, acetylation, and ubiquitination, and it is believed that the sum pattern of such modifications represent a "histone code" for transcription (reviewed in Jenuwein and Allis, 2001; Bannister et al., 2002; Conaway et al., 2002; Dillon and Festenstein, 2002; Hsiao et al., 2002).

The effects of Cdx could, theoretically, positively or negatively influence transcription depending on the nature of the co-regulators recruited. For example, a number of co-regulators possess histone acetyl transferase (HAT) activity, such as p300 or CREB binding protein (CBP). Recruitment of such factors generally positively influences transcription, and this effect is believed to occur, at least in part, through acetylation of histone tails (Bannister and Kouzarides, 1996; Goodman and Smolik, 2000). Conversely, recruitment of histone deacetylase (HDAC) activity by a given transcription factor generally inhibits transcription (Dillon and Festenstein, 2002). In this regard, while there is ample precedent for Cdx members acting as activators of transcription, there are also examples of certain target genes which are inhibited, at least in transfection models, and it may be anticipated that both classes of co-regulators may be recruited. Moreover, as discussed above, Cdx1 and Cdx2 show disparate effects in several model systems, suggesting that they exhibit some degree of functional specificity at least in these particular contexts.

Heterologous transcription assays in transfected cells or *Xenopus* embryos have demonstrated that transactivation can be mediated by a broad region of approximately 180 amino acids N-terminal to the Cdx homeodomain (Epstein et al., 1997; Taylor et al., 1997b; Trinh et al., 1999). In the case of Cdx2, two smaller domains, each of which is capable of conveying transactivation in a cell-type specific manner, have been identified (Trinh et al., 1999). However, these sub-domains may be specific to Cdx2, as similar regions of Cdx1 do not support transactivation in embryocarcinoma cells (our unpublished results). More evidence, albeit circumstantial, for a role for the Cdx N-terminus in transactivation comes from the finding that an Xcad2 mutant lacking key residues of the homeodomain behaves in a dominant-negative fashion in vivo (Epstein et al., 1997); a logical basis for this effect would be to squelch co-activators from endogenous Xcad proteins.

The above data suggest that the N-terminal region of Cdx impacts on transcription, presumably through recruitment of co-regulators. The fact that, with few exceptions, the N-terminal region is poorly conserved between Cdx proteins within a given species suggests that each Cdx member may interact with different co-factor(s), perhaps reflecting regulation of specific subsets of target genes and/or in a cell-type specific manner. However, such a hypothetical functional specificity would only be expected to apply to a subset of target genes, since the phenotype of Cdx1–Cdx2 compound mutants is consistent with their playing overlapping roles in vertebral patterning and posterior specification. Whether these Cdx double mutants also exhibit an exacerbation of the intestinal phenotype characteristic of Cdx2 heterozygous offspring is presently unknown.

To date, one well documented co-activator, CBP, has been shown to associate with Cdx2 and to augment expression from a Cdx-responsive promoter (Lorentz et al., 1999). Although this interaction may be essential to Cdx function, CBP is unlikely to act in a cell-type specific manner as it, or the highly related molecule p300, are widely distributed (Yao et al., 1998; Vo and Goodman, 2001). Moreover, CBP has been shown to interact with the homeodomain of Cdx2, whereas N-terminal sequences appear to be key mediators of transactivation.

While the nature of ancillary factors recruited to the Cdx N-terminus remains an enigma, it is likely that at least certain co-regulators function in a cell-type specific manner. For example, Cdx2 can induce transcription in both Caco2 cells (an intestinal cell line) and NIH 3T3 cells (a fibroblast-derived cell line) when a reporter harbors a CDRE in a promoter configuration (i.e. proximal and 5′ to basal promoter sequences). However, when the response element is juxtaposed to a 3′ configuration, a standard test for enhancer properties, Cdx2 stimulates expression only in Caco2 cells (Taylor et al., 1997b). This observation suggests the potential existence of two classes of co-activators for Cdx2, one functioning in a promoter context and common to NIH 3T3 and Caco2 cells, and a second which conveys enhancer-like properties to Cdx2 and is restricted to Caco2 cells. The possibility of multiple, tissue restricted, co-regulatory molecules is further supported by the finding that specific regions of the N-terminus of Cdx2 can mediate transcription in a cell-type specific manner (Trinh et al., 1999).

2.5. Combinatorial regulation of gene expression by Cdx2

Regulation of a given gene is typically affected by combinations of transcription factors, the net result of which contributes to tissue-specific expression. In the case of Cdx2, interactions with HNF-1α (hepatocyte nuclear factor 1α, a transcription factor of the forkhead class) have been demonstrated on several promoters. The *lactazse-phlorizin hydrolase* gene, which encodes a brush-border enzyme expressed in the small intestine, harbors two Cdx2 binding sites separated by an HNF-1α binding site (Table 1). Cdx2 and HNF-1α can associate directly with these elements, resulting in a synergistic activation of transcription in transfection assays (Mitchelmore et al., 2000). Cdx2 and HNF-1α can also physically interact via their homeodomains, although it is presently unclear if this particular interface is necessary for the observed synergy on the *lactazse-phlorizin hydrolase* promoter.

Cdx and HNF-1α also co-operate on the *claudin-2* promoter. Claudin-2 is a component of tight junctions in the intestine, kidneys, and liver (Heiskala et al., 2001). The proximal promoter of *claudin-2* contains two conserved binding sites for Cdx preceded by an HNF-1α element, and a critical role for HNF-1α in this pathway is supported by loss of *claudin-2* expression in the villus of the ileum and liver in HNF-1α null mice. Interestingly, although both Cdx1 and Cdx2 induce expression from the Claudin-2 promoter in Caco2 cells, HNF-1α potentiates the effects of only Cdx2 (Sakaguchi et al., 2002).

Synergistic interactions between Cdx2 and the paired-box transcription factor Pax6 (Ritz-Laser et al., 1999) and the CBP-related protein p300 (Hussain and Habener, 1999) have also been described on the *glucagon* promoter. In this model, it is interesting to note that Cdx2 may impact on *glucagons* transcription by increasing the interaction between Pax6 and p300, with all three factors contributing to a synergistic activation of transcription. Interestingly, the effect of Cdx2 on Pax6–p300 association does not absolutely require Cdx2 DNA binding, suggesting that scans for consensus Cdx binding motifs may reveal only a subset of target genes.

3. Cdx and vertebral patterning

3.1. Hox genes

In a number of model systems, loss of Cdx function results in phenotypes that can be interpreted as homeotic transformations. In the mouse, mutation of *Cdx1* and/or *Cdx2* leads to homeotic transformation of certain vertebrae. These observations are consistent with the homeotic transformation of A10 to an A9 identity seen in cad deficient *Drosophila*. However, in contrast to *Drosophila*, there is now substantial evidence that defects in the mouse and other model organisms reflect a role for Cdx proteins in direct regulation of expression of some members of the *Hox* gene family. There is also considerable data suggesting that Cdx members serve to relay signals by other factors involved in vertebral patterning to the *Hox* genes.

Hox genes encode homeodomain transcription factors, and arose from an ancestral group of homeotic genes related to the *HOM-C* genes of *Drosophila* (Lewis, 1978; Lewis, 1994; Akam, 1998; Gellon and Mcginnis, 1998; Ferrier and Holland, 2001; Trainor and Krumlauf, 2001). In the mouse, the 39 *Hox* genes are arrayed in four separate clusters, *Hoxa–Hoxd*. Based on relatedness to their *HOM-C* homologues, and to their relative physical location in each of the four clusters, the vertebrate *Hox* genes have been arranged into 13 paralogue groups.

Hox genes within a given cluster are expressed in a temporally controlled manner along the developing axis, while members within a given paralogue group generally exhibit similarities in onset of expression and in their rostral boundary of expression along the A–P axis (Mcginnis and Krumlauf, 1992; Krumlauf, 1994; Duboule, 1998). This temporal pattern of expression, termed co-linearity, was first noted in *Drosophila* (Lewis, 1978) and was later demonstrated in the developing mouse (Duboule and Dollé, 1989; Graham et al., 1989). Briefly, *Hox* genes situated at the 3′ end of a given cluster tend to be expressed at earlier stages, and with a more rostral limit of expression, than *Hox* genes located in 5′ regions of the locus. In the mouse, this results in a nested set of expression domains such that distinct axial levels exhibit a unique compliment of *Hox* transcripts, which has been referred to as a "Hox code" (Kessel and Gruss, 1991). This implies that the specific combination of *Hox* gene products expressed in a given region of the developing CNS, vertebral column, limbs or organs regulates the developmental fate of that particular structure. Such a

model is supported by a wealth of gain- and loss-of-function experiments, the outcome of which frequently manifests as a homeotic transformation(s) (Burke et al., 1995; Conlon, 1995; Gavalas and Krumlauf, 2000). *Hox* expression must therefore be strictly controlled, both spatially and temporally, for correct patterning of the embryo.

3.2. Regulation of Hox gene expression

There are three general phases of *Hox* gene expression in the developing mouse embryo; initiation, establishment, and maintenance (reviewed in Deschamps et al., 1999). Expression of the 3′ most *Hox* genes is typically initiated around E7.5 in the primitive streak during gastrulation, with sequential activation of progressively more 5′ *Hox* genes continuing in the posterior embryo until E9.5. Following initiation, the expression domains spread forward in the CNS, paraxial mesoderm, and lateral plate mesoderm until a predetermined rostral limit is reached. This forward spreading mechanism is thought to be intrinsic to the cells that have completed gastrulation, occurs in the absence of cell mixing, and can traverse a physical barrier implanted into the embryo (Gaunt et al., 1999; Gaunt, 2001). Once the appropriate boundary has been reached, expression is further reinforced by other transcription factors as well as through auto- and cross-regulation by the *Hox* gene products themselves. In Drosophila, *HOM-C* expression is maintained through chromatin remodeling by *Polycomb*-group and *Trithorax*-group proteins, and a similar mechanism of long-term maintenance appears to have been conserved in vertebrates (reviewed in Gebuhr et al., 2000; Mahmoudi and Verrijzer, 2001).

3.3. Hox genes and A–P vertebral patterning

In vertebrates, among other functions, the *Hox* gene products are involved in the A–P patterning of metameric structures, such as the rhombomeres of the hindbrain and the vertebrae. Vertebrae arise from paraxial mesoderm formed by epiblast cells ingressing through the primitive streak (Christ and Ordahl, 1995; Tam et al., 2000). Paraxial mesoderm, deposited along both sides of the midline, subsequently condenses and segments off to form bilaterally-paired somites. Somites subsequently differentiate into sclerotome ventrally, and dermamytome dorsolaterally. The sclerotome gives rise to the vertebrae, occipital bones and ribs, whereas the dermamyotome contributes to the dermis and trunk and limb musculature.

Although somites are indistinguishable, most vertebrae exhibit unique morphological characteristics. The anterior-most somites gives rise to the occipital bones, while more posterior somites gives rise to the vertebral column. In the mouse, the vertebral column is normally composed of 7 cervical (C1–C7), 13 thoracic (T1–T13), 6 lumbar (L1–L6), 3 or 4 sacral (S1–S4), and 31 caudal vertebrae. The first cervical vertebra (C1, or atlas) has thick neural arches, lacks a vertebral body, and exhibits a ventrally located tubercle, the anterior arch of the atlas (AAA). The neural arches of C2, although not as broad as those of C1, are thicker than the more posterior cervical vertebrae. C2 also possesses two vertebral bodies, the second of which

(the dens axis) is composed of material derived from C1. Vertebrae C3 to C5 are virtually identical, while C6 is distinguished by ventrally protruding anterior tuberculi. C7 resembles C3 to C5 but lacks transverse foramen. The thoracic vertebrae are characterized by the presence of ribs the first seven of which (T1–T7) attach to the sternum. The second thoracic vertebra is further distinguished by a large dorsal spinous process. Several of the five lumbar and four sacral vertebrae also exhibit unique characteristics.

These distinct vertebral morphologies implies a molecular basis for establishing their identity along the A–P axis, and this information is imparted early in their genesis. In the chick, if unsegmented paraxial mesoderm containing presumptive cervical somites is transplanted to the thoracic region, the resultant vertebrae do not develop ribs, and the prevertebrae from such a manipulation continue to express the *Hox* genes that are characteristic of cervical somites (Kieny et al., 1972; Nowicki and Burke, 2000). Therefore, despite their uniform morphology, molecular differences exists in the somite early in their ontogenesis that imparts their distinct A–P vertebral identity; a large body of data demonstrates that this information is encoded by the *Hox* gene products (Krumlauf, 1994; Burke et al., 1995).

3.4. Regulation of Hox expression by Cdx

Vertebral homeotic transformations are a frequent outcome of *Hox* gene inactivation. A link between Cdx function and *Hox* gene expression is therefore inferred by the finding of vertebral homeosis in *Cdx1* null and *Cdx2* heterozygous offspring (Subramanian et al., 1995; Chawengsaksophak et al., 1997). The nature of several of these vertebral defects are close phenocopies of certain *Hox* mutants and correlate with posteriorized expression of these particular *Hox* genes (Table 2). However, it should also be noted that *Hox* transcripts are lost in only an anterior region of their normal expression domain typically spanning one somite. Although this posteriorized expression is slightly exacerbated in *Cdx1–Cdx2* compound mutants, posterior *Hox* expression remains unaffected (van Den et al., 2002). Additional mechanisms must therefore exist to sustain *Hox* expression in more caudal domains in the face of reduced Cdx levels. Whether this is due to residual Cdx function, or reflects the contribution of other pathways, is presently unknown.

The above data suggest that Cdx1 and Cdx2 are epistatic to *Hox*. A role for Cdx in directly affecting *Hox* expression is supported by the finding of potential CDREs in numerous *Hox* loci (Subramanian et al., 1995). For example, the *Hoxa7* promoter contains two putative Cdx binding sites in a region necessary for the correct specification of the anterior limit of *Hoxa7* expression in vivo (Knittel et al., 1995; Subramanian et al., 1995). Deletion of one of these two elements reduces the response of a reporter gene to Cdx1 by 50% in transfection assays (Subramanian et al., 1995). Further evidence for a direct relationship comes from the finding of functional Cdx binding sites in a regulatory region of the *Hoxb8* locus necessary for normal spatial expression of a transgenic reporter gene in vivo (Charité et al., 1998). Consistent with this latter observation, gain or loss of Cdx function in the mouse

Table 2
Phenotypic relationship between *Hox* and *Cdx1*/*Cdx2* mutants

Gene	Skeletal phenocopy	Expression in Cdx[a] mutants	Hox null mutant reference
Hoxd3	AAA assimilated into basioccipital bone Partial C2 to C1 transformation	Shifted from somite 5 to 6	Condie and Capecchi, 1993
Hoxd4	Partial C2 to C1 transformation Abnormal neural arches C1, C2, and C3. Extensive rib anlage on C7	Shifted from somite 6 to 7	Horan et al., 1995
Hoxc6	T2 to T1 transformation	Shifted from pre-vertebra 8 (weak) and 9 (strong) to 9 (weak) and 10 (strong)	Garcia-Gasca and Spyropoulos, 2000
Hoxa7/b7	Thoracic rib fusion	Hoxa7 shifted from pre-vertebra 10 to 11	Chen et al., 1998
Hoxb8	Absence of full rib on T1 Fusions between first pair of ribs	Shifted from pre-vertebra 7 to 8 in $Cdx1^{-/-}$ and to pre-vertebra 9 in $Cdx1^{-/-}Cdx2^{+/-}$	van Den et al., 1999
Hoxc8	T8 to T7 transformation	Shifted from pre-vertebra 11 (weak) and 12 (strong) to 12 (weak) and 13 (strong)	Le Mouellic et al., 1992
Hoxb9	Fusion of the first and second ribs T8 to T7 transformation	Shifted from somite 13 to 14 in Cdx1 null and from somite 13 to 15 in $Cdx1^{-/-}/Cdx2^{+/-}$	Chen and Capecchi, 1997

[a] $Cdx1^{-/-}$ and/or Cdx1/Cdx2 compound mutants (Subramanian et al., 1995; van Den et al., 2002).

results in anteriorized or posteriorized expression of *Hoxb8*, respectively. Taken together, these findings strongly suggest that Cdx members directly regulate the expression of certain *Hox* genes with subsequent impact on vertebral patterning along the A–P axis.

As discussed above, in addition to vertebral homeosis, Cdx2 heterozygotes exhibit intestinal defects which may be interpreted as a transformation of colon epithelium to a more anterior (esophageal) fate. A number of *Cdx2* target genes, expressed in the intestine, have been described, and it conceivable that this intestinal phenotype is dictated by the loss of expression of this constellation of genes. Alternatively, however, a number of *Hox* genes are expressed along the A–P of the digestive tract in a manner reminiscent of their pattern of expression along the developing vertebral column (Beck et al., 2000; Grapin-Botton and Melton, 2000). It is therefore tempting to speculate that the intestinal phenotype exhibited in Cdx2 heterozygous offspring is the result of loss of expression of a cohort of *Hox* genes.

3.5. Cdx gene products function in the initiation phase of Hox gene expression

Hox gene expression is first initiated during gastrulation in the primitive streak region. As heterotopic transplantation studies indicate that vertebral A–P identity is conferred at, or shortly after this time, events related to this early phase of *Hox* expression would appear to be essential to vertebral patterning. Consistent with this, Cdx members and relevant *Hox* genes are co-expressed in the nascent mesoderm as it emerges from the streak or tail bud. This supposition is further supported by the finding that the anterior boundary of a *Hoxb8* reporter gene is posteriorized at E9 in Cdx1–Cdx2 compound mutants (van Den et al., 2002). As *Cdx* expression has regressed from this anterior region of *Hoxb8* expression by this time, the effects of loss of Cdx1 and Cdx2 must have occured during an earlier window.

3.6. A conserved role for regulation of Hox expression by Cdx?

In *Drosophila*, cad has properties consistent with a function as a homeotic selector gene in itself. However, in a number of other species, Cdx members appear to elicit at least part of their function through effects on *Hox* gene expression. In addition to the mouse, work in *Xenopus* supports a role for Xcads in patterning the A–P axis and in the case of Xcad2 and Xcad3, these events correlate closely with altered *Hox* expression. For example, a dominant negative Xcad3 elicits strong defects of the posterior embryo and suppresses expression of *Hoxc6*, *Hoxa7*, *Hoxb7*, and *Hoxb9*. Conversely, over-expression of Xcad3, or an activated Xcad3-VP16 fusion protein, results in ectopic expression of the same cohort of *Hox* genes, concomitant with suppression of anterior development (Isaacs et al., 1998, 1999). Similar observations have been made as regards Xcad2 overexpression, which anteriorizes *Hoxc6* and *Hoxb9* (Epstein et al., 1997), and chicken Cdx-B, which can activate *Hoxa7*, *Hoxc6*, and *Hoxc8* (Ehrman and Yutzey, 2001). The same cohort of *Hox* genes affected in these frog and chick models are also altered in *Cdx1* null and

Cdx1–Cdx2 compound mutants (Subramanian et al., 1995; van Den et al., 2002), suggesting that specific Cdx targets are conserved across vertebrate species.

The relationship between Cdx function and *Hox* gene expression also appears to be conserved in at least some invertebrates, as the Cdx homologue Pal-1 is upstream of the *Hox* gene *mab-5* in *C. elegans* (Hunter et al., 1999). Although additional models need to be evaluated, it is tempting to speculate that this association will hold in a number of other species. This may suggest an ancestral function of Cdx which has been lost in organisms such as *Drosophila*, or alternatively, may be indicative of a function for which Cdx members have been co-opted.

4. Regulation of Cdx1 expression

4.1. Players in posterior patterning

A number of factors are known to be essential for specification and/or patterning of the caudal embryo. Of particular interest are the vitamin A metabolite retinoic acid (RA) and certain Wnt and fibroblast growth factor (FGF) family members. In a variety of model systems, these signaling molecules can suppress markers of anterior identity concomitant with induction of genes indicative of more posterior fate, such as certain *Hox* members, an outcome generally referred to as posteriorization (reviewed in Sasai and De Robertis, 1997; Nieuwkoop, 1999; Altmann and Brivanlou, 2001; Stern, 2001). As discussed above, Cdx1 and Cdx2 (and likely Cdx4) are key players in development of the posterior embryo as regards both vertebral A–P patterning and specification of the caudal embryo. A number of studies now suggest that Cdx members act, in part, to relay information from signals involved in posterior patterning.

4.2. Retinoid signaling

RA, the carboxylic acid derivative of vitamin A, is the principle biologically active form of the vitamin. RA signals by binding to the RA receptors (RARα, β, and γ and their isoforms). RARs belong to the family of ligand-inducible nuclear receptors and modulate the expression of target genes via heterodimerization with a retinoid X receptor (RXRα, β, and γ) partner. These heterodimers bind to *cis*-acting regulatory sequences (RAREs) present in the promoter region of target genes. RAREs usually consist of direct repeats (DR) of the consensus sequence PuG(G/T)TCA with 2 or 5 nucleotides intervening the repeats (denoted a DR2 or a DR5 element, respectively) (Mangelsdorf et al., 1995; Lazar, 1999; Hansen et al., 2000). However, RAREs are highly polymorphic, and a number of other functional motifs have been described.

Retinoid signaling is essential for numerous ontogenic programs (Lohnes et al., 1995; Eichele, 1997; Niederreither et al., 1999; Maden, 2000), a general discussion of which is beyond the scope of this review. Of particular relevance, however, is the role for RA in regulation of *Hox* gene expression. A key observation

suggesting this relationship came from the finding that RA is able to activate *Hox* genes in embryocarcinoma cells in culture (Simeone et al., 1990; Boncinelli et al., 1991). Remarkably, this induction mimicked the co-linear activation of *Hox* genes seen during development, with 3′ genes induced by RA more rapidly than 5′ genes.

A relationship between RA, *Hox* gene expression and axial patterning was first evidenced by the finding that, in the mouse, treatment with RA at E7.0–8.5 results in homeotic transformations, primarily posteriorizations, of vertebral identity. These transformations occur concomitant with rostral shifts in the expression domains of a number of *Hox* genes, suggesting that RA reprograms A–P values by altering the vertebral *Hox* code (Kessel and Gruss, 1991; Conlon and Rossant, 1992; Krumlauf, 1994). This response is also temporally regulated, with the 3′-most *Hox* genes being insensitive to RA after E7, while more 5′ *Hox* genes remain responsive at progressively later times up until day 9 of gestation. Extensive work has also shown that RA exerts similar effects on *Hox* expression and patterning of the CNS at the level of the hindbrain (Marshall et al., 1992; Maden et al., 1996; Marshall et al., 1996; Kolm et al., 1997; Dupé et al., 1999; Gavalas and Krumlauf, 2000; Niederreither et al., 2000).

In agreement with studies employing exogenous RA, certain RAR null mice, or mice lacking the RA synthesizing enzyme RALDH2, also exhibit axial malformations and hindbrain patterning defects which correlate with altered *Hox* gene expression patterns (Lohnes et al., 1993, 1994; Dupé et al., 1999; Niederreither et al., 1999, 2000). A direct role for RA in regulating *Hox* expression is further attested by the finding of functional RAREs in the promoter/enhancer regions of *Hoxa1*, *Hoxa2*, *Hoxa4*, *Hoxb4*, and *Hoxd4* genes (Pöpperl and Featherstone, 1993; Marshall et al., 1994; Frasch et al., 1995; Marshall et al., 1996; Morrison et al., 1996; Dupé et al., 1997; Langston et al., 1997; Zhang et al., 1997; Huang et al., 1998; Packer et al., 1998; Studer et al., 1998). However, these RAREs appear to play a role in interpreting the RA signal as regards patterning of the CNS, and a definitive role for any of these elements in A–P vertebral patterning has not been presented.

4.3. Cdx1 is a direct RA target gene

The *Hox* RAREs documented to date have not been shown to play a critical role in regulating *Hox* function in paraxial mesoderm, suggesting that RA may impact on *Hox* expression in somites through an indirect mechanism; Cdx family members are a logical target for such a role. In investigating this hypothesis, we found that RA has a profound effect on *Cdx1* transcripts in the caudal embryo (Houle et al., 2000). This effect occurs during the window (E7.5–E9.5) when exogenous RA can impact on *Hox* expression and reprogram vertebral identity along the A–P axis. Subsequent analysis led to the identification of an RARE in the Cdx1 proximal promoter. Although this motif is an atypical element, it nevertheless behaves in a manner indistinguishable from a conventional DR5 RARE in mobility shift assays and transfection analysis (Houle et al., 2000). Moreover, these

sequences are absolutely conserved in the human *CDX1* promoter, further supporting a critical role for this element.

Further evidence for a role for RA as a direct regulator of *Cdx1* comes from analysis of RAR null mutants. Although the RARs are functionally redundant, vertebral homeosis are observed in some RAR single mutants, being most prevalent in RARγ null offspring and increasing in incidence and severity in RARα1γ and RAR$\alpha\gamma$ double mutants (Lohnes et al., 1994, 1995). Moreover, in agreement with the supposition that retinoid signaling affects vertebral patterning through *Cdx1*, certain of the homeotic transformations observed in these RAR null offspring bear strong resemblance to some of the defects seen in Cdx1 null mutants. Consistent with these observations, RARα1/γ double null embryos exhibit a reduced level of *Cdx1* expression in the primitive streak region at E7.5. However, *Cdx1* is not overtly affected in embryos derived from any RAR mutant background at E8.5 or later (Houle et al., 2000).

The finding of a specific window for RA-dependent regulation of *Cdx1* is in agreement with several observations. In the mouse, transgenic reporter assays suggest that biologically active retinoids are detected in the primitive streak at E7.5 (Rossant et al., 1991; Balkan et al., 1992), a stage which correlates closely with the onset of *Cdx1* expression (Meyer and Gruss, 1993). From E8.5, however, RA is undetectable in the caudal embryo although *Cdx1* expression continues in the primitive streak remnant. Moreover, the cervical vertebrae are most sensitive to RAR disruption, and it is this region of the axial skeleton which is presumably patterned at E7.5. Together, these data suggest that RA is involved in early stages of expression of *Cdx1* with subsequent impact on expression of *Hox* genes initiated during this period and involved in patterning the somites giving rise to anterior vertebrae.

While the above findings clearly support a role for retinoid signaling in regulating *Cdx1* expression, it is entirely possible that the RARE isolated in these studies is not functional in vivo, and/or that retinoid-response is conveyed by other means. To assess this, we have recently disrupted the *Cdx1* RARE in embryonic stem cells by homologous recombination and derived mice homozygous for this mutation. Offspring lacking this element exhibit a subset of the vertebral defects seen in Cdx1 null mutants, clearly demonstrating a critical role for this motif in regulating *Cdx1* expression.

4.4. Cdx1 is not the sole player in RA-dependent vertebral patterning

To further assess the relationship between RAR signaling and Cdx1 function, a complete allelic series of *Cdx1–RAR*γ compound null mutants was analyzed (Allan et al., 2001). This study illustrates a strong synergistic relationship between these transcription factors, with *RAR*γ*–Cdx1* double heterozygotes exhibiting vertebral defects that are not observed in either single heterozygous background. There are several mechanisms that could potentially underlie this interaction. First, RARγ and Cdx1 may converge on a common *Hox* target gene(s), with both factors contributing

to transcription. In this regard, a putative Cdx binding site is found in close proximity to an RARE in the 3′ region of the *Hoxd4* gene (Zhang et al., 1997, 2000). However, *Hoxd4* expression is not detectably altered in *RARγ* mutants (Folberg et al., 1999) and the posteriorization of *Hoxd4* expression, inherent to *Cdx1* mutants, is not further affected in *RARγ–Cdx1* double null offspring (our unpublished results). A second possibility is that RARγ and Cdx1 regulate the expression of distinct, separate, target genes which themselves converge on vertebral patterning in a synergistic manner. However, RA is unable to rescue the C2 to C1 transformation seen in *Hoxd4* mutants (Folberg et al., 1999), although it does so in RARγ null offspring (Iulianella et al., 1999). This suggests that *Hoxd4* and retinoid signaling regulate parallel pathways which converge on C2 morphogenesis. Given the interactions between *Hox* paralog group 4 genes in patterning C2 (Horan et al., 1995), these paralogs are a logical target. A final mechanism would be that loss of RARγ results in a reduction in *Cdx1* expression from the remaining wild-type allele, consistent with our demonstration that *Cdx1* is a direct retinoid target. Although *Cdx1* expression is not detectably altered in the RARγ null background, this does not exclude subtle differences in expression which may escape detection. Moreover, as both Cdx1 and Cdx2 heterozygotes exhibit vertebral defects, small alterations in Cdx1 are anticipated to have overt phenotypic consequence.

Offspring null for both *Cdx1* and *RARγ* exhibit a significant increase in the penetrance of certain vertebral transformations relative to either single mutant. This clearly demonstrates that RARγ must regulate the expression of factors involved in vertebral patterning in addition to *Cdx1*. Further evidence to this effect is provided by the finding that *Cdx1* null mutants remain sensitive to certain of the effects of exogenous RA on the vertebral axis (Allan et al., 2001).

As discussed above, RA–Cdx1 interactions could conceivably occur by regulation of a common target gene by both Cdx1 and the RARs, or through different target genes that both converge on vertebral patterning. As regards the former, *Hoxd3* is posteriorized by one somite in *Cdx1* null embryos at E9.5, and both *Cdx1* and *Hoxd3* mutants exhibit a fusion of the first cervical vertebra (C1) and the exoccipital bone (Condie and Capecchi, 1993; Subramanian et al., 1995). One of the most striking effects of administration of exogenous RA in *Cdx1* mutants is the restoration of a relatively normal cervical region, including this particular C1 defect. Consistent with this, *Hoxd3* expression is anteriorized by RA treatment at E7.5, thus normalizing its expression in *Cdx1* mutants (Allan et al., 2001). These data clearly demonstrate that RA can affect expression of some *Hox* genes in the absence of Cdx1, and further supports the contention that RARs function both upstream of, and in parallel to, Cdx1, in vertebral A–P patterning.

4.5. Regulation of Cdx1 expression by Wnt3a

The Wnt/wingless family of signaling molecules are involved in diverse ontogenic processes. In the canonical pathway, the Wnt signal is transduced

from receptors of the Frizzled family to cytosolic β-catenin. Cytosolic β-catenin is part of a multiprotein complex that includes APC, Axin and GSK3, among other components. In unstimulated cells, this complex contributes to the rapid degradation of β-catenin through a phosphorylation-dependent mechanism. Wnt signaling via Frizzled results in stabilization of β-catenin which then enters the nucleus, forms a complex with transcription factors of the LEF/TCF family and activates target genes (reviewed in Yamaguchi, 2001; Brantjes et al., 2002; Moon et al., 2002) . A number of Wnt target genes have been characterized, several of which are involved in axial patterning. Among these are *Drosophila ultrabithorax* (Riese et al., 1997), *Xenopus siamois* (Brannon et al., 1997) and the murine *brachyury* (T) transcription factor (Yamaguchi et al., 1999; Arnold et al., 2000).

Among *Wnt* family members, the pattern of expression of *Wnt-3a* closely resembles that of *Cdx1* in the caudal embryo. Loss of Wnt3a results in dose-dependent axial truncation varying from the absence of the tail to complete lack of axial structures caudal to the forelimb (Takada et al., 1994; Greco et al., 1996). A similar phenotype is observed in *LEF1/TCF1* compound null mutants (Galceran et al., 1999), suggesting that LEF1 and TCF1 interpret the Wnt3a signal critical to the normal development of posterior embryo.

Consistent with a role for Wnt signaling in regulating *Cdx1* expression, LEF/TCF binding sites are present in the proximal *Cdx1* promoter, and are necessary for LEF/β-catenin transcriptional response in F9 embryocarcinoma and epithelial cells (Lickert et al., 2000; Prinos et al., 2001). A role for Wnt signaling in regulating *Cdx1* expression in vivo stems from the finding that the Wnt3a hypomorph *vestigial tail* (*vt*) exhibits a reduction in *Cdx1* transcript abundance in the caudal embryo where the two genes are co-expressed. Moreover, Wnt3a null offspring, and to a lesser degree, *vt* hypomorphs, exhibit cervical vertebral homeosis reminiscent of certain of the defects associated with Cdx1 disruption (Ikeya and Takada, 2001). A more detailed evaluation of the role of Wnt3a in regulating Cdx1 expression is complicated by the loss of caudal tissue inherent to *Wnt3a* mutants, and the residual Wnt signal in *vt* hypomorphs.

The axial truncation phenotype observed in *Cdx2* heterozygotes suggests that this gene may also lie in the Wnt3a pathway. However, *Cdx2* transcripts are not altered in *vt* offspring (Ikeya and Takada, 2001; Prinos et al., 2001). In this regard, however, Cdx1–Cdx2 compound mutants exhibit a more pronounced caudal truncation than Cdx2 heterozygotes, and it is therefore conceivable that Wnt3a regulation of *Cdx1* may play a role in specification of the posterior embryo.

A relationship between Wnt signaling and *Cdx1* expression is likely conserved in other target tissues and in other species. For example, *Cdx1* abundance is reduced in intestinal crypt cells in *TCF4* null offspring (Lickert et al., 2000), while in *C. elegans* a genetic link between Wnt function and the nematode *caudal* homologue *pal1* has been demonstrated (Hunter et al., 1999; Zhang and Emmons, 2001). Also consistent with this latter observation is the finding that Wnt signaling affects the expression of several *Hox* genes in the nematode (Maloof et al., 1999;

Hoier et al., 2000), although a role for *pal1* in this cascade has not been formally demonstrated.

4.6. Wnt–RA interactions

Although *Wnt* signaling is widespread during embryogenesis, *Cdx1* is expressed only in a subset of tissues known to be dependent on this pathway. In this regard, both Wnt and RA signaling are involved in embryonic posteriorization events and can interact on these processes (Altaba and Jessell, 1991; Sasai and De Robertis, 1997; Altmann and Brivanlou, 2001). To assess possible interplay between these signaling pathways, we derived embryocarcinoma cell lines harboring a reporter gene under the control of the proximal *Cdx1* promoter. This reporter responds to RA or to conditioned media from a Wnt3a-expressing cell line. Remarkably, the addition of RA and Wnt3a-conditioned media together results in a profound induction of the reporter gene (Fig. 4). This interaction is likely due to direct regulation of the promoter, as it requires the presence of both the RARE and the LREs (Prinos et al., 2001). This observation suggests that in vivo interactions between retinoid and Wnt signaling pathways may occur, in part, through convergence on *Cdx1* (Fig. 5) with concomitant effects on target genes such as certain *Hox* members.

Whether Wnt–RA synergy is observed in other lineages is presently unknown. However, it is interesting to note that RA increases crypt formation and proliferation in intestinal grafts, and that this effect is paralleled by an induction of *Cdx1* (Plateroti et al., 1997). As *Cdx1* is also regulated by TCF4 in the intestine (Lickert et al., 2000, 2001), it is tempting to speculate that retinoid and Wnt

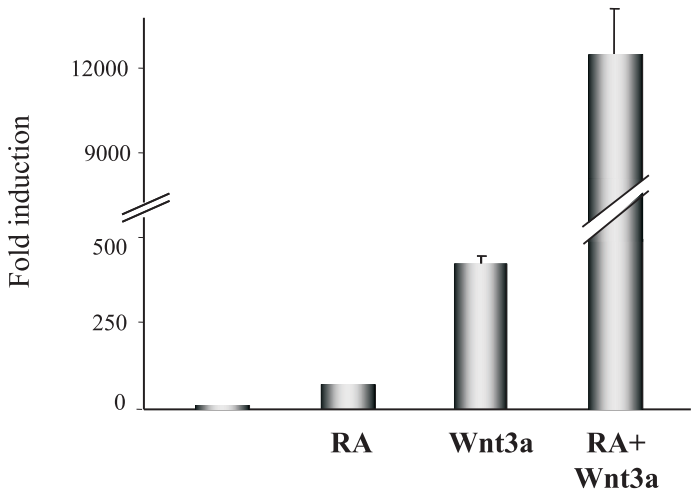

Fig. 4. Synergy between Wnt3a and RA on the *Cdx1* promoter. Stable cell lines harboring a *Cdx1* reporter vector (Prinos et al., 2001) were treated with RA (10^{-6} M), Wnt3a conditioned media, or the two together and luciferase activity assessed 24 h post-treatment. Note the strong synergistic interaction between RA and Wnt3a.

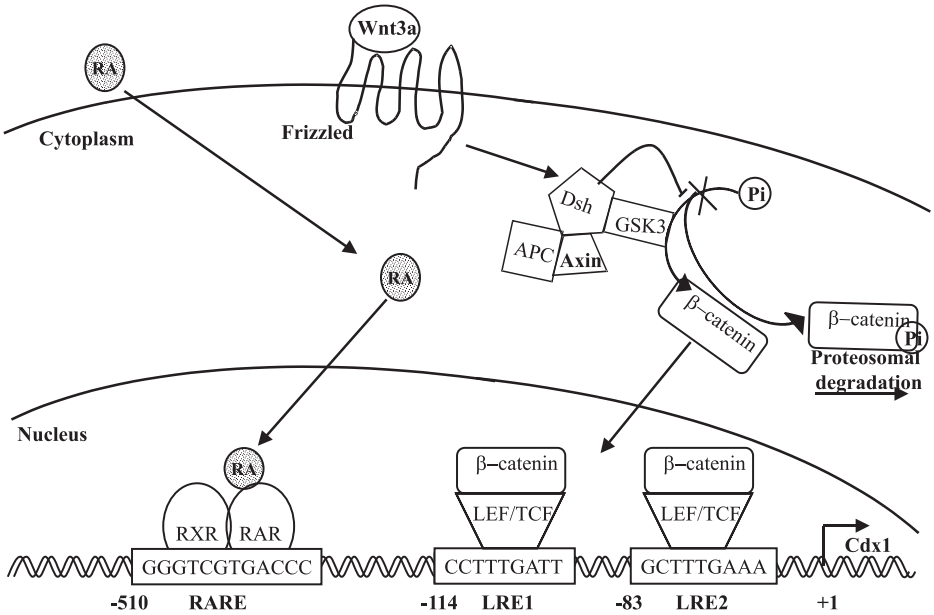

Fig. 5. Schematic representation of RA and Wnt3a on the *Cdx1* promoter. Wnt 3a interacts with surface frizzled receptors, resulting in a cascade of events in the cytosol, leading to stabilization of *β*-catenin. *β*-catenin then translocates to the nucleus, where it presumably interacts with LEF/TCF transcription factors resident on two LREs on the Cdx1 promoter, inducing Cdx1 expression. RA interacts with the RAR moiety of RXR-RAR heterodimers, and regulates *Cdx1* through an RARE situated 5′ to the transcriptional start site, as indicated.

pathways may also converge during differentiation of this tissue. The lack of an intestinal phenotype in *Cdx1* null offspring may be due to functional compensation by Cdx2, as suggested by their overlapping roles in vertebral patterning and posterior specification (van Den et al., 2002).

4.7. Cdx autoregulation

Autoregulation and cross regulation mechanisms contribute to establishment of *Hox* expression domains (reviewed in Deschamps et al., 1999), and *Cdx1* would appear also to be subject to some form of autoregulatory loop. This conclusion stems from the finding that *Cdx1* transcripts are essentially absent in the cognate null background commencing at E8.5. This attenuation is not due to destabilization of the *Cdx1* mutant message by the insertional mutagenesis approach used to disrupt the locus (Subramanian et al., 1998), since both mutant and wild-type transcripts can be detected in equimolar ratios in heterozygous embryos. This autoregulation also appears to be stage-specific, since *Cdx1* expression is unperturbed in the null mutant background at E7.5 (Prinos et al., 2001).

It is interesting to note that most of the *Hox* genes known to be direct RA targets also exhibit autoregulation (Pöpperl and Featherstone, 1992; Wu and Wolgemuth, 1993; Pöpperl et al., 1995; Nonchev et al., 1997; Packer et al., 1998). Moreover, the *Hoxa4* autoregulatory element is required for maintenance of the effects of RA (Packer et al., 1998). These observations are suggestive of a cohort of retinoid target genes which are regulated by a temporally limited RA pulse at E7.5 in the primitive streak region, and their expression propagated to later stages of development by autoregulation in the absence of retinoid signaling.

While the precise mechanism by which Cdx1 maintains its own expression is unknown, it is notable that Cdx2 also exhibits autoregulation, at least in tissue culture models. In this case, two putative motifs governing this effect have been documented; one a TATA box-like element, and the second in the 5′ untranslated region of the gene (Xu et al., 1999). Whether this is a specific modality of regulation in the cell lines used in these studies, or whether such regulation extends to other tissues in vivo, is presently unknown. This autoregulation does, however, provide a logical mechanistic basis for the loss of expression seen in the intestinal lesions in Cdx2 heterozygous offspring (Chawengsaksophak et al., 1997).

Finally, although data regarding regulation of *Cdx4* is limited, expression of a dominant-negative *Xcad3* (the *Xenopus Cdx4* homolog) reduces expression of endogenous *Xcad3* in vivo (Isaacs et al., 1998). Thus, it is possible that all three Cdx members regulate their own expression.

4.8. Regulation of Cdx by FGF

Among other roles, FGF signaling is essential for proper patterning and specification of the posterior embryo in vertebrates, including the mouse (Amaya et al., 1991; Yamaguchi et al., 1994; Ciruna et al., 1997; Dubrulle et al., 2001; Schier, 2001; Vasiliauskas and Stern, 2001). Data from *Xenopus* demonstrates that FGF signaling positively affects the expression of a number of *Xhox* genes (Cho and De Robertis, 1990; Lamb and Harland, 1995), and may do so through *Xcad3* (Pownall et al., 1996; Isaacs et al., 1998). These findings suggest a pathway from FGF to Cdx with subsequent impact on *Hox* gene expression involved in patterning the A–P axis, at least in *Xenopus*.

In the mouse, attenuation of FGFR1 function results in aberrant mesodermal patterning and vertebral homeotic transformations associated with altered *Hox* expression (Yamaguchi et al., 1994). Although these data are consistent with a role for FGF signaling upstream of *Cdx*, as in *Xenopus*, *Cdx* expression has not been reported to be affected in FGFR1 mutants. However, it is conceivable that slight alterations in *Cdx* levels occur which escape detection. Such a possibility is supported by our finding that disruption of the *Cdx1–RARE* has subtle effects on *Cdx1* expression at late streak stages, yet these mutants exhibit highly penetrant vertebral defects (our unpublished observations). Alternatively, FGF signaling may be upstream of *Cdx* expression in other tissues, such as the placenta. Consistent with such a possibility, both FGF and Cdx2 are implicated

in trophoblast function (Chawengsaksophak et al., 1997; Tanaka et al., 1998; Rossant 2001).

5. Conclusions

The vertebrate *Cdx* genes are emerging as key players in a number of ontogenic processes, including A–P vertebral patterning, posterior specification, trophoblast function and intestinal differentiation. Our understanding of the scope of these functions is presently unresolved, and will likely necessitate generation of a comprehensive panel of Cdx null mutants. In a similar vein, although *Hox* genes are emerging as direct Cdx targets as pertains to axial patterning, the molecular basis for Cdx function in the trophoblast, intestine and posterior embryo are presently unknown. A large void also exists as regards to our understanding of the means by which Cdx proteins affect transcription. Hopefully, the ensuing years will lead to resolution of these, and other, facets of Cdx function.

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Dlx genes in craniofacial and limb morphogenesis

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Contents

1. Introduction

Cladistic analyses suggest that vertebrates evolved from a common ancestor of chordates through a stepwise increase in complexity of the body plan. This process involved the appearance of a number of "vertebrate innovations" both at an anatomical and a molecular level (Shimeld and Holland, 2000). Morphologically

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these include neural crest cells (NCCs) and their derivatives, neurogenic placodes, a segmented brain, and a mineralized endoskeleton; molecularly, the expansion of gene families by gene duplication on the vertebrate phylogenetic lineage. *Dlx* genes, a family of homeobox-containing transcription factors related to *Drosophila Distal-less* (*Dll*), might have played a central role in the appearance of vertebrate novelties and gain of complexity of the body plan as their expression and function has been associated with each new vertebrate morphological character (Stock et al., 1996; Neidert et al., 2001). In this chapter we will summarize the major findings on the role of *Dlx* genes in the control of craniofacial, and limb skeletal development.

1.1. Evolution of the Distal-less gene family

The *Dll* gene of *Drosophila* encodes for a homeodomain protein that is the first to be expressed specifically in leg primordia of the thoracic segments and in various appendages of anterior regions of the Drosophila embryo (Cohen et al., 1989; O'Hara et al., 1993; Panganiban and Rubenstein, 2002). During insect limb development *Dll* is expressed in the center of the outgrowing leg primordium and in the distal segments of the leg, throughout the entire larval stage (Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997). *Distal-less* mutant *Drosophila* show various extents of size reduction and dysmorphogenesis of distal segments of the legs in the adult fly, indicating that *Dll* activity is required during early larval stages for the development of the entire limb and for correct proximo-distal (PD) organization (Cohen et al., 1989). The function of *Dll* in determining PD organization is more general as its function is not only exerted in legs, but also in antennae, mouth appendages (Cohen and Jurgens, 1990), and the anal plate (Gorfinkiel et al., 1999). Interestingly, in addition to specifying PD growth-differentiation patterns, *Dll* is also essential to specify antennal identity (Cohen et al., 1989; Dong et al., 2000): hypomorphic mutants that result in leg truncation also lead to antenna-to-leg homeotic transformations. In insects, *Ultrabithorax* and *abdominalA*, two homeotic genes of the *Bithorax* complex, repress *Dll* transcription in the abdominal segments (Vachon et al., 1992), this negative regulation in not present in other arthropods (Panganiban et al., 1995; Grenier et al., 1997). Presumably this mechanism is at the basis of the absence of legs in posterior *Drosophila* abdominal segments (Carroll et al., 1994; Panganiban et al., 1997).

In anterior regions of the *Drosophila* embryo, *Dll* is expressed in the antennal, maxillary and labial primordia. *Dll*-mutant flies show abnormalities of these appendages consistent with a PD growth and morphogenesis defect (Cohen, 1990; Cohen and Jurgens, 1990). It has been shown that *Dll* is activated by the HOM gene *deformed* in the maxillary primordium (O'Hara et al., 1993). In insects (and presumably in vertebrates), therefore, there is a different regulation of *Dll* expression in head regions compared to thoracic segments.

Dll-related genes have been identified and cloned in several species, from Hydra to man (Schummer et al., 1992; Di Gregorio et al., 1995; Holland et al., 1996; Caracciolo et al., 2000; Myojin et al., 2001; Neidert et al., 2001). In mouse and in man, there are six *Dlx* genes arranged as pairs facing each other through the 3′ end

and located near *Hox* clusters (Porteus et al., 1991; Price et al., 1991; Robinson et al., 1991; Robinson and Mahon, 1994; Simeone et al., 1994; Weiss et al., 1994; Scherer et al., 1995; McGuinness et al., 1996; Stock et al., 1996; Liu et al., 1997; Quinn et al., 1997). The spatial expression of these genes in vertebrates is somehow reminiscent of that observed in insects. Namely they are expressed during limb development, in head structures (branchial arch derivatives) and in sensory organs (olfactory epithelium, vestibular organ). In addition, *Dlx* genes in mammals play a role in forebrain development and bone tissue differentiation (Panganiban and Rubenstein, 2002). In this chapter, we will focus on the role of *distal-less*-related genes in limb and craniofacial skeletal development.

1.2. Sequence, structure, and organization of Dlx genes in vertebrates

All vertebrate *Dlx* genes share a highly conserved homeodomain, homologous to that of *Drosophila Distal-less*. The *Dlx* genes in zebrafish, mouse and man are linked in pairs, in a tandem convergent configuration, in the following order: *Dlx*-1 and *Dlx*-2; *Dlx*-5 and *Dlx*-6; *Dlx*-3 and *Dlx*-7 (the latter is also named *Dlx4* (Panganiban and Rubenstein, 2002)). Within each pair, one member shows a higher degree of homology to one gene of another pair, rather than the cognate gene on the same pair. This has led to a subdivision of the *Dlx* gene family in two subfamily, one including *Dlx*-1, -6, -7 (4), the other includes *Dlx*-2, -3, -5. These data can be interpreted as an indication of an initial *Distal-less* duplication event, that occurred in early chordates and yielded an ancestor tandem, followed by a series of subsequent duplications of the entire tandem to yield the mammalian configuration. The analysis of *Distal-less* related genes in different vertebrate species substantially supports this hypothesis (Stock et al., 1996; Ellies et al., 1997).

Further support for this notion comes from the finding that the Drosophila *Distal-less* gene is located near the HOM-C complex. In human, the *DLX-3* and *DLX-7(4)* genes are located on chromosome 17q21 (Scherer et al., 1994; Nakamura et al., 1996; Quinn et al., 1997) near the *HOX-B* homeobox gene cluster. The *DLX1* and *DLX2* genes are linked to the *HOX-D* gene cluster on chromosome 2 (McGuinness et al., 1996), while the *DLX5* and *DLX6* genes are linked to the *HOX-A* cluster on chromosome 7 (Simeone et al., 1994). The same linkage of *Dlx* genes to *Hox* clusters is respected in the mouse genome.

It has been observed that the expression pattern of couples of linked *Dlx* genes is in general very similar and often indistinguishable at the level of spatio-temporal resolution used so far in embryonic analysis (Simeone et al., 1994; Chen et al., 1996; Ellies et al., 1997; Eisenstat et al., 1999; Sumiyama et al., 2002). Although valid in many instances this concept cannot be generalized. For example, within the *Dlx5/6* tandem, only *Dlx5* and not *Dlx6* is expressed in the subventricular zone (SVZ) of the embryonic mouse brain. The similar expression suggests that linked *Dlx* genes may share cis-acting sequences that control their pattern of expression in the embryo and in the adult (Zerucha et al., 2000; Sumiyama et al., 2002). All vertebrate *Dlx* genes share a similar exon–intron organization with three coding exons separated by two introns (Price et al., 1991; McGuinness et al., 1996; Ellies et al., 1997; Pfeffer et al.,

2001). The first exon of human and mouse *DLX6* contains a CAG/CCG (poly-glutamine/poly-proline) repeat region with high homology to the trinucleotide repeat present in the Huntington's disease gene. The length of the CAG-repeat region is polymorphic in the normal human population ($CAG_{12\text{-}20}$). In addition to this repeat a short CGG (poly alanin) repeat and a CAC (poly-histidine) repeat are found (Pfeffer et al., 2001).

2. *Dlx* genes in craniofacial development

2.1. Origin of the skull

The skull is a complex structure. It serves vital functions like eating and defense and it harbors the brain and the otic, optic and nasal capsules, which are indispensable for perception of the environment. The earliest skeletal elements seen during mammalian skull development are cartilage structures, evolved from modification of ancient elements of more primitive vertebrates (for a review see Morriss-Kay, 2001; Wilkie and Morriss-Kay, 2001), collectively known as chondrocranium. Part of the chondrocranium gives rise to the skeleton around the nose, eye, inner ear, and the base of the brain, and is known as neurocranium. The skeletal elements derived from the branchial arches (BA) give rise to most of the facial, mouth and pharingeal skeleton and is known as splanchnocranium. Most of the chondrocranial elements undergo ossification, but some regress (i.e. the Meckel's cartilage of the first arch). A third component of the skull, which appears later and originates by intramembranous ossification, is known as dermatocranium. This type of bone formation is characteristic of the calvaria but is seen also around preexisting chondrocranial elements. Thus, craniofacial development requires the coordinated migration, determination and tissue organization of cells derived from multiple embryonic origins.

While the posterior part of the skull is mostly derived from somitic and cephalic mesoderm, the anterior part, comprising the facial skeleton, is derived from cranial neural crest (CNC) derived mesenchyme (Couly et al., 1993). Indeed, fate-mapping studies in the chicken embryo have shown that the dermato- and splanchnocranium originate from CNC-derived mesenchyme (see for example: Couly et al., 1993; Kontges and Lumsden, 1996; Le Douarin et al., 1997). The complex genetic control of CNC migration and differentiation is gradually being elucidated through the analysis of mutant mice. Members of the *Dlx* gene family are expressed early in CNC cells and later in craniofacial mesenchyme (Dolle et al., 1992; Bulfone et al., 1993; Akimenko et al., 1994; Robinson and Mahon, 1994; Simeone et al., 1994; Ellies et al., 1997; Qiu et al., 1997; Yang et al., 1998; Acampora et al., 1999; Depew et al., 1999; Neidert et al., 2001; Beverdam et al., 2002; Depew et al., 2002). Interestingly expression of "*AmphiDll*" (Dll) in the primitive chordate Amphioxus has led to speculations on its possible role in the evolutionary origin of migratory NCCs (Holland et al., 1996).

Facial development starts with the emergence of the facial primordia, the frontonasal process and the branchial arches, which are arranged around the primitive mouth, the stomodeum. These are a series of bulges containing mostly CNC derived mesenchymal cells covered by a layer of ectoderm (externally) and endoderm (internally). The facial primordia undergo complex morphogenetic interactions involving growth and fusions and eventually give rise to the facial skeleton (Thorogood, 1988). These developmental processes are governed by the many gene families, among which the *Dlx* genes, which are expressed in the facial ectoderm, mesoderm, and endoderm (for review see Francis-West et al., 1998).

2.2. Distribution and functions of Dlx genes in the craniofacial primordia

During craniofacial development, all six murine *Dlx* genes are expressed in the craniofacial primordia. In general, it appears that the expression patterns of physically linked *Dlx* gene pairs overlap extensively (Qiu et al., 1997; Panganiban and Rubenstein, 2002). At 9.5 days of the mouse development *Dlx1* and *Dlx2* genes are expressed in the first BA in the mesenchyme of both the maxillary (proximal) and the mandibular (distal) component, whereas the *Dlx3*, *Dlx5*, and *Dlx6* are expressed only in the mandibular portion of the first BA (Simeone et al., 1994; Merlo et al., 2000; Panganiban and Rubenstein, 2002).

2.2.1. Dlx1 and Dlx2

Dlx1 and *Dlx2* are expressed in migratory NCCs. Within the craniofacial primordia they are expressed from E9.5 onwards in the mesenchyme and epithelium of first and second BA (Bulfone et al., 1993). Later, the genes are also expressed by the nasal process epithelium and by posterior BAs (Dolle et al., 1992; Bulfone et al., 1993; Qiu et al., 1997; Thomas et al., 2000). *Dlx1* and *Dlx2* single and double mutants have craniofacial abnormalities. In general, it appears that *Dlx1* and *Dlx2* are both required for development of the splanchnocranial skeleton, whereas *Dlx2* alone is essential for proper morphogenesis of dermal bones in the lateral skull wall (Qiu et al., 1995; 1997). In *Dlx1* mutants the alisphenoid, the palatine, and pterygoid bones are affected (Qiu et al., 1997). In *Dlx2* mutants many structures derived from proximal regions of BA1 and –2 are malformed (Qiu et al., 1995). Notably, no distal elements (first arch Meckel's cartilage, malleus, dental, tympanic and gonial; second arch: upper hyoid horns) are affected by this mutation (Qiu et al., 1995). The lack of distal defects has been interpreted as the indication of a functional redundancy of the various *Dlx* genes in the mandibular portion of the first BA, a location where all of them are co-expressed. *Dlx1/2* double mutants exhibit a collection of defects that can be attributed to individual single mutants, and novel defects in dental formation (Qiu et al., 1997). Prior to the initiation of tooth development, *Dlx1* and *Dlx2* are expressed in the odontogenic mesenchyme of the maxillary and mandibular divisions of the first arch (Bulfone et al., 1993; Thomas et al., 1995; Thomas and Sharpe, 1998; Thomas et al., 1998a). Loss of *Dlx1* and *Dlx2* function results in a failure of upper molar development at the epithelial thickening stage. This goes together with loss of *BarX1* expression in the maxillary molar

odontogenic mesenchyme, which implicates *BarX1* in a pathway downstream of *Dlx1* and *–2* responsible for upper molar development (Thomas et al., 1997). *Fgf8* on the other hand controls *Dlx2* expression in the BA1 mesenchyme, whereas it prevents *Dlx2* expression in the epithelium (Thomas et al., 2000). In contrast, the lower molars and the incisors are not affected in the *Dlx1/2* double mutant. Most likely other *Dlx* genes compensate for the loss of *Dlx1* and *–2* in these regions (Thomas et al., 1997).

2.2.2. Dlx5 and Dlx6

Dlx5 and *Dlx6* are expressed with an onset of E8.5 in the distal portion of BA1. At E9.5 all BAs express both genes. Interestingly, the maxillary portion of BA1 does not express *Dlx5* and *–6* up to E10.5, but then expression gets increasingly stronger (Acampora et al., 1999; Depew et al., 1999; Merlo et al., 2000; Charite et al., 2001). *Dlx5* single mutants have abnormalities of the first, second, third and fourth arch derived skeleton (Acampora et al., 1999; Depew et al., 1999). The lower jaw was proximally reduced, the upper jaw displayed abnormalities causing a cleft palatine, the nasal capsule was affected to varying degree and abnormalities were observed in the skull base region and the skull vault. In addition, defects were observed in the otic capsule (Acampora et al., 1999; Depew et al., 1999; Kraus and Lufkin, 1999; Merlo et al., 2002b). The presence of defects in derivatives of the mandibular arch of $Dlx5^{-/-}$ animals, where also *Dlx1*, *2*, *3,* and *6* are expressed at 10.5 dpc suggests that redundancy between *Dlx* genes is not generalized, but occurs only in specific cases. The results of *Dlx1* and *Dlx2* knock-outs have led to the proposition that a PD pattern of nested *Dlx* gene expression might be the basis of PD specification of splanchnocranial skeletal elements (Qiu et al., 1997).

The critical role of *Dlx* genes in dictating the fate of jaws and craniofacial development can, however, be only appreciated analyzing the striking phenotype of *Dlx5/6* double mutant mice which has been recently reported by two separate groups (Beverdam et al., 2002; Depew et al., 2002) with very similar results. In both cases deletion of the coding and intergenic regions of *Dlx5* and *Dlx6* was obtained with a single targeting event in the mouse ES cells. Homozygous mutant mice die shortly after birth. They have severe hindlimb malformations (see later) and exencephaly together with a unique craniofacial lesion. Both upper and lower jaws are severely affected and seem mirror images of each other causing their snouts to be symmetric both along the right–left and antero-posterior planes (summarized in Fig. 1). Strikingly, whiskers pads with vibrissal follicles are visible both on the upper and the lower jaws. Moreover, structures resembling palatine rugae, a series of ridges associated with the inner surface of the palatal shelves, are present on the inner surface of both upper and lower jaws. In *Dlx5/6* mutants, the mandible became unrecognizable and was transformed in a structure indistinguishable from the deformed maxillary bone complex. Moreover, the transformed lower jaw seemed to articulate with structures that may be interpreted as distorted and duplicated pterygoid processes, rather than with the squamosal bone in normal skeletons. Besides the abnormalities in the first arch derived skeleton, also

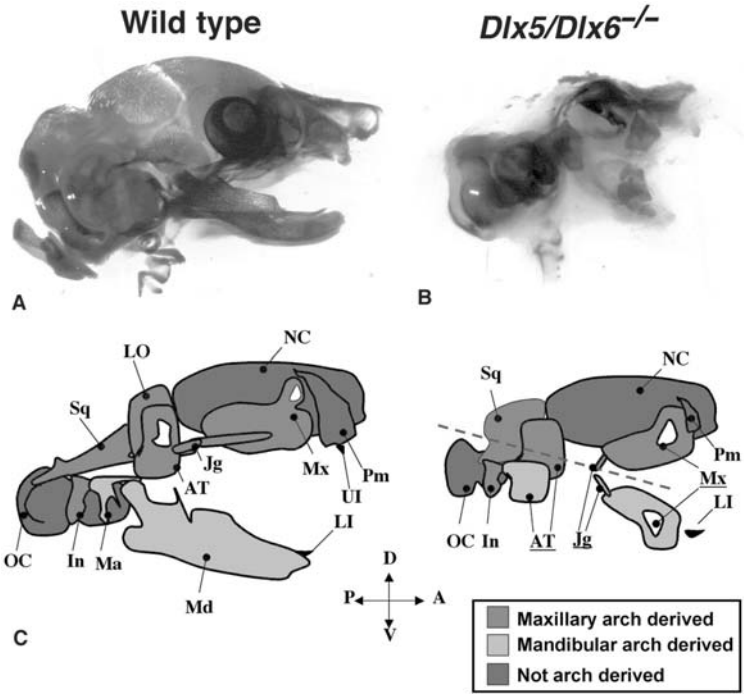

Fig. 1. Homeotic transformation of the mandibular arch into a maxillary arch in *Dlx5/Dlx6* double mutant mice. (A, B) Skeletal staining of newborn heads of normal (A) and *Dlx5/Dlx6* null (B) pups. Ossified structures are colored in red, cartilages in blue. (C) Schematic comparison of lateral view of normal and *Dlx5/Dlx6* null cranial skeleton. The main duplicated structures are indicated by underlining the symbol of the respective bone. P, posterior; A, anterior; D, dorsal; V, ventral. AT, Ala Temporalis; In, Incus; Jg, Jugal; LO, Lamina Obturans; LI, lower Incisor; Ma, Malleus; Mx, Maxillary; OC, Otic Capsule; Pm, Premaxillary; NC, Nasal Capsule; Sq, Squamosal; UI, upper Incisor. (See Color Insert.)

second and third arch derived structures are affected in *Dlx5/6* homozygous double mutants.

The striking morphological similarity of the upper and the lower jaw of *Dlx5/6* double mutants suggests a transformation of the mandibular process into a maxillary process early during craniofacial development. This was demonstrated, at a molecular level, through the analysis of the expression of a number of genes involved in the differentiation of mandibular and maxillary arch. In general, genes involved in the differentiation of the mandibular arch (*Alx4*, *dHAND*, *Dlx3*, *Bmp7*, and *Pitx1*) are absent or strongly reduced in Dlx5/6 mutants, while the expression of genes usually expressed in the maxillary arch (*Dlx1*, *Dlx2*, *Msx1*, *Msx2*, and *Prx1*) maintain their expression (Beverdam et al., 2002; Depew et al., 2002). For example, in E10.5 embryos, *Pitx1* is normally expressed in the mesenchyme of the mandibular process and in the ectoderm of the stomodeum (Lanctot et al., 1997). In E10.5 and E11.0 double mutant embryos *Pitx1* expression was present in the ectoderm of the mandibular and the maxillary process, but was completely absent from

the mandibular mesenchyme. Moreover, *dHAND*, which in E10.5 pharyngeal regions is usually expressed in the mandibular process and is activated by *Dlx6* (Charite et al., 2001), was silenced in the BA of the double mutant, but was still expressed in its distal-most part. These molecular data corroborate the hypothesis that in these mice the mandibular process has acquired a maxillary identity. Both morphological observations and molecular data support the hypothesis that combined inactivation of *Dlx5* and *Dlx6* results in a transformation of the lower jaw into an upper jaw. Using the classical definition of homeosis given by Bateson as an event in which "something has been changed into the likeness of something else" (Bateson, 1894), this can be called a homeotic-like transformation of the mandibular into a maxillary portion of BA1. Homeosis of jaw elements has previously been shown only after inactivation and forced expression of *Hox* genes in post-migratory NCC (Rijli et al., 1998; Pasqualetti et al., 2000). *Dlx5* and *Dlx6* can act, therefore, as homeotic genes essential for antero-posterior patterning of BA1 in modern mammals.

Both the upper and the lower jaws derive from BA1, which is colonized by NCCs arising from the mesencephalic neural fold and the segmented anterior hindbrain (Couly et al., 1993; Kontges and Lumsden, 1996). It has been elegantly shown that, in the chick, most NCCs which colonize BA1 do not express *Hox* genes and get patterning clues from the endoderm. The endoderm instructs NCCs as to the size, shape, and position of all the facial skeletal elements, whether they are cartilage or membrane bones (Couly et al., 2002). However, Hox-expressing NCCs of more posterior regions do not respond to these cues. Actually, more recent results from the same group support the notion that the absence of Hox gene expression in the anterior part of the chordate embryo was crucial in the vertebrate phylum for the development of a face, jaws and brain case, and, hence, also for that of the forebrain. If the expression of *Hoxa2*, *Hoxa3*, and *Hoxb4* is selectively targeted to the Hox-negative neural folds of the avian embryo prior to the onset of NCC emigration, the development of the entire facial skeleton is either completely precluded (in the case of *Hoxa2*) or shows severe defects (*Hoxa3*, *Hoxb4*) (Creuzet et al., 2002). Although several other classical grafting experiments would suggest that NCCs are pre-programmed before migration, it appears that in general a more complex integration of cell and tissue interactions is needed for the morphogenesis of cranial structures (reviewed in: Trainor and Krumlauf, 2001). As *Dlx5* and *Dlx6* do not seem to be expressed in the endoderm, but only in pre- and post-migratory NCC it would seem plausible that these genes are conferring to NCC the competence to interpret correctly signals coming either from the endoderm or from other signaling structures (Fig. 2).

Apart from sharks, where the upper and lower teeth are relatively similar in shape and number, the upper and lower jaws of modern jawed vertebrates (gnathostomes) generally differ in the shape and number of their teeth or tooth-bearing dermal bones. In bony fishes (osteichthyans), and land vertebrates (tetrapods), this difference disappears as one considers early, Paleozoic groups, whose upper jaw bones are almost a mirror image of those of the lower jaw. This curious symmetry has been pointed out long ago by the American paleontologist A.S. Romer in early

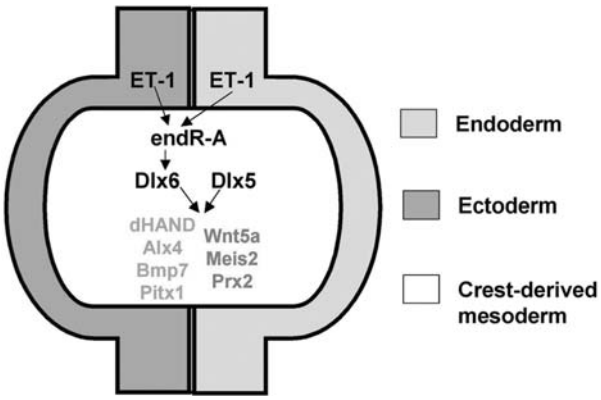

Fig. 2. Possible endothelin-1-dependent regulatory cascade taking place in the mandibular arch. Endothelin-1 (ET-1) is secreted both by the endoderm and by the ectoderm and binds to the endothelin receptor A (endR-A) in the crest-derived mesoderm of the arch. This leads to the activation of *Dlx6* which in turn activates (green) or suppresses (red) a number of genes in the mandibular arch. In the case of *dHAND* the activation of *Dlx6* is direct, for all the other it might be either direct or indirect. *Dlx5* is not induced by ET-1, but it might still play a role in the regulation of the downstream genes. (See Color Insert.)

amphibians, but has never received any explanation other than merely functional (Romer, 1940). The generalized osteichthyan condition, in this respect, can be observed in a Devonian tristichopterid fish (*Eusthenopteron*), a close piscine relative to the tetrapods. In living osteichthyans, this bone pattern is profoundly modified in most ray-finned fishes (actinopterygians) and, among tetrapods, in mammals, in which the lower jaw is represented by the dentary alone. But important modifications of the jaw bones, such as the loss of the maxillary or coronoids also occur in the living piscine sarcopterygians, i.e. the coelacanth and the lungfishes. *Dlx5/6* gene inactivation in the mouse leads to a homeotic-like transformation of the lower jaw into an upper jaw and generates a symmetric mouth. The transformed structure is, in a sense, reminiscent of the jaw pattern of early osteichthyans, including early tetrapods. More generally, one could suggest that *Dlx* genes were needed to allow asymmetry when required by the rise of complex anatomical structures during evolution reinforcing the concept that *Dlx* genes are associated with the appearance of morphological novelties in vertebrates (Neidert et al., 2001).

2.2.3. Dlx3 and Dlx7(4)

Dlx3 expression is first detected in the distal tips of E9.5 BA1 and −2. Later its expression becomes restricted to the caudal portion of BA1 and –2. In addition, *Dlx3* expression was detected in the epithelium of the nares (Robinson and Mahon, 1994; Sumiyama et al., 2002). The role of *Dlx3* during craniofacial patterning remains unclear as *Dlx3* mouse mutants die between E9.5 and E10.0 due to placental defects (Morasso et al., 1999). The expression domain of *Dlx7(4)* overlaps that of *Dlx3* in the BAs, but no function for *Dlx7(4)* during craniofacial development has yet been described (Zhao et al., 2000).

3. Dlx genes and the control of vertebrate limb development

The expression of the *Dll* or *Dlx* homeoproteins is a common feature of appendage outgrowth from arthropods to man. Their expression has been analyzed in protostomes and deuterostomes (Panganiban et al., 1997) leading to the finding that *Dll* is expressed along the PD axis of developing polychaete annelid parapodia, onychophoran lobopodia, ascidian ampullae, and even echinoderm tube feet.

Morphologically, vertebrate limb buds are simple structures consisting of mesoderm covered by specialized ectoderm extending from the lateral surface of the embryonic body wall. Outgrowth is controlled by two signaling centers. The first is the apical ectodermal ridge (AER), a specialized thickened region of the ectoderm along the rim of the limb bud, marking the border of dorsal–ventral symmetry axis. The AER is responsible for proximo-distal outgrowth of the limb by keeping the underlying mesodermal cells (the progress zone) in an undifferentiated, proliferating state. It does so by producing morphogens such as *Fgf-2*, *-4* and *-8,* which embody the proliferating signal. The second signaling center is the zone of polarizing activity (ZPA), a region of mesenchyme in the posterior margin of the limb bud. This region is responsible for antero-posterior patterning of the limb by producing Shh. Expression of *Shh* by the ZPA and of Fgfs by the AER appears to be interdependent. Loss of either of them causes limb development arrest. Later during development, apoptosis plays a critical role in determining the shape of limbs. Programmed cell death takes place in four regions: the anterior and posterior necrotic zones, the opaque patch, and the interdigital necrotic zones. Bmp's play a key role in triggering apoptosis in the limb mesoderm (Macias et al., 1997). In vertebrate embryos all *Dlx* genes are co-expressed in the AER and in the underlying mesenchyme of the progress zone of the developing limb buds (Dolle et al., 1992; Bulfone et al., 1993a, b; Simeone et al., 1994; Zhao et al., 1994; Ferrari et al., 1995; Zhang et al., 1997; Acampora et al., 1999; Ferrari et al., 1999).

In spite of their expression during early and late phases of limb outgrowth restricted to distal limb territories, suggestive of a role in PD development, targeted inactivation of *Dlx1*, *Dlx2*, *Dlx1* + *Dlx2* and *Dlx5* failed to produce any appreciable limb phenotype (Qiu et al., 1995, 1997; Acampora et al., 1999; Depew et al., 1999). Recently, two groups have reported that the combined disruption of *Dlx5* and *Dlx6* in the mouse causes severe distal limb defects (Merlo et al., 2002a; Robledo et al., 2002). Furthermore it has been suggested, but not yet shown in detail, that other *Dlx* compound mutants such as *Dlx2*/*Dlx5* have malformations of the distal limb (Panganiban and Rubenstein, 2002). These novel observations, which support a conserved function of these genes through evolution, deserve to be better detailed and commented.

Beginning at around E11.5–E12, the hindlimb of mice null for *Dlx5* and *Dlx6* show defect in the morphogenesis of the central portion of the limb bud. Mutant hindlimbs have a variable phenotype characterized by a more or less severe reduction of the central wedge of the palette. The hindlimb skeleton of double mutant mice at E14.5 and at E18 reveals a distal defect of the third and fourth metatarsal and

phalanxes, resulting in shorter or missing digits, often accompanied by deformation and syndactyly of the remaining digits, and a profound medial cleft. A similar malformation is rarely observed in the forelimbs of the mutant animals (Robledo et al., 2002), reinforcing the notion that the developmental programs of fore- and hindlimbs are not identical. Little or no malformation of tarsal bones or other proximal elements has been observed.

The mechanism by which loss of *Dlx5–Dlx6* expression in the developing limb bud leads to malformation of the central rays of the mouse limb is not yet completely elucidated. As mentioned above, a limb defect is first visible at around E11.5–E12 of development. This has prompted the search for downstream *Dlx*-dependent genes implicated in the genesis of the ectrodactyly at these early stages or before. At later stages the expression of many genes in the central digit region is likely to be affected as an indirect consequence of the primary lesion.

The AER is essential for limb outgrowth and patterning. Its activity consists in the expression of key morphogenetic molecules that induce and control proliferation in the progress zone and maintain the zone of polarizing activity (reviewed in Capdevila and Izpisua Belmonte, 2001). A number of such molecules have been examined in the *Dlx5–Dlx6* mutant mice. The AER is normally formed and functioning up to E10.5, an indication that AER induction takes place normally. However, starting at E11.5 onward, the expression of *Fgf8*, *Msx2*, and of the knock-in reporter *LacZ* is strongly reduced (Robledo et al., 2002). No alteration in *Shh* expression is seen, an indication of integrity of the ZPA. Interestingly, the lack of expression of *Fgf8* and *Msx2* is restricted to the region of the limb bud affected in the ectrodactyly phenotype, namely the central wedge of the AER. Thus, the loss of *Dlx5* and *Dlx6* leads to the inability of the central portion of the AER (and underlying mesenchyme, Merlo et al., unpublished data) to maintain its integrity and function. The discrepancy between the wider expression territory of *Dlx5* and *6* (the AER in its entire antero-posterior length, plus a region on the antero-proximal margin) compared to the morphogenetic defect which is restricted to the middle region of the palette has not yet been explained (see Fig. 3). However, it appears that: (a) in *Dlx5–Dlx6* null mice the central portion of the AER is defective and is unable to carry its morphogenetic function via expression of signaling molecules, and (b) an intrinsic regional difference in the function and tissue interaction of AER cells must exist, independently of *Dlx* gene expression. Understanding of this later finding might lead to new insight on the molecular mechanism of limb development.

The limb defect observed in double mutants is strongly reminiscent of a human congenital dysmorphogenesis of the hands and feet known as ectrodactyly or Split hand/foot malformation (SHFM). SHFM is characterized by a profound median cleft of the hands and/or feet, typically associated with absence or fusion of the remaining fingers. Linkage studies and analyses of chromosomal abnormalities have permitted to associate SHFM to loci on 7q21.3 (SHFM1, Scherer et al., 1994; Crackower et al., 1996), 10q24-q25 (SHFM3, Nunes et al., 1995; Raas-Rothschild et al., 1996), Xq26 (SHFM2), 3q27 (SHFM4, Celli et al., 1999), and 2q31 (SHFM5) (OMIM183600).

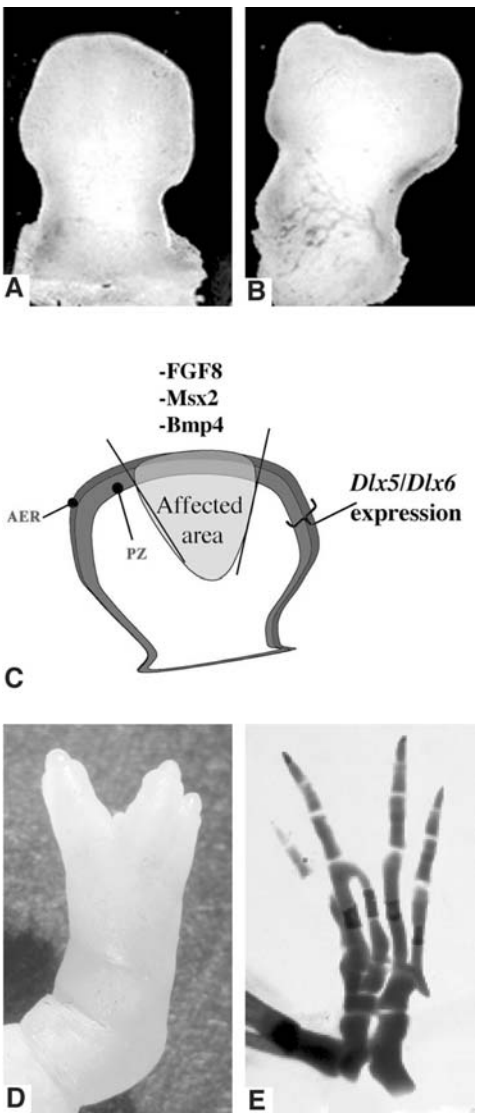

Fig. 3. Phenotype of *Dlx5*/*Dlx6* inactivation in the limb bud. (A, B) Limb buds from a normal (A) and a *Dlx5*/*Dlx6* null mouse (B) at 11.5 dpc, the defect in the middle ray of the limb is already visible. (C) Schematic drawing of a limb bud and the defects observed in *Dlx5*/*Dlx6* double mutant mice. The developmental defect leading to this peculiar dysmorphology is restricted to cells and tissue of a central area of the limb bud (between the two black lines). Both the apical ectodermal ridge (AER) and the underlying mesenchymal cells (progress zone, PZ) in this wedge fail to express important morphogenetic markers, such as *FGF8*, *Msx2* and *Bmp4*. Note that expression of *Dlx5* and *Dlx6* genes extends to the entire length of the AER (dark blue), where defects are not observed. The basis for the restriction to the middle ray of the limb remain, for the moment, unknown. (D, E) Hindlimb of newborn *Dlx5*/*Dlx6* double mutant mice seen *in toto* (D) or as skeletal preparation (E) ossification centers are red, cartilages are blue. (See Color Insert.)

Physical mapping of the SHFM1 locus on chromosome 7 led to define a critical interval of 1.5 Mb which includes *DLX5* and *DLX6*, and *DSS1* (Scherer et al., 1994; Crackower et al., 1996). Although *dss1* is expressed in the AER of the limb bud and has been proposed as candidate gene for SHFM1 (Crackower et al., 1996), no evidence exists for its role in limb development or in SHFM1 etiology. In addition, *dss1* expression was unchanged in the Dlx5/6 null limb buds (Merlo et al., 2002a). Thus, *DLX5* and *DLX6* appear to be the best candidate disease genes for SHFM1. Furthermore, although the peculiar craniofacial lesion observed in mutant mice is never observed in patients of SHFM1, an etiological association has been established between SHFM1 and syndromic ectrodactylies in which cleft lip and/or palate, hearing loss, and genito-urinary anomalies are present. It has been suggested that at least a subset of this complex family of human birth defects results from disruption of a single gene or group of tightly clustered developmental genes at 7q21.3-q22.1 (Scherer et al., 1994).

In most cases, hereditary SHFM1 appears to be transmitted as an autosomal dominant trait in man. The human disease is further complicated: approximately 30% SHFM1 obligate carriers show no phenotypic abnormalities (Scherer et al., 1994) and it has been suggested that other alleles and/or genetic mechanisms might be involved in the origin of the human lesion (Palmer et al., 1994). Variable expressivity, segregation distortion, locus heterogeneity and syndromic association with other anomalies have so far prevented the definition of the genetic lesion at the molecular level (Palmer et al., 1994; Zlotogora, 1994; Sifakis et al., 2001). None of the candidate genes located on 7q21.3 is interrupted directly by any of the deletions, inversions or translocations on 7q21 associated with the disease (Crackower et al., 1996). Furthermore, no mutations have as yet been found in the coding region of these genes in cases of SHFM with involvement of 7q21 (Tackels-Horne et al., 2001). In man, the proposed disease mechanism remains the haploinsufficiency of gene(s) present in 7q21, resulting either from gene deletion or from disruption of *cis*-acting regulatory elements located in the proximity (see for example Zerucha et al., 2000).

A very interesting recent observation is that an imprinted domain has been newly defined in the region of human chromosome 7q21-q31 (which contains *DLX5* and *DLX6*) using human–mouse monochromosomal hybrids (Okita et al., 2003).

Some forms of SHFM can increase in severity in successive generations. We have recently found (Pfeffer et al., 2001) that the first exon of human and mouse *DLX6* genes contain a CAG/CCG (poly-glutamine/poly-proline) repeat region with high homology to the trinucleotide repeat present in the Huntington's disease gene. This CAG repeat is polymorphic in the normal human population suggesting that *DLX6* could have a role in the control of limb patterning. Mutation analysis of *Dlx6* will possibly contribute to answer this question.

The *Dlx5*/*Dlx6* null mouse phenotype differs from the human SHFM1 in two respects. First, although reports on SHFM linked to 7q abnormalities suggest a preferential involvement of the feet, hand defects are frequent as well. In the mouse forelimb defects are rare (Robledo et al., 2002). Second, the limb phenotype in the mouse is transmitted in autosomal recessive fashion, while SHFM1 is segregated

as an autosomal dominant trait. This apparent discrepancy could be explained with the potential complexity of the control of expression of these genes. Indeed splice variants mRNAs and antisense transcripts of *Dlx5* and *Dlx6* have been reported, suggesting multiple transcriptional and post-transcriptional regulations (Liu et al., 1997). Furthermore, it is conceivable that a dominantly inherited cytogenetic abnormality on 7q21 may unmask sub-active allelic variants present in the sister chromatid. This latter possibility will have to be investigated on DNAs from sporadic and familial SHFM with involvement of chromosome 7q21. Although there is no obvious evolutionary relation between insect appendages and mammalian legs, the similarity in terms of territory of expression is striking. *Dll*/*Dlx* expression in such diverse appendages could be convergent, but this would have required the independent co-option of *Dll*/*Dlx* several times in evolution. Alternatively, appendicular *Dll*/*Dlx* expression might have been originated in a common ancestor and used subsequently to pattern body wall outgrowths in a variety of organisms, including vertebrates. In this regard, it is interesting to note that other non-limb appendages express *Dlx* genes. For example, the *Dlx5* and *Dlx6* are strongly expressed in the external ear lobes (Merlo and Levi, unpublished observations) and in the distal part of the genital bud of the mouse embryo, with a gradient PD pattern reminiscent of that of *Hox* genes (Merlo et al., 2000). This later observation is interesting if we consider that *Dll* is expressed in the Drosophila genital disk, and that the overall pattern of expression of *Dll* and of the morphogens *wingless* and *decapentaplegic* is to a great extent similar to the leg imaginal disk (Gorfinkiel et al., 1997, 1999). No overt phenotypic defects in the genitalia of *Dlx* mutant mice has been reported as yet.

4. Upstream and downstream regulation of *Dlx* genes

4.1. Dlx6 is an endothelin-1-dependent activator of dHAND

Deciphering the upstream and downstream regulations of *Dlx* gene expression and function is essential for understanding of their role in craniofacial development. Although little is known on the subject, one possible regulatory pathway that include *Dlx5* and *Dlx6* gene has been recently uncovered.

Endothelin-1 (ET-1) signaling has been shown to be essential for expression of the basic helix-loop-helix (bHLH) transcription factors *dHAND* and *eHAND*, which are expressed in partially overlapping territories in the distal portion of the developing BAs (Cserjesi et al., 1995; Thomas et al., 1998b; Clouthier et al., 1998, 2000). In the promoter region of *dHAND* a heart-specific and a BA-specific enhancer, located respectively 4 Kb and 7 Kb upstream of the transcription start, are present (Charite et al., 2001). The BA enhancer is exquisitely dependent on signaling from the ET-1/endothelin Receptor-A (endR-A) system for its activation, and such sensitivity is maintained in transgenic animals carrying the enhancer/promoter fragment linked to the β-gal reporter.

This BA enhancer contains a series of ATTA/TAAT sequences, regarded as general consensus elements for binding of homeobox-containing proteins. The search for homeodomain proteins binding to this site has revealed the presence of Dlx6 in complex with other proteins at this location (Charite et al., 2001). Using the same analysis Dlx2, Dlx3, and Dlx5 proteins were excluded from the complex. Thus, Dlx6 appears to be directly and specifically involved in the *cis*-regulation of *dHAND* expression exerted by ET-1 signaling.

A number of related observations further indicate that the ET-1/Dlx6/dHAND cascade is indeed an important regulatory pathway essential for BA development. The expression of both *Dlx5* and *Dlx6* mRNA was greatly diminished in the mandibular arch of *endR-A* null mice (Charite et al., 2001) suggesting that expression of these two linked genes is under ET-1 regulation. Furthermore, *dHAND* expression is strongly reduced in the mandibular arch of double *Dlx5–Dlx6* null mice but not in single *Dlx5* null mice (Beverdam et al., 2002; Depew et al., 2002). The lesion due to the disruption of *Dlx6* alone has not been reported yet. It seems that both *Dlx5* and *Dlx6* are required to maintain *dHAND* expression in the first BA. However, only Dlx6 protein, but not Dlx5, is found in the regulatory complex. It is possible that Dlx5 may exert its transcriptional control over *dHAND* expression by binding to a different (non ET-1 dependent) region of the *dHAND* promoter–enhancer sequences.

Development of BAs and vasculature derivatives of the BA neural crest critically depends on ET-1 signaling, as indicated *in vivo* by targeted disruption of components of the endothelin signaling cascade (Kurihara et al., 1994, 1995a, b; Clouthier et al., 1998; Yanagisawa et al., 1998a, b; Miller et al., 2000). NCCs migrating in the BAs express endR-A (Clouthier et al., 1998) while ET-1 is expressed in a complementary fashion in the mesodermal core and the surface ectoderm of the arches, in the pharingeal endoderm and in the endothelium of the BA arteries (Maemura et al., 1996; Clouthier et al., 1998).

Putting all these information together it seems reasonable to propose the following regulatory pathway (see Fig. 2): signaling from the endR-A is required for expression of *Dlx6* (and possibly *Dlx5*) in the mandibular portion of the first BA. The Dlx6 homeoprotein binds to the ET-1-dependent BA enhancer of *dHAND*, while *Dlx5* is required for binding to other regulatory sequences. dHAND is required for ventral/mandibular identity of the first BA (see below). Targeted disruption of both *Dlx5* and *Dlx6*, but not the single disruption of *Dlx5*, results in near complete loss of *dHAND* expression in the mandibular portion of the first BA (Beverdam et al., 2002; Depew et al., 2002). Expression of *dHAND* in mice with disruption of *Dlx6* has yet to be reported. If this cascade will prove to be correct, at least in those territories where expression of these genes overlaps, one should expect that the knock-out of *dHAND* results in a phenotype that is a subset of that seen in the *Dlx5-Dlx6* mutant mice. Regrettably, these mice are embryonic lethal (Yamagishi et al., 2000) and only a conditional BA disruption of *dHAND* will answer this interesting question.

A number of observations would indicate that ET-1 might be an inducer of ventral (mandibular) cell fate for NCCs migrating in the first BA. The analysis of cranial

cartilage morphogenesis of the zebrafish mutant *sucker* has led Kimmel and co-workers (Miller et al., 2000; Kimmel et al., 2001) to suggest that one effect of ET-1 signaling could be the specification of ventral identity of skeletogenic NCCs, these cells would become dorsal by default in the absence of proper ET signaling. Another example of the capacity of ventral BA environment to determine the fate of NCCs is seen in the chick embryo where dorsal NCCs give rise to ventral cartilages when allowed to migrate in the ventral BA (Baker et al., 1997). ET-1 could be at least partly responsible for this tissue-mediated environmental control of cell fate. The experiments reported in Charite et al. (2001), suggest that *dHAND* could be a key downstream selector gene for ventral cell fate. The phenotype of *Dlx5/6* null mice is well consistent with the proposed role of Dlx6 in the ET-1-dependent *dHAND* expression in the BA.

4.2. Hierarchical transcription regulation of Dlx1/2 on Dlx5/6

As mentioned above, *Dlx* genes in vertebrates are organized as pairs of duplicated genes *[Dlx1–Dlx2*; *Dlx5–Dlx6*; *Dlx3–Dlx7(4)]*. The relatively short distance (few kilobases) between the two members of each pair and their similar expression pattern has suggested that the two genes of a tandem might share common regulatory sequences, possibly located in the intergenic region (see for example Sumiyama et al., 2002). Short stretches of conserved sequences have been identified in the intergenic region of human, mouse and zebrafish *Dlx5–Dlx6* (in zebrafish they are named *dlx4-dlx6*) tandem locus. These DNA segments as well as the *col1A1* promoter, another *Dlx* regulated gene, contain the sequence TAATTA/ATTAAT, which is also a general consensus binding site for homeodomain proteins (Zerucha et al., 2000; Panganiban and Rubenstein, 2002). When placed in front of a minimal (inactive) promoter driving the β-gal reporter, the zebrafish-derived conserved fragment was sufficient to recapitulate most of *Dlx5–Dlx6* forebrain expression in a transgenic mouse model. Instead, when the mouse conserved sequence was examined by the same transgenic assay, β-gal expression was observed not only in the expected forebrain territories but also, at low frequency, in the BAs, in the olfactory placodes and in the AER of the limb buds (Zerucha et al., 2000), all known *Dlx5–Dlx6* expression territories. The same enhancer fragment is a site of *Dlx1–Dlx2*-dependent transcriptional regulation of the *Dlx5–Dlx6* locus.

Expression of *Dlx5* and *Dlx6* is strongly reduced in the subventricular zone (SVZ) and lateral ganglionic eminence (LGE) of the basal ganglia of double *Dlx1–Dlx2* knock-out mice (Anderson et al., 1997), however the cells which should express the *Dlx* genes in these territories are still present. Similarly, the activity of zebrafish intergenic enhancer on the β-gal reporter is greatly reduced in the *Dlx1/Dlx2* null mice. Therefore, *Dlx1* and *Dlx2* seem to be intimately involved in the regulation of *Dlx5* and *Dlx6* expression in the forebrain. The regulation appears to be direct, since the enhancer fragment contains potential homeodomain binding sites and *Dlx* genes can function as transcriptional activators (Liu et al., 1997; Zhang et al.,

1997). An indirect type of regulation could also be envisioned, in this case regulation should be mediated by recruitment of other transcription factors, or both mechanisms could operate in this regulation. *In vitro* co-transfection experiments indicate that Dlx1 and Dlx2 proteins can activate transcription of the zebrafish enhancer, and that mutation of the putative binding sites abolishes this response. Together these data are strongly in favor of a direct regulation of *Dlx1* and *Dlx2* on the zebrafish (and likely the mouse) enhancer in the intragenic region of *Dlx5* and *Dlx6*. *Dlx1*, *Dlx2*, *Dlx5*, and *Dlx6* are all expressed in an overlapping pattern in subcortical telencephalon, and in particular in the developing basal ganglia region. Single disruption of *Dlx2* and *Dlx5* show subtle defects in forebrain development (Qiu et al., 1995; Anderson et al., 1997; Eisenstat et al., 1999). In double *Dlx1*/*Dlx2* mutant mice, a severe block in late neurogenesis results in reduced production and differentiation of basal ganglia GABAergic neurons as well as several other GABAergic cells (Anderson et al., 1997; Marin et al., 2000). Late-born subcortical neurons derive from proliferative precursor cells located at the SVZ, where *Dlx5* but not *Dlx6* is also expressed. As the expression of *Dlx5* is reduced in *Dlx1–Dlx2* knock-out mice, this raises the possibility that the loss of at least three *Dlx* genes may be necessary for an overt SVZ defect. A tempting speculation is that hierarchical *Dlx* gene regulation may also take part in the development of olfactory bulbs. In the *Dlx1–Dlx2* knock out there is a complete loss of GABAergic and dopaminergic interneurons (Anderson et al., 1997; Marin et al., 2000). Likewise, *Dlx5* null mice show a partial loss of GABAergic and Dopaminergic neurons (Levi et al., 2003).

4.3. Fgf8 and induction of Dlx expression in the face mesenchyme

While the disruption of *Dlx1* or *Dlx2* alone does not result in evident tooth abnormality, the targeted inactivation of both *Dlx1* and *Dlx2* results in the arrest of the upper molar development at early stages (Qiu et al., 1997). Partial redundancy of function of *Dlx5* and *Dlx6* could explain why no tooth defect is observed in the mandibular molars of these mice as the two genes are expressed in similar territories in the mandibular primordium. Indeed, in *Dlx5* null mice no teeth abnormality has been described, with the exception of the occasional absence of one molar tooth (Depew et al., 1999). Tooth induction and development is a paradigm of well-studied epithelium–mesenchyme interaction. Both tissue compartments express signaling molecules whose activity leads to the formation of properly positioned teeth germs. *Dlx* genes may participate in such complex signaling.

Dlx2 is expressed in the first BA in distinct non-overlapping domains in the mesenchyme and in the epithelium, prior to tooth bud initiation. In the mesenchyme of the first arch *Dlx2* is expressed proximally in both the maxillary and the mandibular division (Thomas and Sharpe, 1998), while in the epithelium is expressed distally in the same territories. Given the proposed function of *Dlx* genes in tooth development, it is important to elucidate the signals that restrict the *Dlx2* expression boundaries in the mesenchyme. A fragment of 3.8 Kb upstream of *Dlx2* contains

regulatory elements sufficient to drive expression of the gene in the epithelium, but not in the mesenchyme. This element responds to signaling of *Bmp4*, which is co-expressed in the oral epithelium. Mesenchymal expression is regulated by another mechanism involving *Fgf8*, expressed in the overlying epithelium. *Fgf8* seems to serve the dual function of inducing mesenchymal expression and inhibiting epithelial expression (Thomas et al., 2000). In spite of these observations, conditional targeting of the *Fgf8* gene resulting in reduced *Fgf8* expression in the BAs does not seem to affect *Dlx* gene expression (Trumpp et al., 1999), and thus the *Fgf8* regulation on *Dlx* needs to be further studied.

The same promoter fragment that governs expression of *Dlx2* in the oral epithelium is also able to drive expression in the AER of the limb bud (Thomas et al., 2000). In this location, however, it is unlikely that *Fgf8* may repress *Dlx2* expression since the two genes are co-expressed in the flank epithelium (early) and in the AER (later).

4.4. Dlx protein–protein interactions in control of gene transcription

Dlx proteins share similar DNA-binding properties *in vitro*, bind to specific DNA sequences via their homeodomain and act as transcriptional regulators via an independent activation domain (Zhang et al., 1997; Feledy et al., 1999; Masuda et al., 2001). Transcriptional activation by Dlx3 protein on reporter construct *in vitro* depends on two subdomains located on either sides of the homeobox (Feledy et al., 1999). Similarly, Dlx5 can regulate transcription through a *col1A1* response element via an activation domain positioned at the N-terminus (Masuda et al., 2001).

Dlx proteins appear to participate in transcriptional regulation in complex with other proteins. Up to now, two proteins have been recognized to be part of Dlx trascriptional complexes and thereby modulate gene transcription: the homeoproteins of the Msx family (Zhang et al., 1997) and Dlxin-1 (Masuda et al., 2001).

Dlxin-1 was identified by yeast-two-hybrid screening of mammalian proteins binding to the N-terminus of Dlx5. This domain is recognized as a transcription activation domain active on the *col1A1* promoter *in vitro*. Dlxin-1 binds to Dlx5, Dlx7, and Msx2 proteins both *in vitro* and *in vivo*, and has a promoting function on Dlx5-dependent *col1A1* transcription (Masuda et al., 2001). Expression of *Dlxin-1* mRNA is rather ubiquitous, although it is higher in developing skeletal elements and osteoblasts which are also sites of *Dlx5* and *Dlx6* gene expression. The requirement of the role of *Dlxin-1* as a molecular modulator of *Dlx* genes remains to be specifically tested in other tissues.

The other class of proteins recruited into Dlx transcription complexes is likely to be represented by the Msx family. Proteins of the Dlx and of the Msx families contain an homeodomain, are capable of binding DNA and their preferred DNA binding sequence is essentially the same (Catron et al., 1993; Feledy et al., 1999). Msx proteins have been shown to bind to Dlx *in vitro*. The binding is mediated by

interaction of their homeodomains and results in a mutual functional antagonism. Using appropriate *in vitro* reporter assays, Msx proteins are potent transcription repressors (Catron et al., 1993; Semenza et al., 1995; Zhang et al., 1996, 1997; Newberry et al., 1997a, b), while Dlx proteins are transcription activators (Zhang et al., 1996; Newberry et al., 1998; Feledy et al., 1999; Masuda et al., 2001).

Protein–protein interactions, with homo- and heterodimer formation, as well as competition for the same binding sites, have been demonstrated (Yang et al., 1998). Upon binding, each of the homeoprotein functions as an inhibitor of the other partner's function. It should be noted that these findings are obtained *in vitro* using purified recombinant proteins. The Msx-Dlx interaction raises the interesting possibility that at least some of the defects observed in *Msx* and *Dlx* null animals (Satokata and Maas, 1994; Acampora et al., 1999; Depew et al., 1999) might be the result of the loss of inhibitory function exerted on the cognate partner. There is experimental evidence suggesting, for instance, that Dlx5 can antagonize the transcriptional repression function of Msx2 (Newberry et al., 1997). Should this be a more general case, double inactivation of both *Msx* and *Dlx* could result in rescue of (at least some) phenotypes in the regions where they are co-expressed. To directly approach this interesting question it will be necessary to examine the craniofacial and limb defects in mice deficient for both *Msx* and *Dlx*. It is important to note that this effect can only take place *in vivo* in cells in which the two classes of proteins are co-expressed such as the AER and underlying mesenchyme and the pharingeal arches (Zhang et al., 1997; Bendall and Abate-Shen, 2000). If the hypothesis of mutual inhibition is correct then some of the defects observed *Msx* (Satokata and Maas, 1994; Houzelstein et al., 1997; Satokata et al., 2000) or *Dlx* mutants might be due to improper activation of partner genes.

5. Conclusions

During evolution *Dll* and *Dlx* genes have maintained a strikingly similar pattern of expression and reminiscent functions. *Dll* and *Dlx* appear to be both involved in correct limb and head morphogenesis and share roles in the development of sensory organs and of the central nervous system (Panganiban and Rubenstein, 2002). Mutations of these genes in *Drosophila* and vertebrates lead to homeotic transformations in the head (antennae; jaw) and to abnormal appendage outgrowth. The sum of data summarized in this chapter reinforces the notion that *Dll*/*Dlx* genes are involved in the emergence of innovation during vertebrate evolution while maintaining a memory of their ancestral roles.

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Prx, *Alx*, and *Shox* genes in craniofacial and appendicular development

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Contents

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1. Introduction

Paired (Prd)-type homeobox genes make up one of the major classes of the homeobox superfamily (Galliot et al., 1999; see also Bürglin, 2002). Within this Prd class, a number of diverse sub-classes can be distinguished on the basis of sequence similarities, including the presence of additional conserved domains encoded by these genes. One such group of similar genes are those that encode a short (approx. 16-amino acid) domain that has been referred to as aristaless domain, c-tail, OAR domain, paired tail, SLAK domain etc. Since the aristaless domain is only found in paired type homeodomain proteins it seems likely that (1) all aristaless related genes have one common ancestor gene that encoded a protein containing both domains and (2) that the aristaless domain has a function that is directly related to the way these homeodomain proteins exert their function. The nature of this function is most likely in attenuating the activity of these transcriptional activators presumably entirely or in part through modulating DNA binding (Amendt et al., 1999; Norris and Kern, 2001; Brouwer et al., 2003).

Previously we have distinguished three groups of vertebrate "aristaless-related genes," including (1) the *Prx*/*Alx* subclass, (2) a group of genes with predominant functions in the CNS and PNS, and (3) the Pitx genes (Meijlink et al., 1999). Apparently the aristaless domain is evolutionary ancient, because in insects, in addition to the aristaless gene itself, clear homologues can be recognized for Otp, Pitx, Rx, and Chx10. Aristaless-related genes are widespread throughout the animal kingdom and are also found in for instance C. elegans and also in the diploblastic polyp Hydra (Gauchat et al., 1998). In agreement with their ancient history they have been linked to extremely diverse functions, ranging from early functions in eye development to left–right determination and from diverse functions in limb development to pituitary function.

Knowledge of the function of Drosophila homologues of developmental vertebrate genes from the mouse is often helpful in studying the latter genes. In the case of the *Prx*/*Alx* and *Shox* genes it remains however unclear whether the Drosophila aristaless gene can be considered a true homologue. While there is an intriguing analogy in their involvement in proximo–distal patterning of structures that protrude from the main body axis (appendages, facial primordia) it remains to be seen whether this is more than a coincidence.

The Prx/Alx genes and the somewhat elusive Shox genes are the subject of this review. They are, compared to the diversity within the entire set of aristaless-related genes, structurally and functionally a rather homogeneous group. This classification and its further ramifications is based on a combination of structural and functional comparisons. *Prx*/*Alx* genes include the "*Prx* genes" *Prx1* and *Prx2*, the "*Alx* genes" *Alx3*, *Alx4*, and *Cart1*, and the "*Shox* genes" include *SHOX* and *Shox2*. All these genes are typically expressed during embryogenesis in limb, craniofacial, and genital primordia. In addition they have often other sites of expression usually of unclear significance.

Figure 1 shows the results of phylogenetic analysis of the seven genes that are discussed in this review, based on the primary protein sequences of either the

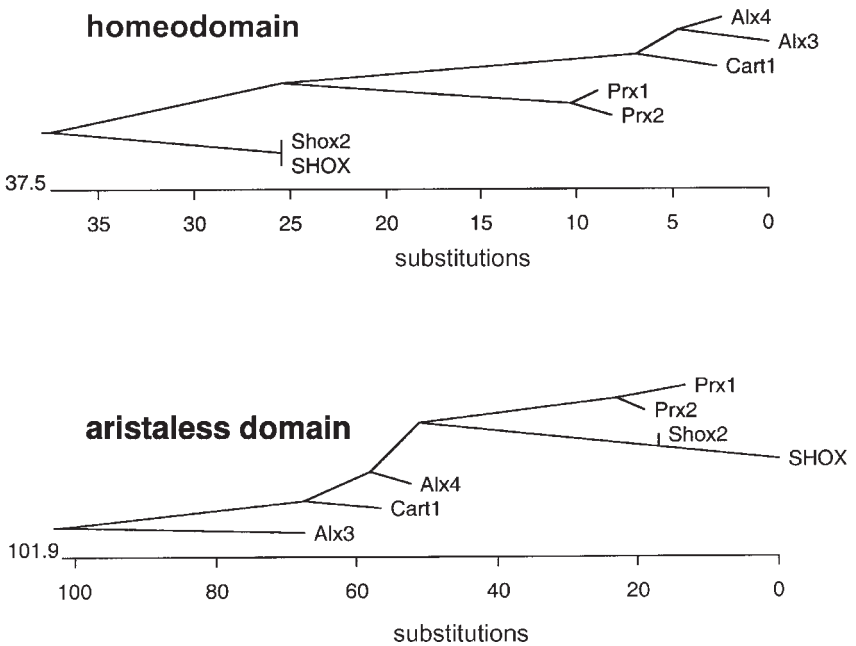

Fig. 1. Phylogenetic trees for the groups of *Alx*, *Prx*, and *Shox* genes based on amino acid sequences of the homeodomains (top) and aristaless domains (below). DNAstar/Lasergene software was used to generate these trees, using Clustalv (PAM250). Shown as "unbalanced branches," forcing branch distances to correspond to sequence divergence.

homeodomain or the aristaless domain, and assuming that these genes arose through duplication events from a common ancestor.

2. The *Prx* genes: *Prx1* and *Prx2*

2.1. General

Two vertebrate *Prx* genes, *Prx1* and *Prx2* have been described. Previously we and others referred to a "Prx3" gene that we now discuss as Shox2 in the section on Shox genes. Alternative names for *Prx1* include *Pmx1*, *Mhox*, *K2*, *Phox1* (for the human gene), and *Prrx1*; for *Prx2*: *S8*, *Pmx2*, and *Prrx2*. *Prx1* and *Prx2* share structurally nearly identical homeo- and aristaless domains, as well as additional sequence similarity including a so-called Prx domain, a stretch of amino acids of unknown function located close to the N-terminus.

2.2. Embryonic expression

Cloning and basic expression features of *Prx1* and *Prx2* in mouse embryos were first described by Cserjesi et al. (1992) and Opstelten et al. (1991), respectively.

These genes are widely expressed in complex and mostly overlapping patterns that dynamically evolve during development. Expression is most prominent in mesoderm and in neural crest derived mesenchyme (Kern et al., 1992; Leussink et al., 1995). Both genes are expressed starting at the early head fold stage and become later restricted to the neural crest derived mesenchyme of the facial processes including the branchial arches, and the lateral mesoderm with highest expression in the limb buds and the genital tubercle. Limb expression is initially throughout the limb bud, but is downregulated in the condensing mesenchyme in the core of the limb bud as chondrocyte differentiation begins.

Leussink et al. (1995) performed a rather detailed comparative analysis between the two genes, showing marginal differences in many areas of the embryo, but also a number of striking differences (see also Beverdam and Meijlink, 2001b). Examples of the latter are the expression of *Prx1* early on in the dermomyotome and later in embryonic and postnatal brain, and the expression of Prx2 in spleen. In addition, both genes are expressed in overlapping but distinct patterns in the heart. Particularly intriguing were the expression of *Prx1* in the atrioventricular valves and the unique expression of *Prx2* in the ductus arteriosus (Bergwerff et al., 1998).

2.3. Functions of Prx genes

Targeted gene deletion of *Prx1* (Martin et al., 1995) demonstrated for the first time the importance of a member of this gene family for correct morphogenesis of the skeleton. Newborn homozygous *Prx1* mutants died from the consequences of cleft palate. More in general, the abnormalities in homozygous *Prx1* mutants were confined to the skeleton, the affected skeletal elements being of diverse nature.

Bone formation may involve either endochondral ossification as in most long bones, or membranous ossification as in the calvaria and the dentaries. The *Prx1* defects were seen in both types of bones and the affected bones were also diverse in their embryonic origin, as they included facial bones that are derived from neural crest, limb bones that originate from lateral plate mesoderm, and vertebrae that originate from paraxial (somitic) mesoderm.

The most dramatic defects were observed in the skull, especially in bones that form from neural crest derived mesenchyme of the first two branchial arches. These defects included the reduction, deformation or abnormal fusion of the squamosal, and zygomatic bones, the tympanic ring as well as severe abnormalities in the middle ear ossicles. Furthermore, in the limbs the zeugopodal bones (ulna, radius, tibia, and fibula) were truncated and abnormally bowed, and finally in about 12% of the homozygous mutants spina bifida was observed as a result of abnormally shaped neural arches in the vertebral column. The earliest embryonic stage when these defects were seen was E13.5 for the cranial as well as the limb phenotypes. Martin et al. (1995) speculated that compensation by the *Prx2* gene explained why merely rather mild effects were observed in the limbs where *Prx1* is highly expressed. This was confirmed by analysis of double mutants (see below), but strikingly, no

defects in *Prx1* single mutants were observed in tissues where only *Prx1* and not *Prx2* are expressed.

The *Prx2* gene was knocked out in the groups of Kern (Lu et al., 1999a) and Meijlink (Ten Berge et al., 1998b). No skeletal defects or affected health were found in these mice, but analysis of *Prx1*/*Prx2* double knockouts revealed much more dramatic phenotypes than were seen in the *Prx1* single knockout. The nature of the defects in the double mutants was in most cases an aggravation of those already present in the *Prx1* mutant; some defects that were absent in the single mutant also correlated with high embryonic expression of both genes. This strongly indicates that both genes act through regulation of an identical set of target genes, also because *Prx1* and *Prx2* encode structurally very similar proteins therefore implying that the $Prx1^{-/-};Prx2^{-/-}$ phenotype best demonstrates the key functions of the *Prx* genes and for this reason we focus here on the double mutants.

2.4. Prx genes and craniofacial morphogenesis

Ten Berge et al., (1998b); Lu et al., (1999a) carried out rather extensive analyses of the craniofacial aspects of *Prx1*;*Prx2* double mutants. Especially the former group reported a high degree of variability in the severity of the phenotypes. A number of differences between the average severity of the phenotypes described by both groups should undoubtedly be mostly contributed to differences in the genetic backgrounds in which these mice were bred, since the various knock-out mice studied by both groups all carried very likely, complete null-alleles.

While *Prx1* null mutants die within a day after birth due to cleft palate and the resulting incapability to breath and feed normally, the *Prx1*;*Prx2* double mutants die within one hour (Ten Berge et al., 1998b). This is most likely caused by the extreme severity of the cleft palate phenotype.

Cleft palate is caused by the failure of the palatal shelves to grow out, position appropriately and fuse in the midline. A host of mutants of different nature suffer from this defect, which can also have environmental causes. Cleft palate may occur as an indirect consequence of growth abnormalities elsewhere in the skull but is in other cases directly caused by insufficient outgrowth of the palatal shelves or by defects in the epithelium of the fusing shelves (e.g. see Sun et al., 1998; Schutte and Murray, 1999). The *Prx1*/*Prx2* double KO is rather distinctive in that the palatal shelves are entirely or almost completely absent. Since the mandible of these mutants is very small, the tongue, which is of nearly normal size, penetrates into the nasal cavity where it obstructs the airway (Ten Berge et al., 1998b). Apparently the double mutants described by Lu et al., (1999a) live longer, presumably because the genetic background of their mice causes a less severe phenotype. Another apparent discrepancy was that Lu et al. reported that $Prx1^{+/-};Prx2^{-/-}$ mice survived and were normal, while in the background used by Ten Berge et al., only a minority survived more than 24 h after birth. A genetic basis for this was

confirmed by the observation that this minority gave rise to viable offspring (not published).

Differences in the mice of the two groups are also seen in the peculiar phenotype that affects the incisors of the lower jaw. Lu et al. describe how these incisors are arrested at the bud stage, failing to progress to the bell stage that is normally reached at E12.5; in addition there was only one such tooth bud. In contrast, Ten Berge et al. report that the double mutant mice are usually born with a single incisor in the mandible.

The lower jaw develops from the posterior or mandibular part of the mandibular arch. It has since long been clear that reciprocal interactions between epithelium and the underlying neural crest derived mesenchyme play a major role in the development of the mandibular arches and in particular in tooth development (e.g. Kollar and Fisher, 1980). Development of the incisor teeth is first perceptible at E11.5–E12 as a local thickening of oral ectoderm. This thickened ectoderm then becomes a tooth bud when it invaginates into the underlying condensing mesenchyme. In due course, after the subsequent ''cap'' and ''bell'' stages (Peters and Balling, 1999; Jernvall and Thesleff, 2000), the enamel secreting ameloblasts form from the ectoderm, while the odontoblasts and alveolar bone derive from the mesenchymal component. Growth factors that are known to signal at early stages from the mandibular oral epithelium include several *FGF*s, Sonic hedgehog (*Shh*), and *BMP4*. The gene encoding the paired-box transcription factor Pax 9 is essential for tooth development (Peters et al., 1998), and an early marker of dental mesenchyme. Its expression is already observed at E10.5, prior to ectodermal thickening. Neubüser et al. (1997) showed that an antagonistic balance between *FGF8* and *BMPs* determines where in the underlying mesenchyme Pax9 is induced: expression is seen in mesenchyme adjacent to *FGF8* positive epithelium, but not where *BMP2* or *BMP4* are co-expressed in the epithelium.

Ten Berge et al. (1998b) deduced an explanation for the single-incisor phenotype of *Prx1*/*Prx2* knockouts from a comparative analysis of molecular markers in the mandibular arch of wildtype versus double mutant embryos. Mandibular processes were normal until E10. Using the observations of Neubüser et al. as a model, they compared expression of *Pax9*, and its regulators *FGF8*, *BMP2*, and *BMP4* in the course of the aberrant mandibular development in *Prx1*;*Prx2* double mutants and wildtypes. At E10.5, and more clearly at E11.0, *FGF8* expression had shifted medially. *BMP4* was not expressed in this medial ectopic location, and, in confirmation of the Neubüser et al. model, *Pax9* was expressed in the medial mesenchyme at an abnormal medial position. As a result, upon complete fusion of the mandibular processes into the mandibular arch, instead of two *Pax9* domains on either side of the midline, one fused domain is generated, which explains the appearance of a single incisor.

The basic mechanistic causes of the mandibular abnormalities must be upstream from ectopic *FGF8* and *Pax9* expression, because the embryos in which the above molecular analysis were done had anatomical abnormalities already. Analysis of proliferation by measuring BrdU incorporation in vivo in the mandibular processes of *Prx1*;*Prx2* double mutants at E10.5 indicated a slightly lower proliferation

locally in the distal mesenchyme of the processes, which correspond to the middle part of the future arch. A spatially restricted inhibition in the outgrowth of fast developing structures like the mandibular processes at this stage should lead to a deformation or rotation of that structure, and this deformation was confirmed by expression of the homeobox genes *Alx3* and *Dlx2* which were used in this context as markers of distal and proximal mesenchyme, respectively. Their near-complementary expression boundaries were tilted over in a way consistent with the suspected deformation of the mandibular arch.

This raised the question of the direct origin of this decreased proliferation. The medial location of the mesenchyme with lower proliferation rate suggested that it could be under the influence of *Shh* present in the epithelium overlaying it. In situ expression analysis of *Shh* in oral epithelium between E9 and E11.5 confirmed this hypothesis. In the medial area of oral-epithelial Sonic hedgehog expression was normal at E9.5, but was strongly decreased at E10.5. The more lateral/proximal epithelial expression of Sonic hedgehog was normal.

A model that would accommodate these observations is depicted in Fig. 2. Lower proliferation of mesenchymal mandibular arch cells in a restricted area underneath Shh expressing oral epithelium is caused by downregulation of Sonic hedgehog. Since *Prx1* and *Prx2* encode transcription factors and are not expressed in the epithelium, the simplest explanation is that they regulate a positive factor that signals to the epithelium to induce *Shh*. *Shh* then signals back to the mesenchyme to stimulate proliferation. In the mutant, lower proliferation leads to the deformation with the consequences outlined above.

Other abnormalities found in the cranium include defects of the inner ear. The otic capsule was smaller, and the lateral semicircular canal was entirely absent (Ten Berge et al., 1998b).

2.5. *Functions of* Prx1 *and* Prx2 *in limb development*

In spite of high expression of *Prx1* and *Prx2* in early limb bud mesoderm, *Prx2* has no and *Prx1* a rather mild limb phenotype (see above). In contrast, in $Prx1^{-/-}$;$Prx2^{-/-}$ double mutant neonates, the zeugopodal bones, and especially those of the hind limbs are strongly reduced in size. The remaining radius and tibia elements are much more strongly bent than in $Prx1^{-/-}$ mice. In its most severe phenotypic manifestation the tibia has a disk-like appearance caused by bulging out of the tibial diaphysis (Ten Berge et al., 1998b) and histological analysis of the growth plates demonstrates disorganization of the proliferating and hypertrophying chondrocytes as well as defects in endochondral ossification (Lu et al., 1999b). This aspect of the phenotype is not yet apparent in E12.5 embryos, but at E13.5 a clear retardation in growth is visible, and at E14.5 the difference between wildtype and mutant zeugopodal cartilage elements is already striking (see Fig. 3). The basic classical events that determine antero-posterior and dorso-ventral patterning are thought to take place prior to E12.5–E13.5. In concert with this Lu et al. did not find changes in expression of factors like

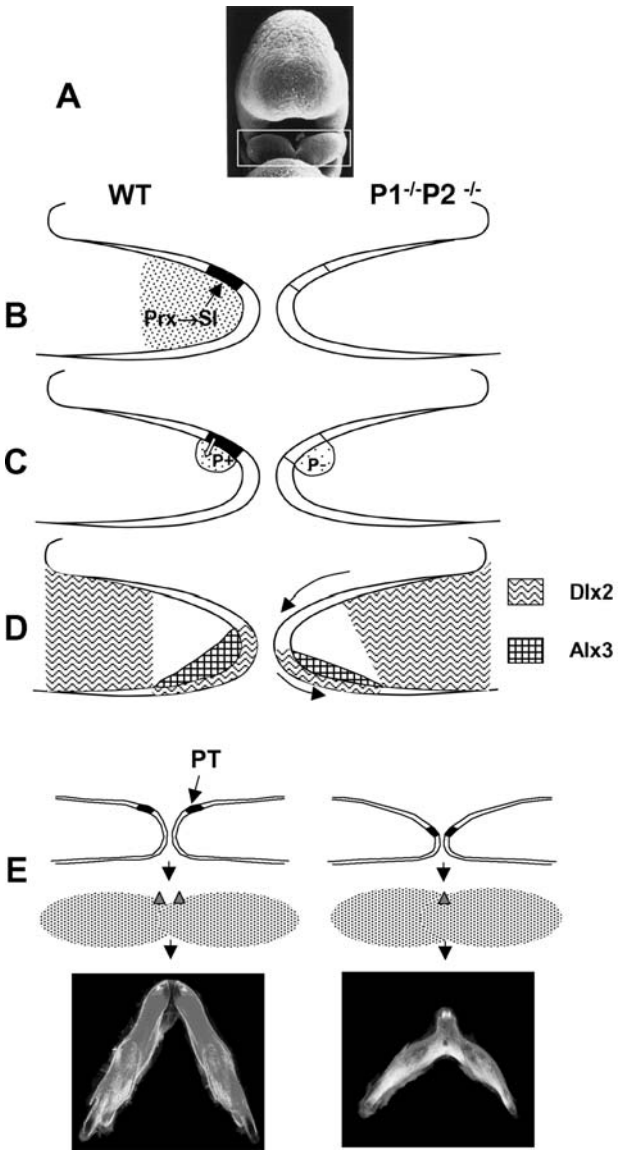

Fig. 2. Model explaining mandibular defect of $Prx1^{-/-};Prx2^{-/-}$ mice. Cellular and molecular abnormalities in the $Prx1^{-/-};Prx2^{-/-}$ mandibular arch. (A) Picture of an E10.5 embryo to indicate area (in rectangle) of the mandibular arch, which is schematically depicted in the remainder of the figure. To the left the wildtype situation is shown, to the right, the situation in the mutant. (B) Expression of *Prx1* and *Prx2* is indicated by shading. Shh expression in oral ectoderm (upper part in scheme) that presages incisor is indicated in black; a more proximal area of Shh expression corresponding to the future molars is omitted. Prx induces an as yet elusive factor "SI" (Shh inducer) in the mesenchyme, which signals to the oral ectoderm. (C) Proliferation of an area of mesenchyme (shaded) is influenced by epithelial Shh, but this effect is lacking in the mutant. (D) Proximo-distal markers *Dlx2* and *Alx3* (as indicated) demonstrate deformation of mandibular processes. (E) Diagram indicating how deformation of mandibular arches leads to fusion of a single medial incisor. PT, presumptive tooth. The presumptive tooth domains fuse as a consequence of the deformation of the mandibular arch.

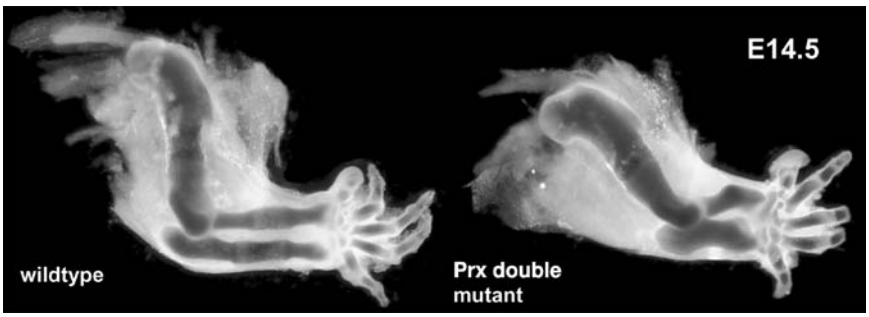

Fig. 3. Limb defect in $Prx1^{-/-};Prx2^{-/-}$ embryos. Shown are wildtype and double mutant forelimbs of E14.5 embryos, stained with Alcian blue (cartilage) and Alizarin red (bone; virtually absent in this case). At E 12.5 the defect is entirely undetectable, while here, 48 h later it is already very clear. Note that only ulna and radius are affected.

Shh and Hoxd11. Outgrowth of the limb from E12.5 is contingent on populations of proliferating mesenchyme that depend on an early-phase of expression of the Indian hedgehog (Ihh) gene (St-Jacques et al., 1999). It is therefore interesting that Lu et al. (1999b) reported a decrease in the expression level of the hedgehog target Ptc in the perichondrial layer surrounding the proliferating mesenchyme at E12.5.

Unlike the single mutants, the double mutants have defects of the hand plate. The penetrance of this phenotype was incomplete and its severity highly variable, and consists of a mixture of pre-axial (anterior) and post-axial (posterior) polydactyly, as well as oligodactyly. At E10.5 this forelimb-specific phenotype is foreshadowed by truncation of the Apical Ectodermal Ridge (AER) (Lu et al., 1999b), a transient early structure that is a source of signals to the underlying proliferating mesenchyme. FGF8 and BMP4 expression in the AER were downregulated locally in the corresponding position and so were the known downstream BMP4-targets Msx1 and Msx2 in the adjacent mesoderm.

An interesting observation in the appendicular phenotypes of the *Prx* double mutants was that frequently left/right changes were observed, notably a different number of digits in left and right hands without a left/right preference. This demonstrates that variability in the strength of the phenotype is not purely genetic, but presumably also dependent on stochastic events: the abnormal genotype causes a degree of instability in the processes that normally govern limb development resulting in different morphogenetic outcome even in genetically identical context.

Abnormalities of skeletal structures are also found at a number of other locations in the skeletons of $Prx1^{-/-};Prx2^{-/-}$ mutants, for instance in the pectoral girdle the pubic symphysis fails to form (Ten Berge et al., 1998b). In view of the high expression of both genes in the genital tubercle throughout mid-gestation, it was not surprising that defects in the os penis (baculum) were found (Ten Berge et al., unpublished).

3. The Alx genes: *Alx3*, *Alx4*, and *Cart1*

3.1. Introduction; history

The classification of *Alx3*, *Alx4*, and *Cart1* as a subgroup of related genes is based on

(1) very high similarity within the homeodomains encoded, as well as sequence similarity in the region separating the homeodomain and the aristaless domain; however *Alx3* is much richer in prolines than *Alx4* and *Cart1*, while otherwise highly similar. This proline enrichment affects the aristaless- but not the homeodomain and leads to an unusual form of the aristaless domain which is functionally deteriorated (Brouwer et al., 2003).
(2) similarity of their expression patterns, as well as to a large extent phenotypes of mutants. Like the *Prx* genes, they show prominent expression in mesenchyme of the facial processes, the limbs and the genital tubercle, but they each have their own idiosyncratic aspects of expression.

Alx4 stands out in this group as it has been linked to the ''classical'' mutant Strong's luxoid (*Lst*) (Strong and Hardy, 1956) of which several alleles are known. P. Forsthoefel described this mutant between 1962 and 1970 in detail and was therefore unknowingly the first to study the embryonic function of a member of this gene group. As early as 1962, Forsthoefel described and appreciated the essential early morphological abnormalities in *Lst* embryos that are at the basis of the craniofacial and appendicular defects found in newborns (Forsthoefel, 1962, 1963). For example, he observed in E11.5 homozygous mutant embryos an anteriorly enlarged apical ectodermal ridge (AER) and a mesodermal protrusion foreshadowing the pre-axial polydactyly, and he recognized that the cranial abnormalities found its origin in defective patterning of the anterior part of the cranial skeleton that we know to be neural-crest derived.

3.2. Embryonic expression

Several groups have described expression patterns of *Cart1*, *Alx4*, and *Alx3* (Zhao et al., 1993, 1994; Qu et al., 1997a; Hudson et al., 1998; Ten Berge et al., 1998a; see also Beverdam and Meijlink, 2001b for a direct comparative analysis of these patterns). Expression of these three genes starts generally in the period E8–E9, and is initially most prominent in neural crest derived mesenchyme in the head region. Most conspicuously and characteristically is the expression in the nasal processes, but there is also clear expression in the distal parts of the mandibular processes. Expression in the second (hyoid) arch is much lower and no expression is seen in more caudal branchial arches. Differences between the patterns of these genes are rather marginal, but in general *Alx4* and *Cart1* are expressed somewhat broader than *Alx3*. For example at E10.5, *Cart1* and *Alx4* are expressed in the maxillary process, the rostral protuberance of the mandibular arch from

which eventually the upper jaw is formed, whereas *Alx3* is absent. Comparison with the *Prx* expression patterns is in line with a more important role of the *Prx* genes in formation of the branchial arches and greater impact of the *Alx* group on the formation or patterning of the nasal processes, as is evident from mutant studies.

All three genes are expressed in the developing limb bud, *Alx3* and *Alx4* having a characteristic restriction to anterior mesoderm. *Alx3* and *Alx4* are also expressed in the flank immediately anterior from the limb buds and in the limb field before and around the time of overt outgrowth of the limb. Expression is then already absent or much lower in the posterior part of the (future) limb field, which suggests that it is subject to early patterning events that polarize the limb field. *Cart1* is expressed at much lower levels in the early limb bud, but was reported by Beverdam and Meijlink (2001b) to be expressed in mesoderm in both the anterior and posterior margin of the bud. *Cart1* stands out in the whole group of genes discussed in this review by its expression in differentiating chondrocytes and cartilage (Zhao et al., 1993). Other genes of the *Prx*/*Alx*/*Shox* group are always downregulated in chondrocytes differentiating into cartilage, and often restricted to the perichondrial layers.

Recently, both *Alx3* and *Alx4* have been found to be expressed in the brain (Asbreuk et al., 2002; Wimmer et al., 2002). The functional impact of this aspect of their expression has not yet been established.

3.3. Alx *genes and craniofacial development*

Newborn Strong's luxoid mutants have skull abnormalities that are caused by deficient development of the anterior part of the head (Forsthoefel, 1963). As a result the nasal septum is shortened and the maxilla and the lower jawbones are reduced. In addition the calvaria, the membranous bones that cover the skull, and more in particular the parietal bone show ossification defects and is smaller than normal, which result in a persistent fontanel. This might be the aspect of the skull phenotype with the highest penetrance, since in the genetic background in which the *Alx4* gene was knocked out (Qu et al., 1997b) this was the only detectable skull phenotype. Abnormal anatomy of the brain and the strikingly bulging eyes that prevent the eyelids from closing appears to be secondary to the defects in the cranial base. According to Forsthoefel, even the persistent fontanel is "partly the result" of excessive lateral growth of the brain, which on its turn would be caused by the earlier skull defects. Interestingly the human congenital disease Parietal Foramina (PFM) has been linked to heterozygosity for *Alx4* (Wu et al., 2000; Wuyts et al., 2000; Mavrogiannis et al., 2001). Patients that suffer from this disease have ossification defects and hypoplasia of the calvaria, notably the parietal bones, which results in abnormally large open fontanels, actually a rather large opening in the brain case. It is not clear whether these patients have other underlying skull defects, but the demonstration by Mavrogiannis et al. (2001) that *Alx4* is expressed in coronal sutures of E16 mouse fetuses suggests a

more direct role of *Alx4* in formation of the parietal bone than suggested by Forsthoefel. The observation that the heterozygous mutation in humans gives rise to a defect that is about as severe as that of the homozygous mutation in the mouse whereas, vice versa, hypodactyly has never been shown for human patients with *Alx4* mutations, is an example of the disparities one can encounter between mouse and human genotype–phenotype relations. It should be recognized that major differences between the human and mouse skull exist: the mouse has for instance an interparietal bone that that does not exist in primates, and obviously the human calvaria had to adapt to the spectacular evolution of the human brain.

Whereas *Alx3* mutant skulls are indistinguishable from those of wildtypes, newborn Cart1 mutants have major defects of the calvaria. They may be born (as usual depending on their genetic background) without the interparietal bone and strongly reduced frontal and parietal bones. The basis of the phenotype has been traced back to abnormal apoptosis in cervical mesenchyme at very early stages, followed by failure of the neural tube to close over the midbrain region. Interestingly, this defect, which has a variable penetrance, can be rescued by high doses of folic acid (Zhao et al., 1996).

Double mutants missing two out of three genes of this group have severe facial clefts and it appears that lacking most combinations of three mutant alleles already causes these defects (Qu et al., 1999, Beverdam et al., 2001a and unpublished). Qu et al. (1999) showed that *Alx4*/*Cart1* mutants are born with cleft nose and palate and without a nasal septum. The anterior skull bones, including the nasal and the basisphenoid, were dramatically reduced or malformed. The mandibles were also shorter and abnormally shaped. The craniofacial phenotype of *Alx3*/*4* newborns is very similar to that of *Alx4*/*Cart1* newborns and is also characterized by anterior reductions leading to nasal clefts, combined with less extreme lower jaw defects (Beverdam et al., 2001b). Analyses of various developmental stages gave an anatomical explanation for the split-nose phenotype: around E10.5, the nasal processes, although not detectably smaller, have an abnormal position. Through development, this malposition cumulates into the severe split-nose phenotype seen at birth. No differences were found in the expression of a number of important genes expressed in the nasal processes. These potential downstream genes included *FGF8* and *Shh*, which are prominently expressed in nasal process ectoderm. *Shh* seemed an attractive candidate by extrapolation of its decreased expression seen in oral epithelium of *Prx1*;*Prx2* double knockout embryos (see Section 2.4; Fig. 2).

Interestingly, abnormal apoptosis was seen at early stages (E10.0) in *Alx3*;*Alx4* double knockouts. The location of the area involved appeared to be consistent with expression of *Alx3* and *Alx4* and with the phenotype observed. It was however not related to the area of abnormal apoptosis seen in the *Cart1* knockout. It seems likely that the characteristics of the *Alx3*/*Alx4* etiology can be extrapolated to the *Alx4*/*Cart1* and *Alx3*/*Cart1* knockouts, although this has not directly been investigated. *Alx3*/*Cart1* double knockouts also have cleft nose (Beverdam and F.M., unpublished observations).

3.4. Function of the Alx *genes in limb development*

The limb is a widely used model system in vertebrate developmental biology because it offers an accessible way to approach the question of how a one-dimensional (genetic) code underlies patterning of a three-dimensional structure. Many reviews (e.g. Capdevila and Izpisua-Belmonte, 2001) give an overview of the role of the various interacting signaling centers in the developing limb bud. Among these, it is the Zone of Polarizing Activity (ZPA) that determines the antero-posterior polarity of the limb bud. The secreted signaling factor Sonic hedgehog (*Shh*) has a key role in the ZPA, and ectopic anterior expression of *Shh* is a crucial aspect in many, but not all polydactyly mutants. Ectopic *Shh* also causes polydactyly when it is brought about by embryological manipulations. Recently, it has become clear that initial antero-posterior patterning of the limb field depends on other genes that act prior to the first expression of *Shh*. Apparently a reciprocal interaction between the posterior gene *dHAND* and the anterior gene *Gli3* polarizes the limb field, and is at least in part responsible for establishing the posterior localization of the ZPA.

Mice homozygous for the Lst mutation usually have polydactyly in all limbs, often accompanied by tibial and radial dysplasia. The mutation is semidominant because heterozygotes usually have mild polydactyly (one extra toe, or a double hallux) in the hind limbs. The severity and penetrance of the phenotype strongly depends on genetic background and *Alx4* heterozygous animals may have normal feet in certain backgrounds and multiple extra toes and even extra fingers in other. Thirty years after the basic work of Forsthoefel (see above), Chan et al., (1995) recognized that an ectopic ZPA was at the basis of the polydactyly in Lst mutants. They drew this conclusion from molecular as well as embryological-experimental data: (1) *Shh*, *Fgf4* and *Hoxd12* are ectopically expressed in the anterior limb bud of Lst mutant embryos, and (2) transplantation of anterior mesoderm from an E11.5 Lst mutant to an anterior position in a host chick limb bud, led to expression of chick Hoxd11 near the site of the graft.

Wisdom and colleagues cloned and characterized *Alx4* and subsequently produced a knockout through targeted gene ablation (Qu et al., 1997b). The phenotype included polydactyly, ectopic ZPA formation and ectopic expression of *Shh*, *Fgf4*, and *Hoxd13*. Shortly thereafter they (Qu et al., 1998) as well as Takahashi et al. (1998) found *Alx4* to be allelic with the Lst mutation. Seeming differences between the phenotypes observed are likely caused by influence of the genetic background (Qu et al., 1998).

Both Takahashi et al. (1998) and Qu et al. (1998) performed molecular analyzes that generally confirmed the findings of Chan et al. (1995) regarding ectopic expression of *Hox d* genes, *FGF4* and *Shh*. A discrepancy seems to be in the stage that ectopic Shh expression is detected by both groups. Both Chan et al. and Qu et al. see anterior *FGF4* expression at E10.5, but ectopic *Shh* is reported at E10.5 by the former group and not until a full day later by Qu et al. This question is of interest for a number of reasons, including the precise relation between ectodermal *Fgf4* and mesodermal *Shh* (Zúñiga and Zeller, 1999; Zúñiga et al., 1999), and the exact

position that *Alx4* has in the molecular genetic cascades that determine the morphogenesis of the autopods. Interestingly, Takahashi et al. (1998) reported that when *Shh* beads are inserted anteriorly in chick limb buds, *Alx4* is rapidly downregulated, implying the existence of a reciprocal negative interaction between *Alx4* and *Shh*. *Gli3* mRNA was downregulated much slower than *Alx4*, but this is presumably caused by the inhibiting effect of *Shh* on processing of the Gli3 transcription factor, which escapes detection by in situ hybridization (Wang et al., 2000; see also Section 3.5).

Alx3 and *Cart1* mutants have been reported to have no polydactyly or other limb abnormalities (Zhao et al., 1996; Beverdam et al., 2001a). Nonetheless *Alx3*;*Cart1* double mutants have weak polydactyly with low but significant penetrance (i.e. higher than in the parent *Alx3* strain) (A. Beverdam and F.M., unpublished observations).

Alx4; *Cart1* double mutants have a much stronger polydactyly than *Alx4* single mutants with characteristically 2–3 extra fingers that are often abnormally long. Surprisingly, in *Alx3*;*Alx4* double mutants the defects observed were barely more severe than those in *Alx4* mutants (Beverdam et al., 2001a). This is paradoxical for a number of reasons: (1) *Cart1* lacks the prominent expression domain in anterior limb field and limb bud mesoderm that is shared by *Alx3* and *Alx4*; (2) deletion of *Alx3* leads to serious aggravation of the craniofacial phenotype seen in *Alx4* mutants (see Section 3.3), showing that *Alx3* is a functional gene; moreover the analyzes of different compound mutants is here strongly suggestive of a dosage effect; (3) we have recently observed polydactyly of weak severity in mice homozygous for a null-mutation in the *Alx3* gene, comparable to that seen in most *Alx4* heterozygotes; this polydactyly is again clearly dependent on genetic background (C.K., S.K., and F.M. unpublished results). In Cart1, at homozygous mutants we observed only a very rare occurrence of soft tissue abnormalities that are possibly a very weak form of a polydactyly (Beverdam et al., 2001a).

Although the high impact of variation in genetic background on the severity of the phenotypes is a complicating factor in the analysis of these genotype–phenotype relations, it is possible that intrinsic, qualitative differences between these homeodomain proteins cause these unexpected observations.

3.5. Alx4 *and the "anterior cascade"*

Gli3 and *Alx4* have in common that they are expressed in anterior mesoderm of the early limb bud and that loss-of-function mutations in these genes lead to anterior ectopic expression of Sonic hedgehog in the limb bud and ultimately to pre-axial polydactyly. They function in a "restrictive way," because loss-of-function mutation of these genes leads to the formation of extra digits from the region where they are normally expressed. The relation between *Gli3* and *Alx4* is therefore of particular interest. Te Welscher et al. (2002a) reported on evidence that early patterning of the limb bud depends on a balance between the transcription factor dHAND in the posterior limb field and *Gli3* anteriorly. This was based in part on expression of either gene in mutant embryos as well as on ectopic expression experiments using

viral vectors. The results show that in dHAND mutant embryos of around E9.5 expression of both *Gli3* and *Alx4* shifts posteriorly in the limb bud, while in *Gli3* mutants dHAND expression shifts anteriorly. However, dHAND expression in *Alx4* (Lst) mutants is indistinguishable from that in wildtype embryos of similar stages, suggesting that dHAND functions upstream from *Alx4* and is required to exclude *Alx4* from posterior limb bud mesenchyme. Similarly, *Gli3* is unchanged in Lst embryos. Expression of *Alx4* in the Gli3 mutant Extra toes (Xt) was only partially downregulated, and especially at around E10.0 mostly in more distal positions. More recently the difference between *Alx4* and *Gli3* was further demonstrated by comparison of double knockout phenotypes involving mutated *Shh*. *Gli3*;*Shh* double mutants have a limb phenotype very similar to that of the *Gli3* single mutant (Litingtung et al., 2002; Te Welscher et al., 2002b). In contrast *Alx4*;*Shh* mutants were indistinguishable from the *Shh* mutant. To explain the surprising *Gli3*;*Shh* phenotype observed, it is important to know that a major function of *Shh* in the limb is to prevent the processing of the *Gli3* protein to form a repressor. Limb development including digit formation does not require *Gli3* or *Shh*, but antero-posterior patterning leading to unique identities of all digits requires the graded concentration of the *Gli3* repressor. In view of the double knockout phenotypes it appears that *Alx4* acts downstream from *Gli3*. The relation between *Gli3* and *Alx4* however remains incompletely understood because of the observation that *Alx4* is far from completely downregulated in *Gli3* mutants and in view of the very early expression of *Alx4* in the limb field; it seems likely that *Alx4* and *Gli3* function at least in part in different cascades. Since *Gli3* as well as compound mutants of the *Alx* group of genes tend to have defects of the pectoral and pelvic girdle, these genes may have overlapping functions in the patterning of these structures.

4. The Shox genes: *SHOX* and *Shox2*

4.1. Introduction: a gene less in mice

The human *SHOX* gene has been located at the "PAR1" (pseudoautosomal region I; Xp22 and Yp11.3). The PARs are the terminal regions shared by the sex chromosomes, which mediate the obligatory crossover during male meiosis (Rappold, 1993). The genetic and evolutionary stability of this region is apparently very low, in view of the major differences between relatively related species. Strikingly, none of the genes in the human PAR1 are found on the mouse X chromosome. Indeed, the *SHOX* gene has no homologue at all in the mouse genome. A strongly related gene, *SHOX2* is located on human chromosome 3 and a mouse *Shox2* gene is on a synthenic region of chromosome 3. Synonyms for *Shox2* include Prx3, Og-12, Og12X, and Shot; we prefer the name *Shox2* because it reflects the high structural similarity with *SHOX*. A potentially important feature of both *Shox* genes is that they encode through differential splicing, multiple RNAs and protein products. Differences in the proteins encoded sometimes include the presence

or absence of the functionally important OAR (aristaless-) domain which is expected to have significant impact on the activity of the gene product.

4.2. Expression patterns

Shox2 (rat and mouse) is expressed during mid-gestation in craniofacial primordia, notably in the medial nasal processes and in the proximal part of the mandibular arch. It is expressed in mesenchyme of the developing limbs in proximal regions with exclusion of the anterior part where *Alx4* is expressed and at higher levels dorsally than ventrally. In addition it is expressed in the spinal ganglia, in the sinus venosus and inflow tract of the heart. Finally, in the embryonic brain it is expressed widely in one dorsal thalamus, pretectum and tectum. In adult brain, expression is seen in nuclei that are part of the subcortical visual system (van Schaick et al., 1997; Blaschke et al., 1998; Semina et al., 1998).

Unlike most other human developmental genes, the embryonic expression patterns of *SHOX* and *SHOX2* have been studied (Clement-Jones et al., 2000). Expression of *SHOX* overlaps at least partially with that of Shox2, but is more restricted. It is seen in the middle part (with respect to the proximo–distal axis) of the limb bud (i.e. at Carnegie stage 14) and in the mandibular and hyoid arch. This fits very well with the regions where the most consistent aspects of *SHOX* deficiency are manifested (see Section 4.3). Unsurprisingly *SHOX2* expression in human embryos seems very similar to that of *Shox2* in rodent embryos.

4.3. Clinical importance of SHOX

Orthology between mouse and human *Shox2*/*SHOX2* is beyond any doubt, and the expression patterns of *SHOX* and *SHOX2* overlap at least partially. This would suggest that *SHOX* and *SHOX2* are genes with overlapping functions, fulfilled in the mouse by the unique *Shox2* gene. It is therefore remarkable that heterozygosity for *SHOX* is associated with severe morbidity (Rao et al., 1997; Kosho et al., 1999).

In a relatively short period *SHOX* has emerged as one of the major genes related to non-growth hormone deficiency related growth disorders. See for reviews Blaschke and Rappold, 2001, and Ogata et al., 2001. Short stature, usually associated with a number of other abnormalities including a range of skeletal anomalies affecting the limbs, heart and kidney defects, and gonadal dysgenesis characterize Turner syndrome. Although Turner syndrome patients may lack a larger part of the X-chromosome than the area containing the *SHOX* gene, it has become increasingly clear over the past five years that mutations in *SHOX* are the major or only factor underlying this syndrome. Several reports concern point mutation in *SHOX* that lead to Leri-Weill Dyschondrosteosis (LWS) (Belin et al., 1998), a defect including the Madelung deformity, a shortening and bowing of especially the zeugopodal bones' radius and tibia. Homozygous mutations are associated with Langer mesomelic dwarfism (MD), which is a much stronger form of basically the same defect, including ulnar and fibular dysplasia. Gain-of-function mutations, as in 47,XXX genotypes seem to be linked with tall stature. It is not

Table 1
Summary of proven and suspected functions of *Alx*/*Prx*/*Shox* subgroups

	Craniofacial		Limb		
	Nasal processes	Pharyngeal arches	Stylopod	Zeugopod	Autopod
Alx genes (*Alx3*, *Alx4*, *Cart1*)	++	+	−	+/−	++
Prx genes *Prx1*, *Prx2*	+	++	−	++	+
Shox genes					
SHOX	+	+	+	++	−
Shox2	++	+	++	+	−

entirely clear to what extent the impact of *SHOX* on growth can be attributed to the embryonic patterning effect and what is the impact of a later role in skeletal maturation during puberty (Ogata et al., 2001). The severity of the morbidity varies enormously between patients with apparently similar genetic defects (Schiller et al., 2000) and it is no exception that haploinsufficiency of *SHOX* leads to prenatal lethality, the stillborn fetuses having characteristic short, webbed neck and limb deformations.

4.4. Shox2

The *Shox2* gene has not yet been linked to a developmental function as no mouse mutants have been described. It is tempting to speculate that it has similar target genes as the *Alx* and *Prx* genes and could have functions in the proximal parts of the limb and mandibular arch. On the basis of genetic mapping data Human *SHOX2* has been proposed as a candidate gene for the "Cornelia de Lange syndrome gene" (Blaschke et al., 1998). This syndrome may involve micrognathia and other craniofacial abnormalities as well as limb defects. The linkage has however not been confirmed.

5. *Prx*, *Alx*, and *Shox* genes as a group

Prx, *Alx*, and *Shox* genes stand out as a group within the much larger and heterogeneous group of aristaless related genes. They are expressed in similar regions of the vertebrate embryo, but all three subgroups have their own characteristic expression and function. Table 1 summarizes the roles of these genes and is based on expression data complemented with results from mutant studies and where necessary some speculation.

While, all genes are expressed in the limb (-bud), each subgroup seems to have a different emphasis, and a similar specialization is seen in the craniofacial processes. Similarly all genes are expressed in the nasal processes and the first two

pharyngeal arches, but *Alx* genes are more prominent in the nasal processes, *Shox2* specifically in the medial nasal processes and proximal arches and *Prx* genes in the distal arch mesenchyme. As for the *Shox* gene(s) no animal model is available, the part of the table concerning these genes is rather speculative. A function of *Shox2* in stylopod is based on its proximal expression and may seem at odds with the zeugopodal defects seen in LWS and LMD patients. However, the expression of the SHOX genes in human embryos suggests that the pathology of the *SHOX* deficient patients may reflect only partially the impact of these genes, as *SHOX2* should well be able to partially compensate for the loss of *SHOX* function.

The similarity of the *Prx*/*Alx*/*Shox* proteins, and in particular of their homeodomains suggests that they control strongly overlapping sets of target genes and downstream pathways. Phenotypes of double mutants strongly suggest dosage effects of these genes, which implies that the homeodomain proteins encoded function as tools with qualitatively similar properties and that the details of their specific expression is decisive for their function. In this respect there might be some analogy between the way these aristaless-related genes function in proximo-distal patterning of various structure, and the way that the *Hox* genes pattern the antero-posterior body axis. In that case complex genetic analysis has also shown in a number of cases that qualitative differences between different genes, even from different "paralogy groups," is less important than previously anticipated (Zakany et al., 1996, 1997, Greer et al., 2000).

Analagously, the *Prx*, *Alx*, and *Shox* subgroups may, mostly by their different expression and to some extent by inherent qualitative differences, coordinately control antero-posterior patterning of protruding structures like limbs, cranio-facial primordial and the genital tubercle. The lack of any physical clustering of these genes shows that their presumably coordinated expression is organized in a different way.

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Hox gene control of neural crest cell, pharyngeal arch and craniofacial patterning

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Contents

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1. Introduction

The vertebrate head is an elaborate assemblage of cranial specializations, consisting of the central and peripheral nervous systems, viscero- and neurocranium, muscle, vasculature and connective tissues. Craniofacial development therefore is a complex process which involves the orchestrated spatio-temporal integration of multiple specialized tissues. Given the complex patterns of cell movement that occur during head development, how do the characteristic facial structures develop in the appropriate locations with their correct sizes and shapes. It is important to understand the mechanisms that control vertebrate head development, as craniofacial anomalies constitute nearly one-third of all human congenital defects. The vertebrate head is shaped by a particular group of cells, called neural crest cells, which are endowed with remarkable differentiative abilities. They are capable of generating diverse components of the head including the skeleton, teeth, peripheral nerves, sensory ganglia, connective tissue, dermis, and pigment cells, among many others. Neural crest cells are at the nexus of several signaling and inducing activities

that act to pattern the features of the cranium and face. This review discusses recent advances in our understanding the dynamic nature of the genetic and tissue interactions that occur during normal craniofacial development and in particular the patterning roles played by the *Hox* gene family of transcription factors.

2. Central nervous system development

Motor co-ordination, sensory perception, memory, learning and the various other diverse functions performed by the mature nervous system, all depend on the intricate network of nerve cell connections that form with exquisite precision during embryonic development. The formation of the vertebrate central nervous system occurs in several steps and is an extraordinarily complex and fascinating process (Figs. 1 and 2). The first step is known as neural induction and this takes place during the gastrulation (the formation of the mesoderm layer) phase of early embryonic development around 7.0–8.0 dpc (days post coitum). During neural induction, the neural plate is recruited from ectodermal cells that have yet to commit to a specific differentiation pathway and forms as a uniform sheet of neuronal progenitors. Neural induction is followed rapidly by neurulation (8.0–9.5 dpc), the process by which the two symmetrical halves of the flat neural plate curl up to form a hollow tube called the neural tube. The process of neurulation is accompanied by further regionalization of the neural tube dorso-ventrally into neural crest cells and various classes of neurons and anterio-posteriorly into the brain and spinal cord. In the final stages of the neurulation (8.5–9.5 dpc), neural crest cells and neurons then migrate from zones of cell proliferation to their final positions where they differentiate and extend connections to their target cells establishing much of the peripheral nervous system.

3. Induction and specification of the neural plate

The neural plate is a thickened columnar epithelial sheet that is derived from uncommitted ectoderm during gastrulation. Single ectoderm cells taken from gastrula stage *Xenopus* embryos and cultured in the absence of any additional factors will differentiate into neural tissue (Wilson and Hemmati-Brivanlou, 1995, 1997). This finding led to the proposal of a model for neural induction whereby ectodermal cells adopt a default neural state when removed from the influence of extracellular signals during gastrulation.

Hans Spemann and Hilde Mangold made the fundamental discovery in amphibian embryos that neural plate induction depended upon extracellular signals emanating from a specialized group of mesoderm cells underlying the prospective neural plate called the blastopore lip, or Spemann's organizer (Fig. 1A) (Spemann and Mangold, 1924). Transplantations of the dorsal blastopore lip to the ventral ectoderm of host amphibian embryos, a region that typically gives rise to epidermal tissues resulted in the formation of a duplicated

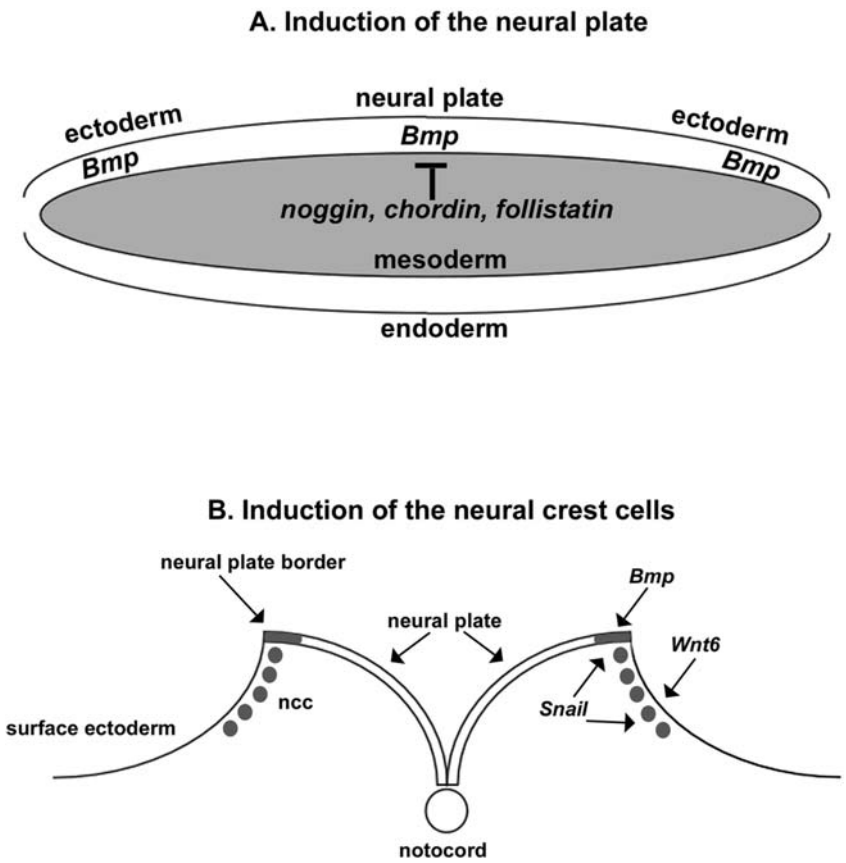

Fig. 1. Neurulation. (A) During the first phase of neurulation, naïve uncommitted ectoderm is induced to form neural plate tissue via BMP inhibitory signals (*noggin*, *chordin*, *follistatin*) secreted from the underlying mesoderm. (B) During the second phase of neurulation the two halves of the open neural plate begin to curl up to form a hollow neural tube. During this time neural crest cells (ncc), which express *Snail* are induced at the neural plate border and begin to migrate in response to *Wnt6* and *Bmp* expression in the surface ectoderm and dorsal neural tube respectively. (See Color Insert.)

body axis including an almost complete second nervous system. Organizer cells are the only cells that exhibit this neural plate inducing capacity. Decades later with the advent of molecular biology came the discovery in amphibian and avian embryos of three secreted factors, *noggin* (Lamb et al., 1993), *chordin* (Sasai et al., 1994) and *follistatin* (Hemmati-Brivanlou et al., 1994), which are expressed by the organizer. These secreted factors all have potent neural inducing activities in *Xenopus* ectodermal explants and each mimics the ability of the organizer to induce and pattern a secondary axis. *Noggin*, *chordin* and *follistatin* all mediate neural induction by binding to and inhibiting a subset of bone morphogenetic proteins (BMPs) (reviewed in Sasai and De Robertis, 1997). *Bmp4* is widely

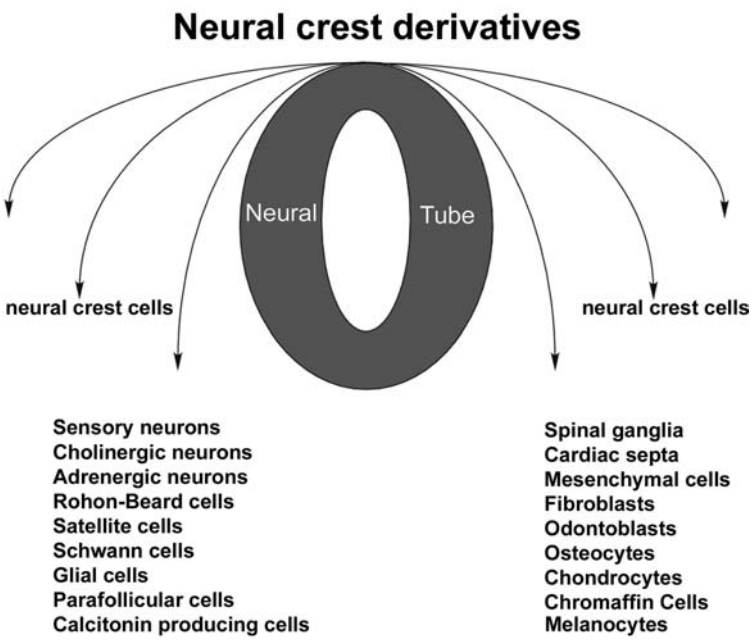

Fig. 2. Neural Crest Derivatives. The neural crest is a pluripotent migratory population derived from the dorsal neural tube that gives rise to an extraordinary number of diverse cell and tissue types. (See Color Insert.)

expressed in the ectoderm of *Xenopus* embryos, prior to neural induction (Dale et al., 1992), however, during gastrulation, *Bmp4* expression is repressed in the portion of the ectoderm fated to become the neural plate in response to signals from the organizer (Fainsod et al., 1994). Therefore, the inhibition of BMP signaling in the ectoderm during gastrulation represses epidermal fate and instead induces neural differentiation (Fig. 1A) (Lamb et al., 1993; Fainsod et al., 1994; Hemmati-Brivanlou and Melton, 1994; Sasai et al., 1994). The neural default model adequately accounts for the majority of experimental data obtained to date in amphibians, however difficulties arise when attempts are made to extrapolate this model to amniotes and mammals.

In chick embryos, although the BMP inhibitors *noggin* and *chordin* are expressed in the organizer (Henson's node), they fail to induce neural cell differentiation (Streit et al., 1998). In addition, the temporal expression of BMP inhibitors in chick embryos does not coincide with the induction of neural cells, suggesting that the roles of BMPs and their inhibitors in chick neurulation are not as clear-cut as that found in *Xenopus* (Streit and Stern, 1999). Furthermore gene-targeting experiments in mice have shown that normal neural differentiation occurs in the absence of BMP inhibitors (Matzuk et al., 1995; McMahon et al., 1998; Bachiller et al., 2000).

The differences between *Xenopus* and amniotes may reflect genuine differences in the embryonic morphologies of each species, however the avian organizer can

substitute for the blastopore lip of *Xenopus* in neural induction assays (Kintner and Dodd, 1991). It would seem unlikely that fundamentally distinct mechanisms for specifying neural fate at the molecular level have evolved in amniotes vs. anamniotes. The differences are more likely to have arisen from differences in the timing and nature of the assays employed but these results also imply that the organizer in avians and mammals produces additional neural inducers that are not BMP antagonists.

Recently in avian embryos, FGF signaling, emanating from cells that are the destined to form the organizer, has been shown to be an essential component in the avian neural induction process (Streit et al., 2000; Wilson et al., 2000). FGF8 can recapitulate the effects of the hypoblast or organizer precursor cells by inducing *ERNI* and *Sox3*, the earliest known markers of neural plate differentiation (Streit et al., 2000). Furthermore, inhibition of FGF8 signaling, using either dominant negative FGF receptors or SU5402 (an FGF-specific inhibitor), decreases the expression of these neural plate specific markers without affecting BMP signaling (Streit et al., 2000). FGF signaling alone however, is insufficient to induce *Sox2* or later neural markers, which implies that although FGF signaling plays a role in neural cell fate specification, other factors are necessary to complete the neural induction process (Wilson and Hemmati-Brivanlou, 1995; Harland, 2000; Streit et al., 2000; Wilson et al., 2000). These results raise the question of whether BMP signaling, which alone is also insufficient for neural induction, plays a combined role with FGF signaling.

Recent in vitro studies have shown that exposure of avian epiblast cells to BMP antagonists is sufficient to promote induction of neural cells when FGF signaling is attenuated (Wilson et al., 2000). Therefore, one possible role for FGF signaling is to attenuate BMP signaling in prospective neural cells. In support of this idea, the inhibition of FGF signaling using SU5042 results in the maintenance of *Bmp4* and *Bmp7* expression, which are normally downregulated in epiblast cells of prospective neural character. This implicates FGFs in the repression of BMP signaling and argues that the acquisition of neural cell fate requires the repression of *Bmp* expression, in contrast to epidermal cell fate which requires maintenance of *Bmp* expression. Thus, as in *Xenopus*, a reduction in BMP signaling coincides with neural induction.

Analysis of the role of Wnt signaling has further helped to clarify the distinct roles played by FGFs and BMPs during neural induction (Wilson et al., 2001). In avian embryos, high levels of Wnt signaling inhibit FGF signaling, allowing increased BMP expression, which in turn directs cells to an epidermal fate (Wilson et al., 2001). These results help to clarify why exposure to BMP antagonists alone is insufficient to induce avian neural differentiation. High levels of Wnt activity block the BMP-independent pathway of FGF transduction which is required for neural differentiation. Hence exposure to Wnt signaling is a key constraint for lateral ectoderm to differentiate into a neural fate.

Collectively, these results demonstrate that neural induction occurs prior to gastrulation in avian embryos, which is much earlier than previously thought (Streit et al., 2000). Furthermore, neural induction is more complex than a

simple neural default mechanism. Neural induction involves the interaction of at least three different gene families. FGFs, BMPs and their associated antagonists and Wnts, all appear to play distinct yet significant roles in the differentiation of neural vs. epidermal fate in the developing CNS. A key feature that remains conserved between frog and chick embryos, however, is the absence of BMP signaling in the prospective neural plate. It remains to be seen if the same is also true in the mouse.

4. Dorso-ventral regionalization of the neural tube: induction and specification of neural crest cells

Shortly after neural plate induction (7.0–8.0 dpc in mice), the process of neurulation begins. During neurulation (8.0–9.5 dpc), the two halves of the neural plate curl up to form a hollow neural tube and during this time the neural plate becomes regionalized dorso–ventrally (Figs. 1B and 2). The dorsal region of the neural tube gives birth to neural crest cells, whereas the ventral region gives rise to the floor plate and numerous distinct classes of neurons.

The neural crest is a pluripotent mesenchymal population that forms the neurons and glia of the peripheral nervous system, and as well as cartilage, bone, pigment cells, and connective tissue in addition to numerous other cell types (Fig. 2). Neural crest cells arise uniformly along almost the entire length of the vertebrate embryo neuraxis and were so named because of their formation at the crest of the closing neural folds (Marshall, 1879; Hall, 1999). This region corresponds to the junction between the non-neural ectoderm (presumptive epidermis or surface ectoderm) and the neural plate (neuroepithelium), a region commonly referred to as the neural plate border (Fig. 1B) (Couly and LeDouarin, 1988). Neural crest cell induction requires contact mediated interactions between the surface ectoderm and neuroepithelium and importantly, each of these tissues contributes to the neural crest cell lineage (Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 1995). Neural crest formation is intimately associated with induction of the neural plate and not surprisingly, many of the same signals, BMPs, FGFs, and Wnts have been implicated in neural crest induction.

Whereas high and low levels of BMP signaling define ectodermal and neural fates respectively, it has been proposed from work performed in *Xenopus* embryos, that an intermediate concentration of BMP induces neural crest cell formation (Marchant et al., 1998). The dynamic pattern of *Bmp4* expression is spatially and temporally consistent with playing a role in neural crest cell induction and migration in avian embryos. *Bmp4* and *Bmp7* are expressed in the surface ectoderm that abuts the open neural plate (Liem et al., 1995; Schultheiss et al., 1997). During neural tube closure, *Bmp4* expression is downregulated in the surface ectoderm, however, it continues to be expressed in the dorsal neural tube (Watanabe and Le Douarin, 1996). This data led to a model whereby BMP proteins, secreted by the surface ectoderm, were thought to act upon the neural plate to induce the formation and migration of neural crest cells. One problem with this model is that intermediate levels of BMP signaling

alone are incapable of inducing neural crest cells in explant cultures of avian neuroepithelium. Therefore, other factors in addition to BMPs are required for neural crest induction.

Recently it was demonstrated that *Wnt6* alone is both necessary and sufficient for inducing neural crest cells in explant cultures of avian neuroepithelium (Garcia-Castro et al., 2002). Conversely, blocking Wnt signaling both in cultured explants and in the whole avian embryos inhibited neural crest cell induction. *Wnt6* is expressed specifically in the surface ectoderm at the time of neural crest induction making it the prime candidate for the ectoderm signal responsible for neural crest formation (Fig. 1B). These new findings are consistent with neural crest phenotypes obtained by inhibition or overexpression of Wnt pathway components in *Xenopus* embryos (Knecht and Bronner-Fraser, 2002). It remains to be determined whether Wnts control BMP signaling or whether synergy between these two pathways is required for neural crest formation (Trainor and Krumlauf, 2002). These results however, demonstrate that BMP, Wnt and FGF signaling which all play critical roles in positioning both the border of the neural plate during gastrulation also play distinct roles later in inducing the formation of neural crest cells (Baker and Bronner-Fraser, 1997; LaBonne and Bronner-Fraser, 1999).

5. Neural crest cell delamination and migration

Concomitant with their induction along the dorsolateral edge of the neural plate, neural crest cells undergo an epithelial to mesenchymal transition and delaminate from the neural tube commencing their migration. The expression of *Slug* and *Snail*, members of the *Snail* family of zinc finger transcription repressors (Nieto et al., 1994; Sefton et al., 1998) is one of the earliest known indicators of neural crest cell formation (Fig. 1B) (LaBonne and Bronner-Fraser, 2000). Ectopic expression of *Snail* in epithelial cell lines causes the downregulation of *E-cadherin*, and a repression in epithelial to mesenchymal cell transformations and cell migration (Cano et al., 2000). Hence *Snail* promotes the epithelial to mesenchymal cell transitions associated with neural crest cell delamination and migration from the neural tube by effecting changes in cell adhesion (Fig. 1B). Further support for this idea comes from, *Slug* antisense mRNA oligonucleotide treatment of both avian (Nieto et al., 1994) and *Xenopus* (Carl et al., 1999) embryos, which results in the inhibition of cranial neural crest cell migration. BMP signaling has been shown to induce the expression of *Slug* and therefore also plays a role in neural crest delamination. This demonstrates that the same signal can fulfill multiple roles during development based on differences in temporal and spatial expression. More recent data implies that *Delta-Notch* signaling promotes *Bmp4* expression while at the same time inhibiting *Slug* expression, and therefore may effectively control delamination of neural crest at the neural–epidermal junction (Endo et al., 2002).

RhoB, which is a GTP-binding protein member of the ras superfamily may also play a role in neural crest cell delamination as it is selectively expressed in the dorsal neural tube and transiently expressed by the neural crest. *RhoB* expression is induced by

BMPs, and the inhibition of *RhoB* prevents neural crest delamination from dorsal epithelium (Liu and Jessell, 1998). The delamination and migration of neural crest cells from the neural tube is a transient event and the competence of the neural epithelium to generate neural crest cells appears to be maintained by *Noelin-1* (Barembaum et al., 2000). *Noelin-1* encodes a glycoprotein that is expressed along the lateral edges of the neural plate and later in migrating neural crest cells. The overexpression of *Noelin-1* in the neural tube results in prolonged neural crest production and migration. Hence, *Noelin-1* may maintain the period during which the neural epithelium is competent to generate neural crest cells (Barembaum et al., 2000).

6. Anterior–posterior regionalization of the neural tube

As neurulation proceeds, the anterior portion of the neural tube becomes partitioned via cell proliferation into a series of swellings and constrictions that define the major compartments of the adult brain: forebrain, midbrain, and hindbrain. The forebrain differentiates anteriorly into the telencephalon and posteriorly into the diencephalon. The telencephalon gives rise to the cerebral hemispheres, which regulate motor performance and memory as well as autonomic and endocrine responses. The diencephalon develops into the thalamic and hypothalamic brain regions, which processes information to the cortex and regulates autonomic, endocrine, and visceral functions, respectively. Similar to the forebrain, the hindbrain also becomes further regionally differentiated into the cerebellum, the medulla oblongata, and the pons. The cerebellum is the specific part of the brain responsible for co-ordinating movements, posture, and balance. The nerves of the medulla oblongata regulate respiratory, gastrointestinal, and cardiovascular movements. The pons conveys movement information from the cerebral hemispheres to the cerebellum. In contrast to the forebrain and hindbrain, the midbrain is not further divided, however, its function is to control many sensory and motor functions and co-ordinate visual and auditory reflexes. Posterior to the head, the simple trunk neural tube that extends towards the tail, ultimately develops into the spinal cord which controls the movement of and receives and processes sensory information from the skin, joints and muscles of the limbs, and trunk.

How does the neural plate become regionalized into the forebrain, midbrain, hindbrain, and spinal cord, since immediately following neural induction, the neural plate is assumed to have a uniformly anterior character. Regionalization of the anterior part of the neural tube is achieved through local organizing centers within the neuroepithelium itself, such as the isthmus, which is the junction between the midbrain and hindbrain (Marín and Puelles, 1995; Crossley et al., 1996a,b); and also the junction between the diencephalon and mesencephalon (Martinez and Alvarado-Mallart, 1990; Itasaki et al., 1991; Martinez et al., 1991). Recent evidence suggests that mechanisms by which the organizing centers regionalize the neural tube involves the convergent actions of graded concentrations of FGFs, RA, and Wnt signals to specify cells of forebrain, midbrain, and hindbrain character (Gavalas, 2002;

Nordstrom et al., 2002). Together, these findings support the concept of an activation–transformation model for the derivation of regionalized neural tissue from naïve embryonic ectoderm (Nieuwkoop, 1952; Toivonen and Saxen, 1968; Saxen and Kohonen, 1969; Nieuwkoop, 1985).

7. Hindbrain segmentation: the blueprint for craniofacial development

The hindbrain provides the essential blueprint or ground plan for establishing many of the characteristic features of craniofacial and central nervous system development (Fig. 3A) (Wilkinson et al., 1989a,b; Wilkinson and Krumlauf, 1990; Hunt and Krumlauf, 1991a; Couly et al., 1993; Kontges and Lumsden, 1996).

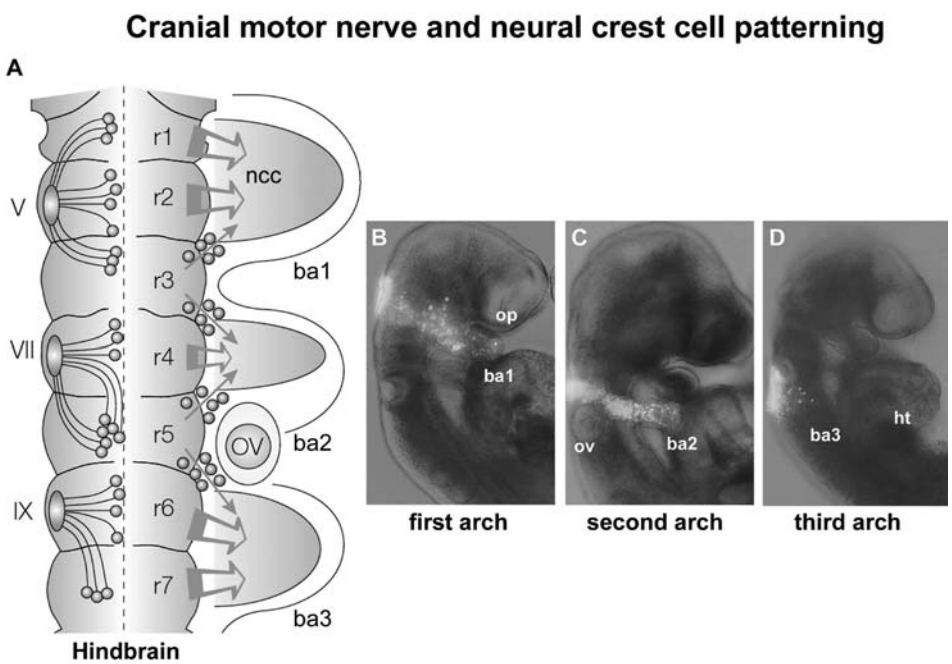

Fig. 3. Cranial Motor Nerve and Neural Crest Cell Patterning. (A). Schematic diagram highlighting that the segmental organization of the hindbrain into rhombomeres (r) exerts a profound influence on the spatial distribution of the cranial nerves (V, trigeminal, VII facial, VII glosso-pharyngeal) and the migration pathways of neural crest cells (ncc) into the branchial arches (ba1, ba2, ba3). Cranial motor nerves are derived from neurons (small red circles) born in multiple rhombomeres, however the axons project from the hindbrain only from exit points (large red circles) contained in the even numbered rhombomeres. Hindbrain derived neural crest cells (small green circles) migrate in streams adjacent to the even numbered rhombomeres (arrows), with clear neural crest free zones being observed adjacent to rhombomeres 3 and 5. (B–D). DiI lineage tracing of murine cranial neural crest cells which migrate ventro–laterally from the hindbrain in three segregated streams into the first, second, and third branchial arches (ba). Ht, heart; op, optic vesicle; ov, otic vesicle (adapted from Trainor et al., 2002). (See Color Insert.)

During neurulation, the hindbrain becomes transiently partitioned into seven cell lineage restricted compartments called rhombomeres (Vaage, 1969; Lumsden and Keynes, 1989; Fraser et al., 1990; Birgbauer and Fraser, 1994; Birgbauer et al., 1995). Morphologically, these transverse periodic bulges are distinguishable during mouse development by 9.0 dpc, although genetically they are identifiable much earlier. Each individual rhombomere gives rise to a unique well-defined region of the mature adult brain (Marín and Puelles, 1995; Wintgate and Lumsden, 1996), such as the cerebellum, for example, which is derived from rhombomere 1. The segmental organization of the hindbrain presages the periodic organization of the cranial motor nerves (Lumsden and Keynes, 1989; Clarke and Lumsden, 1993; Keynes and Krumlauf, 1994; Lumsden and Krumlauf, 1996; Clarke et al., 1998) and the segregated pathways of neural crest cell migration into the branchial arches (Fig. 3A) (Lumsden et al., 1991; Graham et al., 1993; Sechrist et al., 1993, 1994).

Retrograde tracing of motor axons revealed that the cell bodies of individual cranial nerves exhibit a precise relationship to specific rhombomeres (Lumsden and Keynes, 1989; Carpenter et al., 1993). The motor nerves innervating the first three branchial arches (V trigeminal, VII facio-acoustic, and IX glosso-pharyngeal) are respectively derived from nuclei confined primarily within rhombomeres 2, 4, and 6. The facio-acoustic and glossopharyngeal motor nerves are subsequently augmented by neurons developing in the caudally adjacent rhombomere. In contrast the trigeminal nerve is augmented by neurons born in the adjacent rostral and caudal rhombomeres. Although each nerve is derived from neurons born in multiple rhombomeres, the axons leave the hindbrain through exit points contained only within the even numbered rhombomeres and project to the branchial arches and peripheral tissues (Fig. 3A) (Lumsden and Keynes, 1989; Clarke and Lumsden, 1993). Therefore the segmental organization of the hindbrain clearly underpins the metameric pattern of cranial nerves (Keynes and Lumsden, 1990; Keynes and Krumlauf, 1994).

In addition to patterning the cranial nerves, the segmental organization of the hindbrain also plays a role in directing the migration of cranial neural crest cells (Fig. 3A–D) (Lumsden et al., 1991; Serbedzija et al., 1992; Sechrist et al., 1993). In vertebrate embryos, neural crest cells arise at the lateral edges of the neural plate along almost the entire neuraxis, at the junction between the neuroectoderm and ectoderm (Fig. 1B) (Selleck and Bronner-Fraser, 1995). In the mouse the first population of neural crest cells to emigrate from the neural tube do so from the caudal midbrain and rostral hindbrain at the 5–6 somite stage (8.25–8.5 dpc), long before closure of the neural tube (Chan and Tam, 1988). In contrast, the commencement of neural crest migration in the chick coincides with closure of the neural tube (Horstadius, 1950; Tosney, 1982; Le Douarin, 1983). Despite this difference, the migration patterns of neural crest cells in mouse and chick embryos are very similar and the duration of emigration from all axial levels typically lasts between 9 and 12 h (Serbedzija et al., 1992).

Cranial neural crest cells in both mouse and chick embryos migrate ventrolaterally, passing between the surface ectoderm and underlying mesoderm from the dorsal portion of the neural tube into the periphery of the face.

The hindbrain contributes the majority of cranial neural crest cells, which migrate in three, segregated, subectodermal streams lateral to rhombomeres r2, r4, and r6 (Fig. 3B–D). These neural crest streams migrate into the adjacent first, second, and third branchial arches respectively (Lumsden et al., 1991; Sechrist et al., 1993). The neural crest cells that contribute to the branchial arches give rise to a wide variety of cell lineages that are distinct for each branchial arch (Fig. 2) (Noden, 1983; Kontges and Lumsden, 1996; Le Douarin and Kalcheim, 1999). Although, almost the entire cranial neural tube generates neural crest cells, it appears that substantially less neural crest cells delaminate from rhombomeres (r) 3 and 5. In chick embryos but not in mice, this phenomenon is believed to be associated with odd numbered rhombomere specific apoptosis of neural crest precursors (Graham et al., 1993, 1994; Trainor et al., 2002b). Lineage tracing and recent timelapse analysis in numerous vertebrates, show however, that r3 and r5 do generate neural crest cells which ultimately contribute to the proximal most regions of the branchial arches, but rather than delaminating and migrating laterally like the rest of the hindbrain neural crest, odd-numbered rhombomere neural crest cells migrate both rostrally and caudally joining the even-numbered rhombomere streams (Fig. 3A) (Serbedzija et al., 1992; Sechrist et al., 1993; Osumi-Yamashita et al., 1994; Schilling and Kimmel, 1994; Trainor and Tam, 1995; Kulesa, 1998; Kulesa and Fraser, 2000; Trainor et al., 2002b). This implies that the paraxial environment adjacent to the neural tube influences the migration pathways of odd-numbered rhombomere derived neural crest cells (Farlie et al., 1999; Golding et al., 2002; Trainor et al., 2002b). A number of cues have been identified that guide neural crest cells, including molecules that provide a substrate for migration and others that define pathways by acting as inhibitors to migration (Bronner-Fraser, 1993; Robinson et al., 1997; Perris and Perissinotto, 2000). Recently, it was observed that a null mutation in the *neuregulin* receptor *ErbB4* causes the aberrant migration of neural crest cells through the mesenchyme adjacent to rhombomere 3. As a consequence of this aberrant migration, fusions of the cranial ganglia develop (Gassmann et al., 1995; Golding et al., 2000). *ErbB4* is expressed in rhombomeres 3 and 5 and hence it has been suggested that signals from the hindbrain mediated by *ErbB4* pattern the migration of cranial neural crest cells by preventing their migration through the mesenchyme adjacent to the odd-numbered rhombomeres. In contrast, the mechanisms that restrict cell mixing in *Xenopus* embryos involve the *Eph* family of receptor tyrosine kinases and their *Ephrin* ligands. These molecules are the key to guiding the segmental streams of branchial arch neural crest to their targets (Krull et al., 1997; Robinson et al., 1997) and it will be interesting to determine whether the same gene families are involved in patterning the migration pathways of avian and mammalian cranial neural crest cells. Other gene mutations, which also disrupt the segmental patterning of the hindbrain result in fusions of the cranial ganglia, branchiomotor defects, and abnormal patterns of cranial neural crest cell migration (Lufkin et al., 1991; Chisaka et al., 1992; Schneider-Maunoury et al., 1993; Swiatek and Gridley, 1993; Gassmann et al., 1995; Meyer and Birchmeier, 1995; Goddard et al., 1996; Studer et al., 1996; Manzanares et al., 1999; Gavalas et al., 2001).

Collectively these studies argue that the segmental streaming of neural crest cell migration into the branchial arches of the vertebrate head is controlled by a combination of hindbrain intrinsic factors and paraxial exclusion zones in the ectoderm and mesoderm which restrict the migration of neural crest cells through the territory adjacent to the odd numbered rhombomeres (Farlie et al., 1999; Golding et al., 2002; Trainor et al., 2002b). Thus, the segmental organization of the hindbrain into rhombomeric units provides the blueprint for patterning the craniofacial ganglia, the branchiomotor nerves and the pathways of neural crest cell migration into the branchial arches. The hindbrain establishes the foundations for head development and is a conserved strategy used by vertebrates to ensure proper craniofacial morphogenesis.

8. Formation and differentiation of the pharyngeal arches

The evolutionary transition from chordates to vertebrates involved a dramatic shift from sessile feeding to active predatory feeding. The development of jaws and a muscularized pharynx serving the dual roles of feeding and respiration was fundamental to this transition. The origin of the jaw and pharynx can be traced during embryonic development to a series of bulges called the pharyngeal arches, which develop on the ventrolateral surface of the head (Figs. 3 and 4A). The pharyngeal arches are composed of a number of distinct cell types. Each arch consists of a core of mesodermal cells surrounded by neural crest cells (Fig. 4B,C)

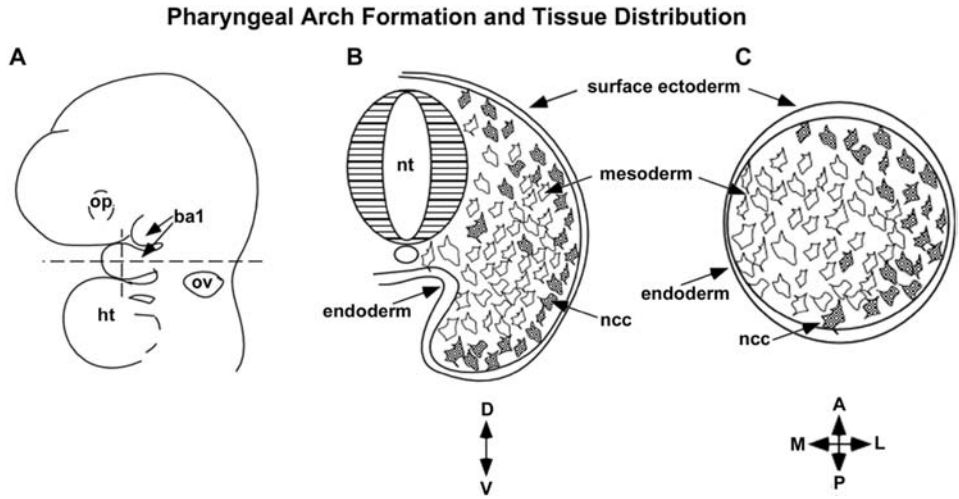

Fig. 4. Pharyngeal Arch Formation and Tissue Distribution. (A). Schematic representation of the reiterated series of branchial arches (ba) which form on the ventral medio–lateral (M–L) surface of the head. (B–C) Schematic dorso-ventral (D-V) and anterior–posterior (A–P) cross-section diagrams showing that each branchial arch is composed of a central core of mesoderm which is enveloped by neural crest cells (ncc). These two populations are then surrounded dorso–laterally by the surface ectoderm and ventro-medially by the endoderm (adapted from Trainor and Tam, 1995).

(Noden, 1986a, 1987; Trainor et al., 1994; Trainor and Tam, 1995). Collectively this arch mesenchyme is enveloped externally by the surface ectoderm and internally by the endoderm. Each of these tissues gives rise to distinct components of the vertebrate head. The mesoderm forms the musculature and endothelium as well as some cartilage (Noden, 1986a; Couly et al., 1992; Trainor et al., 1994), while the neural crest contributes to nervous, skeletal, and connective tissues (Noden, 1983; Couly et al., 1993; Kontges and Lumsden, 1996). The surface ectoderm provides the epidermis and sensory neurons of the epibranchial ganglia (D'Amico-Martel and Noden, 1983; Couly and Le Douarin, 1990) and the endoderm forms the epithelial lining of the pharynx in addition to the thyroid, parathyroid, and thymus (Le Douarin and Jotereau, 1975; Cordier and Haumont, 1980). The pharyngeal arches are separated by a series of ectodermal clefts and endodermal pouches and each pharyngeal arch gives rise to a specific and distinct part of vertebrate head. In all gnathostomes, the first arch forms the jaw, while the second arch differentiates into the hyoid apparatus or jaw support structures. The third and more posterior arches, the number of which varies amongst the vertebrates, gives rise to the gills in fish or are incorporated into the throat in birds and mammals. The generation of a distinct identity for each phayrngeal arch requires each component to be appropriately patterned.

9. Delineating rhombomeres: establishment of a gene expression programme in the hindbrain

The morphological appearance of hindbrain segments coincides with the restricted expression domains of numerous genes including transcription factors (*Krox20*, *kreisler*, *Hox*), signaling molecules (*Eph*/*ephrins*), membrane and nuclear receptors (*RAR*/*RXR*), and enzymes involved in the retinoid biosynthetic pathway. Some genes are expressed in single rhombomeres, or pairs of rhombomeres or even just at rhombomere boundaries, but all exhibit extremely dynamic patterns of activity (Fig. 5).

The zinc-finger transcription factor *Krox20*, is activated in rhombomere 3 (r3) as early as 8.0 dpc in the mouse, followed by expression in r5 by 8.75 dpc (Wilkinson et al., 1989a). Hence *Krox20* is expressed in a two segment periodicity and importantly its expression precedes the appearance of lineage restricted compartments in the hindbrain. By 9.5 dpc, *Krox20* transcripts begin to recede in r3, followed subsequently by r5, suggesting that this regulatory protein plays a highly dynamic role in hindbrain segmentation.

Kreisler, encodes a member of the Maf oncogenic family of basic domain-leucine zipper transcription factors and is one of the earliest markers of presumptive hindbrain tissue and therefore a prime candidate for controlling segmental patterning. *Kreisler* is initially expressed in the presumptive r5 territory at 8.0 dpc, followed by expression in r6 (Cordes and Barsh, 1994; Manzanares et al., 1999). Abundant expression is maintained until 9.0 dpc, but soon thereafter declines rapidly in both rhombomeres as segment boundaries are sharpened.

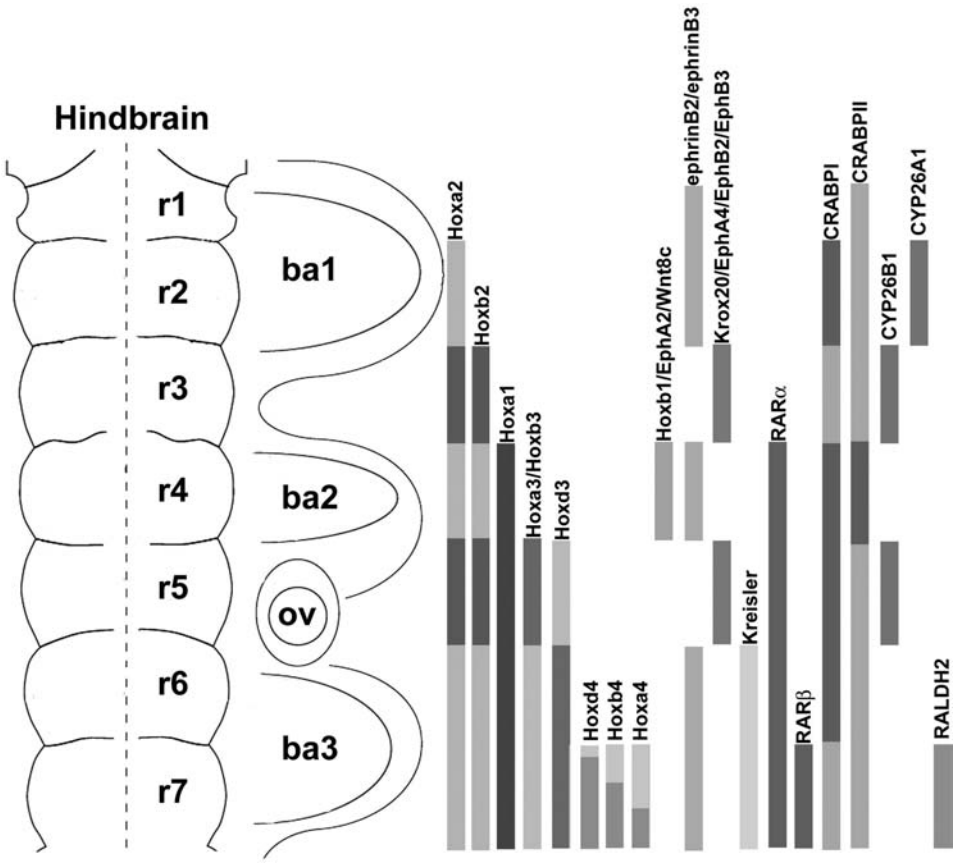

Fig. 5. Segmental Domains of Gene Expression in the Hindbrain. Schematic representation of the subdivision of the hindbrain into seven rhombomeres and their spatial relationship with the branchial arches (ba). The primary segmental domains of gene expression in the hindbrain during 8.5–9.5 dpc are often confined to individual rhombomeres, pairs of rhombomeres or alternating rhombomere segments. *Hoxa1* is not expressed after 7.5 dpc. Darker blocks of color indicate upregulation or higher relative levels of expression in an individual rhombomere. Ov, otic vesicle. (See Color Insert.)

Hox genes are among the earliest markers of presumptive hindbrain neuroepithelium. The *Hox* gene family comprises a set of transcription factors characterized by the presence of a DNA binding domain sequence motif called the homeobox (Krumlauf, 1992). The mouse genome contains 39 *Hox* genes organized into 4 distinct chromosomal clusters (*Hoxa–Hoxd*), which evolved via duplication and divergence from a single invertebrate *Hox* cluster. No single cluster contains members of all 13 paralagous groups presumably due to evolutionary gene loss (McGinnis and Krumlauf, 1992). The most striking feature of the organization of the *Hox* gene transcription factor family is that gene order within the complex is transposed directly in time and space via gene expression (Duboule and Dolle, 1989; Graham et al., 1989; Dollé et al., 1991; Kessel and Gruss, 1991; McGinnis and

Krumlauf, 1992). Genes located at the 3′ end of the cluster are expressed earlier and more anteriorly during development than those found at the 5′ end. Consequently, *Hox* genes exhibit nested domains of expression along the anterior–posterior axis and confer positional information to the embryo during development (Fig. 5).

Hox genes from paralogous groups 1–4 display dynamic patterns of expression in the developing hindbrain particularly in their anterior limits along the anterio-posterior axis (Krumlauf, 1993; Lumsden and Krumlauf, 1996). The group 1 *Hox* genes, *Hoxa1* and *Hoxb1* are activated in the neuroepithelium as early as 7.5 dpc and are initially expressed up to the r3/r4 boundary. While *Hoxb1* transcripts become restricted to r4, *Hox*a1 expression declines significantly by 8.5 dpc (Wilkinson et al., 1989b; Frohman et al., 1990; Hunt and Krumlauf, 1991b; Hunt et al., 1991b; Murphy and Hill, 1991). *Hoxa2* transcripts initially extend uniformly throughout the neuroeipthelium with an anterior limit at the r1/r2 boundary (Hunt et al., 1991c; Prince and Lumsden, 1994). This basal expression is shortly followed by an upregulation within r3 and r5 (Nonchev et al., 1996b). *Hoxb2* expression extends up to the r2/r3 boundary and is subsequently upregulated in r3, r4, and r5 by 8.5 dpc (Sham et al., 1993; Maconochie et al., 1997). Group 3 *Hox* genes are expressed more caudally than group 2 genes, with an anterior limit being set at the boundary between r4 and r5 (Wilkinson et al., 1989b; Hunt and Krumlauf, 1991b; Sham et al., 1992). As with the paralog group 2 *Hox* genes, group 3 *Hox* gene expression levels within individual rhombomerers in the hindbrain is variable. *Hoxa3* is upregulated in r5 and r6, while *Hoxb3* exhibits higher expression levels in r5 only. In contrast, *Hoxd3* exhibits lower expression levels in r5 yet *Hoxd3* transcripts are more abundant in the posterior neural tube. The group 4 *Hox* members *Hoxa4*, *Hoxb4*, and *Hoxd4*, are also expressed in the developing hindbrain, with an anterior limit being set at the r6/r7 boundary by 9.5 dpc (Gaunt et al., 1989; Geada et al., 1992; Morrison et al., 1997). The anterior limit of *Hoxc4* expression is slightly posterior to the other group 4 members, being set at the r7/r8 boundary. These gene expression analysis therefore indicate that *Hox* genes play integral roles in establishing the segmental identity of the hindbrain.

10. Regulation of *Hox* gene expression in the hindbrain

Hox genes exhibit dynamic expression patterns in the hindbrain and establishing these rhombomeric-restricted patterns involves conserved sets of genetic interactions. The expression domains of the *Hox* transcription factors within the hindbrain are initially diffuse, but they subsequently sharpen into specific segments as the rhombomeres develop (Fig. 5). Considerable effort therefore has been spent trying to identify the upstream regulatory factors that impose restricted domains of *Hox* gene expression in order to uncover the mechanisms governing the generation and specification of axial segments. The regulation of *Hox* genes in the hindbrain involves an initiation phase followed by a maintenance phase (Deschamps et al., 1999). Initiation involves the activation of *Hox* gene expression during gastrulation in the tissues ingressing through the primitive streak, including the presumptive

paraxial mesoderm and overlying nascent neuroepithelium in response to retinoic acid and FGF signaling. Maintenance refers to the fine-tuning and continuance of *Hox* gene expression within their characteristic rhombomeric boundaries and this is achieved through a combination of auto-, cross-, and para-regulation and direct regulation by segment specific genes such as *Krox20* and *kreisler*.

11. The role of retinoid signaling in *Hox* gene induction

The vitamin A metabolic derivative, retinoic acid (RA) is a key mediator of the initiation phase of *Hox* gene expression during hindbrain development (Gavalas and Krumlauf, 2000). Retinoids are enriched in the rostral part of the spinal cord immediately adjacent to the caudal hindbrain (Rossant et al., 1991; Hogan et al., 1992; Colbert et al., 1993; McCaffery and Drager, 1994; Horton and Maden, 1995; Maden et al., 1998). The anterior paraxial mesoderm (occipital and cervical somites) and overlying neurectoderm exhibit higher levels of retinoids than more posterior regions, which accurately reflects the expression of *Raldh2*, a major RA biosynthetic enzyme (Niederreither et al., 1997). Furthermore, the RA-catabolizing enzymes *Cyp26A1* and *Cyp26B1* are expressed in the anterior (r2) hindbrain and the anterior plus middle hindbrain regions (r2–r6), respectively (Fig. 5) (Fujii et al., 1997; MacLean et al., 2001). This presumably creates a gradient of RA diffusing from the rostral spinal cord into the caudal hindbrain. RA distribution in the hindbrain region is therefore consistent with it being a planar and lateral mesodermal signal that patterns the neurectoderm according to a concentration gradient.

Consistent with this idea, exogenous RA treatments during the late gastrulation phase of mouse embryo development rapidly induces *Hox* gene expression and causes anterior shifts in *Hox* gene expression along with anterior to posterior transformations of regional fate (Durston et al., 1989; Simeone et al., 1990; Conlon and Rossant, 1992). This response is co-linear, with the 3′-most *Hox* genes being induced earlier, more rapidly and at lower concentrations than genes more 5′ in each *Hox* cluster. Whereas RA treatment at 7.25–8.0 dpc rapidly induces 3′ genes such as *Hoxa1*, *Hoxb1*, and *Hoxa2* there is no corresponding response in the group 4 genes at the same stage. However, if RA is administered between 8.5 and 9.5 dpc the group 4 genes respond rapidly while the 3′ genes are no longer activated (Morrison et al., 1996, 1997). Interestingly, the most 5′ genes in each *Hox* cluster are refractory to excess RA, suggesting that retinoid signaling directly regulates only the *Hox* genes at the 3′ end of each cluster (Conlon and Rossant, 1992).

The retinoid signal is transduced primarily by two distinct ligand-activated transcription factors: the retinoic acid receptors (*RARs*) and retinoid receptors (*RXRs*) (Chambon, 1996). There are three distinct genes in each of the *RAR* and *RXR* gene families (α, β, and γ), all of which are activated by RA. However, not all the members of each gene family are expressed in the hindbrain. *RAR*α is expressed up to the r3/4 border while *RAR*β is expressed uniformly in the neural tube with an anterior limit at the r6/7 boundary (Fig. 5) (Ruberte et al., 1990; Mendelsohn et al., 1991; Ruberte et al., 1991a,b, 1992, 1993; Dollé et al., 1994; Mendelsohn et al., 1994).

RXRα and *RXRβ* are expressed uniformly throughout the entire hindbrain neuroepithelium (Dollé et al., 1994). In addition to the nuclear retinoic acid receptors, the mechanism of RA action is believed to involve cellular retinol and the retinol binding proteins CRBP1 and II, and CRABPI and II, respectively. *CRABP1* is expressed in rhombomeres 4–6 in the mouse hindbrain but also at lower levels in r2 (Fig. 5) (Dencker et al., 1991; Ruberte et al., 1991b; Maden et al., 1992; Lyn and Giguere, 1994). *CRABPII* is also expressed in the hindbrain and more posteriorly along the length of the neural tube (Ruberte et al., 1992; Lyn and Giguere, 1994). These proteins may control the availability of free vs. bound retinoids thereby regulating the regional concentration of RA along the AP axis (Mangelsdorf et al., 1995). Retinoids are enriched in the primitive streak of amniotes, consistent with a role in the activation of early-expressing *Hox* genes (Hogan et al., 1992; Maden et al., 1998).

Robust support for the function of retinoids in *Hox* gene activation comes from the discovery that the retinoid nuclear receptor family comprises sequence specific DNA binding proteins that form heteromeric complexes by recognizing direct repeat motifs called retinoic response elements (RAREs) located within target genes (Mangelsdorf and Evans, 1995; Mangelsdorf et al., 1995). A number of analyzes have identified numerous RAREs in the regulatory control regions of paralagous groups 1 and 4 *Hox* genes, enabling them to respond directly to retinoid signaling (Langston and Gudas, 1992; Moroni et al., 1993; Pöpperl and Featherstone, 1993; Marshall et al., 1994; Studer et al., 1994, 1998; Ogura and Evans, 1995a,b; Langston et al., 1997; Gould et al., 1998; Huang et al., 1998; Packer et al., 1998).

The best characterized RAREs are those associated with the group 1 paralogs *Hoxa1* and *Hoxb1*. In the *Hoxa1* gene a single RARE has been found and it is located within the 3′ regulatory region of the gene (Langston and Gudas, 1992). This enhancer is necessary for establishing *Hoxa1* expression in the neurectoderm up to the presumptive r3/r4 hindbrain boundary, and mediates the RA-inducibility of the gene (Langston and Gudas, 1992; Frasch et al., 1995). Consistent with this, mice lacking the *Hoxa1* 3′ RARE fail to initiate the rostral *Hoxa1* boundary and display reduced levels of *Hoxa1* transcripts (Dupé et al., 1997). Two RAREs have been identified in the 3′ flanking sequence of the *Hoxb1* gene (Fig. 6) (Marshall et al., 1994; Ogura and Evans, 1995b; Langston et al., 1997). These enhancers regulate *Hoxb1* expression during gastrulation and establish an anterior limit in the neuroepithelium that coincides with the r3/4 boundary. In addition another RARE has been identified in the 5′ flanking region of the *Hoxb1* gene which allows RA to make a second regulatory input into *Hoxb1* and it is essential for restricting *Hoxb1* expression to r4 by repressing its activity in r3 and r5 (Studer et al., 1994; Ogura and Evans, 1995b). Similar to the *Hoxa1* RARE, the targeted disruption of the 3′ *Hoxb1* RARE reveals its importance in establishing the early expression of *Hoxb1* in r4, and in directing robust expression levels of this gene (Studer et al., 1998). Although, *Hoxd1* is not expressed in the neuroectoderm of the mouse (Frohman and Martin, 1992; Kolm and Sive, 1995; Kolm et al., 1997), studies in *Xenopus* have shown that RA is

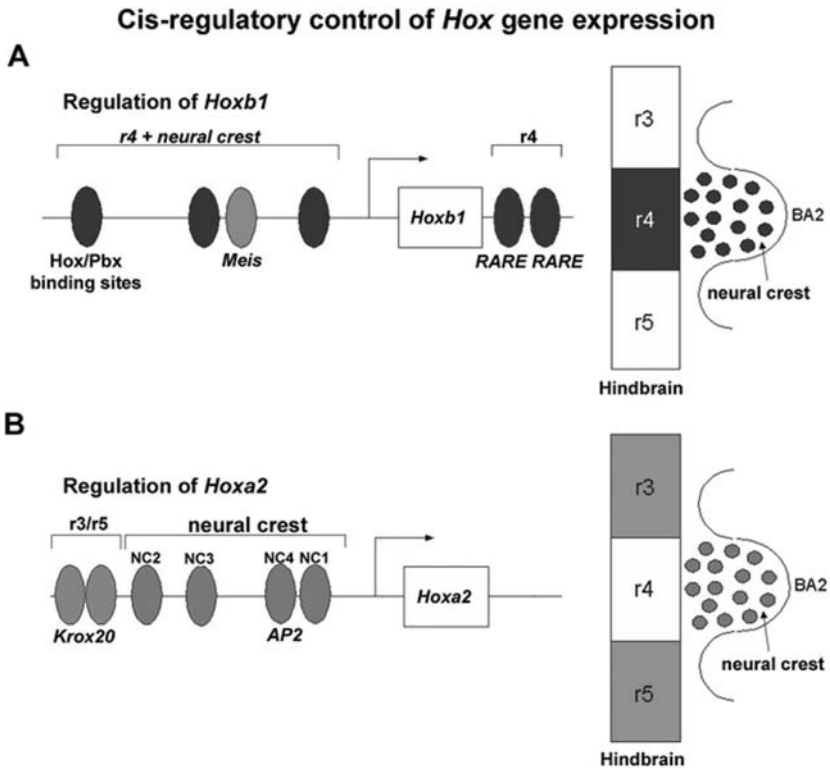

Fig. 6. Independent Regulation of *Hox* gene expression in the Neural Tube and Neural Crest Cells. (A) Schematic diagram of the regulatory modules directing *Hoxb1* expression in the hindbrain and branchial arch neural crest cells. Expression in rhombomere (r) 4 is dependent upon *Hox*/*Pbx* (dark blue circles) and *Meis* (light blue circles) binding sites in the 5′ regulatory region of the *Hoxb1* locus that mediate auto-, para-, and cross-regulatory interactions. Two retinoic acid reponse elements (RAREs; dark blue) in the 3′ flanking regulatory region of *Hoxb1* bind retinoic acid receptor heterodimers and are required to initiate early abundant expression of *Hoxb1* in the hindbrain. The *Hox*/*Pbx* sites also direct *Hoxb1* expression in the neural crest cells (small blue circles) emigrating from r4 into the second branchial arch (ba2). (B) Schematic representation of the essential elements regulating *Hoxa2* expression in the hindbrain and branchial arch neural crest cells. Two-conserved *Krox20* binding sites (green circles) direct *Hoxa2* expression in the odd-numbered rhombomeres (r3/5), while four neural crest-specific regulatory elements (NC1-NC4; red circles) synergize to promote *Hoxa2* expression in the second branchial arch neural crest cells (small red circles; BA2). (See Color Insert.)

involved in regulating *Hoxd1* (Kolm and Sive, 1995; Kolm et al., 1997). This indicates that all three vertebrate *Hox* group 1 members have RAREs that activate their early expression and that these may have been lost or mutated in *Hoxd1* of higher vertebrates such as mice.

Similar to the group 1 genes, RA signaling is clearly involved in regulating the expression of the group 4 paralogs, *Hoxa4*, *Hoxb4*, and *Hoxd4*. The initiation of *Hoxa4* expression in neuroectoderm up to the r6/7 boundary requires an RARE that responds to retinoid signals emanating from the cranial paraxial mesoderm

(Gould et al., 1998). Other similar RAREs have been described for the *Hoxa4* (Packer et al., 1998) and *Hoxd4* (Pöpperl and Featherstone, 1993; Morrison et al., 1996, 1997; Zhang et al., 1997, 2000) loci, which mediate RA-induction and establish the proper rostral expression boundaries of these genes. An interesting corollary to these observations is that no RAREs have been reported for the group 3 paralogous genes, which begin to be expressed in sharp rostral domains a full day later than groups 1, 2, and 4 (Manzanares et al., 2001).

Thus, RAREs convey the universal ability to drive early abundant expression of *Hox* genes within the neurectoderm. Moreover, they set the rostral limits of *Hox* gene expression and are thus able to interpret positional information supplied by posteriorizing signals from either the mesoderm or neural plate. Retinoid signaling therefore helps establish *Hox* gene co-linearity by directly activating the 3′ members of the complex within defined spatial-temporal domains.

12. The role of FGF signaling in *Hox* gene induction

Although, significant evidence exists for the role of RA in initiating the expression of the 3′ *Hox* genes, the mechanism underlying the regulation at the 5′end of the *Hox* clusters has remained elusive. Recent findings suggest that posteriorly-expressed *Hox* genes are regulated differently from the more anteriorly-expressed *Hox* genes within the neuroepithelium. The exogenous application of FGF (FGF2 or FGF4) anteriorizes the expression of several 5′ *HoxB* members (*Hoxb6–Hoxb9*) in the caudal hindbrain of avian embryos, where they are not normally expressed (Bel-Vialar et al., 2002). Furthermore, this effect appears to be mediated at least in part through *Cdx* transcription factors. In support of this idea, a dominant negative Cdx protein is able to block FGF-mediated *Hox* gene induction. *Cdx* genes are not expressed in the hindbrain, possibly accounting for the lack of *Hox* paralog groups 5 to 9 expression in this region (Gamer and Wright, 1993; Meyer and Gruss, 1993; Beck et al., 1995). The ectopic expression of an activated Cdx protein throughout the chick neuropeithelium is however able to drive *Hoxb9* expression up to the level of the otic vesicle, although this effect has to be promoted by exogenously supplied FGF (Bel-Vialar et al., 2002). Interestingly, the 5′ *Hoxb* genes are refractory to RA treatment in the chick neural tube, while the 3′-most *Hoxb* genes fail to respond to FGF signaling. This indicates that *Hox* gene complexes can be broadly classified into two regions with distinct regulatory mechanisms governing their expression: A retinoid-responsive domain encompassing *Hox* paralog groups 1 through 5 at the 3′end of the complex and an a FGF-sensitive domain that includes *Hox* groups 6 to 9 in the 5′ region of the cluster.

13. Auto-, cross-, and para-regulatory interactions control *Hox* gene expression

Auto-, cross-, and pararegulatory interactions are crucial for maintaining and controlling *Hox* gene expression in the hindbrain. This is particularly evident for

Hoxb1 which, after its induction by RA, maintains high levels of expression in r4 (Wilkinson et al., 1989b; Murphy and Hill, 1991). This is achieved via a conserved autoregulatory loop involving three related sequence motifs located 5′ to the *Hoxb1* gene (Fig. 6) (Pöpperl et al., 1995). These sequence motifs represent a bipartite recognition element comprised of overlapping sites for *Hoxb1* and for a mouse homologue of the *Exd* homeobox gene, *Pbx*. *Hoxb1* together with mouse Pbx/Meis proteins are essential for r4 specific expression.

Despite similar domains of expression between various *Hox* gene paralogs, there is frequent variation in their relative levels within specific segments. For example, *Hoxb2* is upregulated in r4, but in contrast *Hoxa2* is not. This type of differential expression indicates that these two genes have distinct modes of regulation. Transgenic deletion analysis of the 5′ flanking region of *Hoxb2* identified a 181 bp element capable of mediating the upregulation of *Hoxb2* expression in r4. Although, this element contained no consensus RARE, a single motif was identified that exhibited high similarity to the *Hox*/Pbx autoregulatory motifs identified in the *Hoxb1* locus (Maconochie et al., 1997). Consequently, it was demonstrated in vitro, that *Hoxb1* binds to the *Hoxb2* motif. Deletions of the *Pbx*/*Hox* site in the *Hoxb2* enhancer confirmed that it is required for normal in vivo r4 activity. Furthermore, it was shown that the *Hoxb2* motif can distinguish between *Hoxb2* and group 1 proteins indicating that r4-specific expression of *Hoxb2* is not a consequence of its own auto-regulation, but results from cross-regulatory interactions with group 1 genes (Maconochie et al., 1997).

Further evidence for the importance of *Hox* auto- and cross-regulation has come from analyzes of the *cis*-acting regions of the group 4 paralogs (Gould et al., 1997, 1998). Enhancers capable of establishing the r6/r7 anterior limits of *Hoxb4* and *Hoxd4* neural expression have been found in the 3′ flanking sequences of these genes (Whiting et al., 1991; Aparicio et al., 1995; Marrison et al., 1995, 1997 Gould et al., 1997, 1998). The neural enhancer of the *Hoxb4* gene sits adjacent to a distal promoter of the *Hoxb3* gene and this *cis*-element is shared by the *Hoxb4* and *Hoxb3* genes. A highly conserved region (CR3) identified from sequence comparisons of pufferfish, chicken, and mouse is alone capable of directing expression with a sharp r6/r7 anterior boundary. Mutations in CR3 abolish the normal expression patterns (Gould, 1997) suggesting that the autoregulation of *Hoxb4* and *Hoxd4* is therefore mediated through this fragment. Hence in a manner analogous to *Hoxb1*, RA signaling directly establishes an early and transient r6/r7 domain of *Hoxb4* expression that directly triggers the *Hoxb4* autoregulatory loop (Gould et al., 1998). This then maintains the proper domains of expression in later stages of embryonic development. *Hoxa4* regulation differs slightly from that of other group 4 paralogs (Behringer et al., 1993). A 3′ enhancer directs neural expression with an r6/r7 anterior boundary but only at later stages. There is a 5′ enhancer that also mediates r6/r7 expression, but it does so at earlier stages, suggesting dual control of *Hoxa4* expression in the hindbrain.

The *Hoxa3* and *Hoxb3* genes exhibit subtle differences in the regulation of their segmental expression in the hindbrain up to the r4/5 boundary. For *Hoxa3*, an auto-regulatory element consisting of two *Hox*/*Pbx* binding sites seems to mediate its

expression in the neuroectoderm, in agreement with its relatively late initiation during development (Manzanares et al., 2001). Similary, *Hoxb3* expression in the neural tube also utilizes an auto-regulatory element containing two *Hox* binding sites. However, in vitro, both *Hoxb3* and *Hoxb4* can interact with these binding sites indicating that *Hoxb3* also uses cross-regulation for the establishment and maintenance of its segmental expression in the hindbrain (Kwan et al., 2001).

Based on the extraordinary volume of information that has been generated from the *cis*-regulatory control of *Hox* gene expression, it is now possible to integrate and model the dynamic expression patterns and interactions in silico which has important implications for predicting the outcomes of disruptions to the *Hox* gene signaling network (Kastner et al., 2002). The above studies highlight the general importance of auto-, cross-, and para-regulatory mechanisms for the functional maintenance of *Hox* gene expression during vertebrate hindbrain development. It also demonstrates how changing the expression of one *Hox* gene can be translated to changes in other *Hox* genes. The fact that some elements are shared has important implications for maintaining the *Hox* complexes themselves. Removing these genes or regions could alter control regions in a particular complex and hence they would be required for proper regulation of multiple genes. This would provide a basis for keeping genes clustered in order to maintain appropriate expression patterns and this necessity is highlighted in the analysis of null mutations in *Hox* genes detailed below.

14. The role of *Krox20* and *kreisler* in regulating *Hox* gene expression in the hindbrain

Krox20 was considered a prime candidate for regulating *Hox* genes by virtue of its early and segmentally restricted expression in r3 and r5 and the observation that group 2 *Hox* genes are upregulated in these odd numbered rhombomeres during hindbrain development. The pivotal regulatory roles of *Krox20* in hindbrain segmentation have now been clearly demonstrated by transgenic analysis in mice. Three *Krox20* binding sites were identified within the 5′ flanking region of the *Hoxb2* gene (Sham et al., 1993). Similarly two *Krox20* binding sites were found in the 5′ flanking region of the *Hoxa2* gene (Fig. 6) (Nonchev et al., 1996a,b). The *Krox20* sites alone are insufficient to induce r3 and r5 specific expression of *Hoxa2* and *Hoxb2* indicating that other as yet unclassified *cis*-regulatory elements are required. However, the *Krox20* binding sites are essential for the upregulation of *Hoxa2* and *Hoxb2* in r3 and r5 and indicate that *Hoxa2* and *Hoxb2* are direct targets of *Krox20*. *Krox20* is also required for *Hoxb3* enhancer activity in r5, demonstrating that this factor functions upstream in the *Hox* regulatory cascade (Manzanares et al., 2002). Thus, the neuroepithelial expression of paralog groups 1 to 3 depend on a dynamic interplay of mutual cross-regulatory interactions, with key regulatory inputs from *Krox20* in r3 and r5.

The cloning of the *kreisler* gene and analysis revealing its expression is restricted to r5 and r6 suggests that it may also play a direct role in the transcriptional regulation of *Hox* genes during hindbrain development (Cordes and Barsh, 1994). Transgenic analysis have revealed that both *Hoxa3* and *Hoxb3* are directly

regulated by *kreisler* (Manzanares et al., 1997, 1999). Two binding sites in the 5′ flanking region of *Hoxb3* are necessary and sufficient for a *kreisler* response. However, these sites mediate expression in r5 only and not in r6 indicating that additional factors probably serve to restrict the expression of *Hoxb3*. Further deletion and mutation analysis identified a second cis-regulatory element that corresponded to an activation site for *Ets*-related transcription factors and which is necessary to potentiate and restrict *kreisler* enhancer activity to r5 (Manzanares et al., 1997). In contrast to *Hoxb3*, *kreisler* directly regulates the expression of *Hoxa3* in both r5 and r6 through the presence of a unique *kreisler* binding site in the 5′ flanking region of *Hoxa3*. Mutations in this site abolish its activity. Hence *Hoxa3* and *Hoxb3* are direct targets of *kreisler*, but they are regulated distinctly in the hindbrain.

Collectively these results indicate that there are intricate, interactive loops between the *Hox* genes, *Krox20* and *kreisler* in the developing hindbrain, all of which are crucial for the control of the segmentation and specification process.

15. Mechanisms for hindbrain segmentation: generating cell lineage restrictions and discrete domains of *Hox* gene expression

Segmentation and the formation of compartments is an integral component of embryonic development. The hindbrain is composed of seven transient rhombomeres that constitute cell lineage restricted territories and each territory exhibits sharp restricted domains of *Hox* gene expression (Fig. 5). Classical cell transplantation experiments performed in avian embryos have demonstrated that cells within one hindbrain compartment are unable to mix with those from adjacent rhombomeres (Guthrie and Lumsden, 1991; Guthrie et al., 1993). Moreover, the differential miscibility or adhesive properties between rhombomeres display a two-segment periodicity. Odd numbered rhombomere cells are able to mix with other odd numbered rhombomere cells but not with adjacent even numbered rhombomere cells. This compartment-specific cellular restriction constitutes the basis of boundary formation in the hindbrain and led to the hypothesis that a hierarchy of cell adhesion molecules could facilitate cell segregation (Wizenmann and Lumsden, 1997). Gene expression boundaries during early hindbrain development are generally diffuse, however concomitant with the generation of rhombomere boundaries, these expression domains are refined and sharpened (Fig. 7). Therefore, the restriction of intermingling between rhombomeres in the developing hindbrain is crucial for the establishment and maintenance of segment identity, both morphologically and genetically.

The best candidates for being molecular mediators of this phenomenon are the *Eph* single pass tyrosine kinase transmembrane receptors and their membrane bound *ephrin* ligands (Wilkinson, 2001; Cooke and Moens, 2002). There are two classes of *Eph* receptors. In general the *EphA* receptors only interact with glycosyl phosphatidylinositol (GPI) linked *ephrinA* ligands while *EphB* receptors only interact with transmembrane bound *ephrinB* ligands (Gale et al., 1996). Little promiscuity

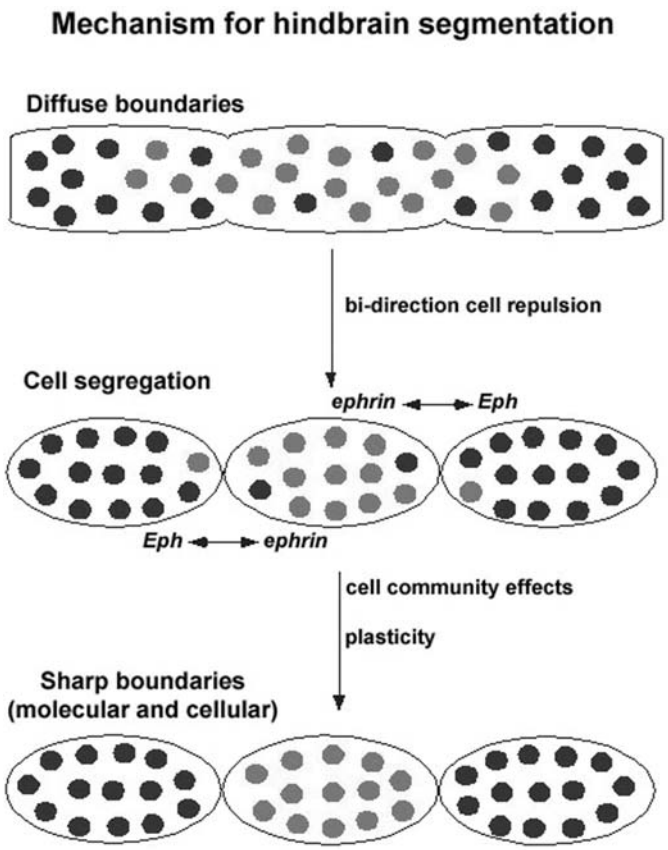

Fig. 7. Mechanism for Hindbrain Segmentation. Hindbrain segmentation and the generation of sharp domains of gene expression is a two step process. Initial gene expression boundaries in the hindbrain are diffuse and bi-directional repulsive signaling mediated the *Eph*/*ephrin* gene families leads to a sorting of cells based on appropriate gene expression. Concomitant with the morphological formation of rhombomere boundaries, cells isolated on the wrong side of the border exhibit plasticity in their gene expression patterns in response to cell community signaling effects. Together this leads to the formation of the sharp molecular and cellular boundaries that are characteristic of vertebrate hindbrain development.(See Color Insert.)

between these groups has been observed, however *EphA4* has been found to bind *ephrinB2* and *ephrinB3* in addition to *ephrinA* ligands (Klein, 1999). Several members of the *Eph* receptor and *ephrin* ligand families are expressed in alternating stripes in the hindbrain (Fig. 5). In particular, *EphA4*, *EphB2*, and *EphB3* are expressed in r3 and r5, co-localizing with *Krox20* transcripts (Flenniken et al., 1996). With the exception of *EphA2*, which is expressed in r4, hindbrain expression of the *Eph* receptors is restricted to odd-numbered rhombomeres. In contrast, *ephrin* transcripts are usually found within even-numbered segments. For example, *ephrinB2*, an *EphA4*-specific ligand, is expressed in rhombomeres 2, 4, and 6, in a manner

strikingly complimentary to that of *EphA4* (Flenniken et al., 1996). As a general rule, the *Eph* receptors and their *ephrin* ligands are expressed in alternating segments which is consistent with a role in defining boundary interfaces within the hindbrain. Recent experiments confirm that *Eph* receptors are required for the segmental restriction of cell intermingling during hindbrain development (Xu et al., 1995, 1999). The mosaic activation of *Eph* receptors such as *EphA4* in rhombomeres 3 and 5 of the hindbrain leads to the sorting of cells to the boundaries of the odd rhombomeres. Conversely the mosaic activation of *ephrins* such as *ephrinB2* in the hindbrain results in the sorting of cells to the boundaries of even rhombomeres. The demonstration that activation of either *Eph* receptors or *ephrin* ligands alters cell sorting properties, indicates that bi-directional signaling at rhombomere boundaries restricts cell intermingling between adjacent segments (Mellitzer et al., 1999).

The cell repulsion that generates the morphological boundaries within the hindbrain correlates with the refinement and sharpening of the initially diffuse *Hox* gene expression domains (Fig. 7). Classically it was believed that there was a direct correlation between the commitment to rhombomere specific fates and the restricted domains of *Hox* gene expression in the hindbrain. This is underscored by numerous gain and loss of function analyzes in several vertebrates which have highlighted the functional importance of the *Hox* genes during hindbrain development. Not surprisingly, rhombomeres transplantated to ectopic locations within the hindbrain generally display molecular and cellular autonomy (Guthrie et al., 1992). In contrast, grafts of neural plate stage tissue acquire the *Hox* identity and neuroanatomy characteristic of their new location (Grapin-Botton et al., 1995). This suggests that during hindbrain segmentation there is a progression towards rhombomere autonomy which occurs concomitantly with the restrictions in cell mixing and the refinement of *Hox* gene expression domains.

Recent analysis have uncovered the mechanistic link between the restrictions in cell movement and the sharpening of gene expression domains which generate segment identity (Figs. 5 and 7). Although current models argue for autonomy of *Hox* gene expression in developing rhombomeres, these analysis were all performed in avian embryos and involved the manipulation of large blocks of tissue. Inter-rhombomeric signaling and cell-community effects could mask any plasticity normally exhibited by individual rhombomere cells. The development of techniques for transplanting small groups of genetically marked cells within the mouse hindbrain overcame this obstacle with surprising results by uncovering a considerable degree of plasticity with respect to patterns of *Hox* gene expression in rhombomeric cells. Small groups of genetically marked cells were isolated from rhombomeres 3, 4, or 5 and heteroptically transplanted into rhombomere 2 (Trainor and Krumlauf, 2000a). The majority of the transplanted cells remained as a cohort in their new location and maintained their original anterior–posterior identity in a cell-autonomous manner as evidenced by their continued characteristic *Hox* gene expression. However, a number of cells, which became separated and dispersed from the primary graft exhibited clear cellular plasticity by failing to maintain their appropriate *Hox* gene expression patterns. Therefore at the level of individual rhombomeric cells, there is an inherent plasticity with respect to *Hox* gene expression

and cell fate (Trainor and Krumlauf, 2000b). The fact that rhombomeric autonomy is maintained in cells that remain as a cohort but not in cells that intermingle with surrounding populations indicates that cell-community effects are important for reinforcing regional identity and has important implications for the generation of sharp segmental rhombomere boundaries of *Hox* gene expression during normal hindbrain development.

The mechanism for generating hindbrain segmentation and establishing *Hox* gene expression domains is therefore a two-step process (Fig. 7) (Trainor and Krumlauf, 2000a,b). First, repulsive signaling between the *Eph* receptors and their *ephrin* ligands serve to generate the distinct rhombomeric territories as units of cell lineage restriction. Second, the plasticity uncovered in recent analysis at the level of the individual hindbrain cells indicates that simultaneously, as rhombomere boundaries are forming, dispersed cells caught on the wrong side of the border change their fate. Together, the two processes of cell repulsion and community induced cellular plasticity provide a mechanism for the progressive generation of precise rhombomeric domains of gene expression in the hindbrain. The morphological and molecular segmentaion of the hindbrain subsequently functions to maintain the appropriate anterior–posterior register between the neural tube, neural crest, and branchial arches, which underscores the functional importance of the blueprint provided by the hindbrain during craniofacial morphogenesis.

16. Mutational analysis of retinoid, *Krox20*, *kreisler*, and *Hox* gene function during hindbrain and craniofacial development

Retinoid signaling mediates the nested expression of *Hox* genes during hindbrain development and it has the potential to modify the anterior–posterior character of the central nervous system. Altering the retinoid gradient therefore should perturb hindbrain patterning in a predictable manner. Indeed, the loss of RA signaling leads to an anteriorization of the hindbrain. The null mutation of the major RA biosynthetic enzyme, *Raldh2*, results in the downregulation or absence of the rostral expression domains of several 3′ *Hox* genes such as *Hoxa1*, *Hoxb1*, *Hoxa3*, and *Hoxb3* (Niederreither et al., 1999, 2000). In the case of *Hoxd4*, its expression is abolished altogether. Since *Hoxd4* is the most caudally expressed *Hox* gene in the hindbrain, its absence is consistent with a role for retinoid signaling in patterning the molecular identity of the posterior hindbrain.

Similarly, avians and rodents raised on a vitamin A deficient diet (VAD) exhibit equivalent disruptions to hindbrain development as assessed by the absence or reduced expression of caudal rhombomeric markers (Maden et al., 1996; Gale et al., 1999; White et al., 2000). Importantly, the *Hox* genes consistently downregulated in these experiments include groups 1, 2, and 4, which is indicative of the fact that they are directly regulated by retinoids (Dupé et al., 1999; Wendling et al., 2001). Furthermore, treating mouse and avian embryos with increasing levels of the retinoid antagonist BMS493 leads to a progressive reduction of posterior rhombomeres along with an enlargement of anterior rhombomeres (Dupé and

Lumsden, 2001; Wendling et al., 2001). Blocking retinoid signaling therefore results in a caudal truncation of the hindbrain, with hindbrain disruptions being progressively more severe the earlier the treatment. In the most extreme form of retinoid deprivation elicited by antagonist treatments, the caudal hindbrain becomes truncated below r4, while the remaining rhombomeres are expanded.

Interestingly, in RAR$\alpha\gamma$ double mutants, the disruptions in hindbrain patterning, are equivalent in severity to those observed in Raldh2 null mutant and VAD embryos. In contrast, RAR$\alpha\beta$ double mutants present a milder loss of posterior rhombomeres, whereby double mutants lack rhombomeres caudal to r6 and have an enlarged r5 (Dupé et al., 1999; Wendling et al., 2001). This indicates that an intermediate degree of retinoid signaling remains during the development of these mutants.

As the absence of retinoid signaling leads to an anteriorization of the hindbrain, increases in retinoid signaling should therefore lead to a posteriorization of the hindbrain. Predictably, inactivation of *Cyp26A1* in the mouse, a RA-catabolizing enzyme expressed in r2, leads to a mild posterior transformation of the anterior hindbrain. Concomitant with a rostral expansion of *Hoxb1* are enhanced levels of *Hoxb1* expression in r4 (Abu-Abed et al., 2001; Sakai et al., 2001).

Collectively, the mutant analysis demonstrate that the developmental role of retinoid signaling is consistent with patterning the hindbrain during embryogenesis. Blocking retinoid signaling leads to a loss of the caudal hindbrain and a respecification of the remaining rhombomeres to a more anterior identity. Conversely, overexpression of retinoid signaling results in a posteriorization of the hindbrain. Hence, there is a sharp gradient of RA patterning within the hindbrain along the anteroposterior axis, with greater levels of retinoids being required to instruct more posterior fates (Gavalas and Krumlauf, 2000). As retinoids are enriched in the neural tube throughout development, RA signaling may therefore continually provide inputs into *Hox* gene regulation in the hindbrain, long after their initiation.

Krox20 plays a pivotal role in hindbrain segmentation. The targeted deletion *Krox20* gene results in fusions of the trigeminal ganglion with the facial and vestibular ganglia as a consequence of a profound perturbation of hindbrain development. There is a progressive loss of r3 and r5 and their derivatives are subsequently eliminated, which demonstrates the importance of this factor in the maintenance of segment identity (Swiatek and Gridley, 1993; Schneider-Maunoury et al., 1993, 1997). *Krox20* function is essential for *Hox* expression within r3 and r5 and consistent with the interactions unveiled by transgenic analysis, there is no upregulation of *Hoxa2*, *Hoxb2*, *Hoxb3*, or *EphA4* in the *Krox20* null mutants. *Krox20*$-/-$ hindbrains also display a reduction of *Hoxb1* in presumptive r4 (Seitanidou et al., 1997; Manzanares et al., 2002).

Similar to *Krox20*, *kreisler* also plays a crucial role during hindbrain development. The classical mutant *kreisler* was identified by virtue of its circling behavior (Deol, 1964). *Kreisler* mutants exhibit inner ear abnormalities as well as defects in neural crest derived skeletal elements such as the hyoid (Deol, 1964; Frohman et al., 1993; McKay et al., 1994, 1997). *Kreisler* is not a null allele. It is a mutation affecting the

regulatory elements responsible for its r5 and r6 domains of expression in the hindbrain and consequently, the hindbrain in *kreisler* mutant embryos is unsegmented posterior to the r3/r4 boundary (Frohman et al., 1993; Cordes and Barsh, 1994; McKay et al., 1994, 1997). Analysis of the expression patterns of *EphA7* and *ephrinB2* indicate that the segmentation defect in *kreisler* mutants is a specific loss of r5. The normal expression domains of *Krox20*, *Hoxa2*, *Hoxb2*, *Hoxb3*, and *Hoxb4* in r5 are absent. Although r6 is formed, it is not properly maintained since the upregulation of *Hoxa3* in r6 is missing (Manzanares et al., 1999). *Kreisler* therefore, similar to *Krox20*, regulates multiple steps during hindbrain development, including the proper formation of r5 and the regulation of the segmental identity of r5 and r6. Hence *kreisler* is a true segmentation gene.

Classical genetic experiments in *Drosophila* identified homeobox genes as the prototypical determinants of body segmentation in animal development (Scott and Carroll, 1987; Carroll, 1995). Based on the co-linear expression of vertebrate homeobox genes in the developing hindbrain and branchial arches, it was hypothesized that the mammalian versions of these genes also function as homeotic selectors, conferring segment identity. This idea has been extensively tested in the mouse via mutational analysis of the *Hox* genes. Two distinct *Hoxa1* null alleles have been generated and both types of homozygous mutants display severe defects in inner ear development along with the loss of facial and abducens nerve motor neurons (Lufkin et al., 1991; Chisaka et al., 1992; Dollé et al., 1993; Mark et al., 1993). Defects in the early hindbrain phenotype differs slightly between the two alleles, ranging from a reduction in the r4 and r5 territories (Lufkin et al., 1991; Dollé et al., 1993; Mark et al., 1993) to a complete absence of presumptive r5, as assayed by the reduced expression of *Hoxb1* in r4 and *fgf3* and *Krox20* in r5 (Chisaka et al., 1992; Carpenter et al., 1993). Thus, *Hoxa1* is required for the formation of the r4/r5 territory, which accounts for the aberrant development of the neural crest-derived cranial nerves originating from this region. Interestingly, the loss of function of *Hoxa1* results in the presence of ectopic r2-like cells within the r3 territory, suggesting that an anteriorization of hindbrain segment identity has occurred (Helmbacher et al., 1998). These results also argue that *Hox*a1 functions as a homeotic selector gene that is not only required for hindbrain segmentation, but also confers identity to r4 and r5. Interestingly, the exogenous application of RA during a very narrow time window is able to rescue the inner ear defects associated with the *Hoxa1* null mutation. This again highlights the intimate relationship between retinoid signaling and *Hox* gene function in the developing hindbrain.

In contrast to the *Hoxa1* mutants, *Hoxb1* null mutants exhibit no obvious defects in hindbrain segmentation, but they do display an aberrant specification of r4 motor neurons, suggesting subtle identity defects within the hindbrain (Goddard et al., 1996; Studer et al., 1996). In *Hoxb1−/−* hindbrains, although r4 patterning and development is initiated, its identity is not maintained during later development. The upregulation of r4-specific markers, such as *wnt8c* and *CRABP1*, is compromised, and *EphA4*, an r2-specifc marker, is abnormally expressed in the presumptive r4 territory of *Hoxb1−/−* hindbrains, suggesting an anterior transformation to an r2-like segment phenotype (Studer et al., 1996). As a consequence, the facial

brachiomotor and contralateral vestibular acoustic neurons are incorrectly specified and fail to migrate to their correct efferent positions, eventually leading to their loss. Thus *Hoxb1* may be required for the proper migration of neuronal derivatives from r4, in addition to conferring r4 segment identity. In support of this, the ectopic expression of *Hoxb1* within the r2 territory of avian embryos transforms it to an r4-like character (Bell et al., 1999).

The different phenotypes exhibited by the *Hoxa1* and *Hoxb1* null mutants suggested little functional overlap would exist between these two genes. Therefore, it was surprising that the double mutants synergize so extensively in hindbrain and craniofacial development (Gavalas et al., 1998; Studer et al., 1998; Rossel and Capecchi, 1999). Although a presumptive r4 territory does appear in *Hoxa1*/*Hoxb1* double null mutants, it fails to induce the earliest r4 marker, *EphA2*, suggesting improper initial specification of the segment. Furthermore, the organization of brachiomotor neurons VII to XI is highly perturbed, owning to the loss of r4 (Rossel and Capecchi, 1999). Further synergy in the *Hoxa1*/*Hoxb1* double mutants is noted in the absence of second branchial arch neural crest derived structures, which implies that *Hox* genes play a vital role in the generation of specific neural crest cell populations (Gavalas et al., 2001).

Recent additional synergistic interactions have been observed between *Krox20* and *Hoxa1* (Helmbacher et al., 1998). In the *Hoxa1* mutants, some cells within the r3 territory acquire an r2-like identity which is manifested in abnomal motor axon migration. This phenotype is greatly exacerbated in *Krox20*/*Hoxa1* double mutants in a dosage dependent manner. Thus, *Krox20* and *Hoxa1* may interact to distinguish odd-numbered rhombomeres from even-numbered rhombomeres and are required together to precisely define the r3 territory.

Null mutations in *Hoxa2* function in the mouse lead to dramatic transformations of the second branchial arch derivatives into structures characteristic of the first arch, a consequence of which is perinatal lethality (Gendron-Maguire et al., 1993; Rijli et al., 1993). These transformations involve the skeletal elements as well as the associated musculature, demonstrating the critical requirement for this gene in the development of the mesenchymal components of the second arch. Most notably, *Hoxa2*−/− mutants display a mirror-image duplication of Meckel's cartilage, along with malleus and incus. These duplicated elements are often fused together and are smaller with respect to their rostral counterparts. This argues strongly for the functioning of *Hoxa2* as a selector gene for second branchial arch mesenchymal neural crest. In contrast to *Hoxb1* mutant embryos, *Hoxa2* null mutants do not exhibit overt defects in hindbrain segmentation (Gendron-Maguire et al., 1993; Rijli et al., 1993). They do, however, display alterations in the anteroposterior and dorsoventral patterning of neuronal derivatives originating from the anterior hindbrain that are consistent with a change of identity of r2 to a more r4-like character (Gavalas et al., 1997; Davenne et al., 1999). There is a reduction in r2 and r3 territories as well as a loss of r2-specific gene expression, accompanied by a concomitant expansion of r1. As a result of these mild segmentation defects, the axons from the trigeminal nerve exit more caudally in these mutants; i.e. from r4 instead of

normally exiting from r2 (Gavalas et al., 1997). Interestingly, *Hoxa2* null mutants also display alterations in motor neuron projections from r2 and r3, suggesting that the dorsoventral patterning of the anterior hindbrain is affected. Thus, in addition to specifying segment identity in the anterior hindbrain, *Hoxa2* also plays a role in dorsoventral patterning of the neural tube.

Hoxb2 null mutants do not exhibit any hindbrain segmentation defects, however the maintenance of r4 identity is severely compromised (Barrow and Capecchi, 1996; Davenne et al., 1999). This is consistent with *Hoxb2* being a direct target of *Hoxb1* in r4. As with *Hoxa2*, *Hoxb2* is required for both the anteroposterior and dorsoventral patterning of neuronal derivatives. In the absence of *Hoxb2*, the r4 territory displays alterations of several dorsoventral neural tube markers, such as *Mash1*, *Math3*, *Nkx2.2*, and *Phox2b*, that are more reminiscent of the r2 programme, suggesting an r4 to r2 transformation (Davenne et al., 1999). *Hoxb2*−/− embryos also display defective development of the facial motor nucleus exiting from r4, as well as a reduction in migrating r4 brachiomotor neurons. Differences between the two mutations include altered neuronal differentiation in the alar and dorsal basal plates in *Hoxb2* of r2 and r3, while the *Hoxa2* mutation specifically interferes with the development of motor neurons in the ventral aspect of the basal plate in r4. Group 2 genes therefore confer segment identity along both the anteroposterior and dorsoventral axes, with *Hoxa2* being specific for r2 and r3, and *Hoxb2* regulating r4 identity.

The combined loss of both *Hoxa2* and *Hoxb2* leads to more severe perturbations in the dorsoventral patterning in r2 and r3 (Davenne et al., 1999). Novel phenotypes present in the double mutants include the absence of *Pax6*-positive ventral interneurons in r3. The group 2 genes also synergize in setting up rhombomeric boundaries in the anterior hindbrain, as double mutants lack inter-rhombomeric broundaries between r1 and r4. The mutation of *Hoxa2* and *Hoxb2* revealed a previously unsuspected role for *Hox* genes in dorsoventral patterning and demonstrate that group 2 *Hox* genes couple anteroposterior specification of the hindbrain with the dorsoventral birth of neurons.

The generation of *Hoxa1*/*Hoxa2* double mutants further illustrates the importance of *Hox* gene cross-regulatory interactions in hindbrain segmentation and patterning (Barrow et al., 2000). As with *Hoxa1* single null mutants, *Hoxa1*/*Hoxa2* double mutants display a failure to maintain the anterior *Hoxb1* expression limit at the r3/r4 boundary, resulting in aberrant hindbrain specification from r2 to r5. These defects are generally more severe in the *Hoxa1*/*Hoxa2* double mutants relative to *Hoxa1*−/− embryos, and demonstrate the importance of cross-regulatory interactions in setting *Hoxb1* expression levels up to the r3/r4 boundary.

Hoxa3 null mutants display abnormalities in both the neural and mesenchymal derivatives of the neural crest emerging posterior to the r4/5 boundary (Chisaka and Capecchi, 1991; Manley and Capecchi, 1995). Defects in the mesenchymal derivatives of the crest include abnormal throat cartilages and an absence of the thymus and parathyroid glands, which are ultimately derived from the third and fourth branchial arches, respectively. Neuronal defects involve the IXth cranial nerve

and range from a loss of the glossopharyngeal branch of the IXth ganglion to a fusion between the IXth and Xth ganglia. Similar cranial ganglion defects are observed in *Hoxb3*−/− mutants, although they occur at a lower penetrance relative to *Hoxa3*−/− mutants (Manley and Capecchi, 1997, 1998). *Hoxd3*−/− embryos, however, do not show any defects in cranial ganglia formation, suggesting a redundancy with the other two group 3 genes (Condie and Capecchi, 1993, 1994). Indeed the generation of various group 3 *Hox* double null mutants (*Hoxa3*/*Hoxb3*, *Hoxa3*/*Hoxd3*, and *Hoxb3*/*Hoxd3*) demonstrate extensive functional overlap within this paralogous group in the patterning of the cranial ganglia (Manley and Capecchi, 1998). Both the penetrance and severity of defects involving the IXth cranial nerve increased in the double null mutants.

Collectively, these mutational analyses point to a conserved role for the *Hox* genes in the pattering of the hindbrain and the branchial arches. In vertebrates, the remarkable patterning properties of the *Hox* genes have evolved to sculpt the unique characteristics of vertebrate head. They function as true selector genes conferring segment identity within the hindbrain, and also play key roles in the process of segmentation itself and in the patterning of various neuronal and mesenchymal derivatives of the branchial arches.

17. Models for craniofacial development: the plasticity vs. pre-programming neural crest paradox

Craniofacial morphogenesis is an elaborate process involving complex patterns of cell movements. An important issue is how the characteristic facial structures develop in their appropriate locations with the correct sizes and shapes during head development. The patterning programme could be intrinsic to each individual tissue precursor or alternatively reciprocal interactions between neighboring tissues could regulate vertebrate head development. The classical model describing vertebrate head development proposes that craniofacial morphogenesis is generated from patterning information imparted by the migrating neural crest cells (Noden, 1983; Hunt and Krumlauf, 1991a; Hunt et al., 1991b). In terms of the viscerocranium, fate mapping analyses in chick revealed that first arch neural crest cells give rise to Meckel's cartilage and the quadrate amongst other derivatives whereas second arch neural crest cells form the columella, the retroarticular process and part of the hyoid cartilage. When first arch (mandibular) neural crest cells are transplanted posteriorly in place of second (hyoid) or third (visceral) arch neural crest, the transplanted neural crest cells migrate into the nearest arch but therein form duplicate first arch skeletal elements, such as the quadrate and Meckel's cartilages (Noden, 1983). Not only are these ectopic crest-derived structures inappropriate for their new location, but their associated muscle cell types and connective tissue attachments are also characteristic of a first arch pattern. This led to the proposal of the neural crest pre-patterning hypothesis. This model argues first, that neural crest cell fates may be pre-programmed or determined prior to their emigration from the neural tube and

second, that myogenic populations and other cell types receive spatial cues from the invading neural crest-derived connective tissue.

The majority of the cranial neural crest cell population is derived from the hindbrain. The observation that the same combinatorial patterns of *Hox* gene expression found in the hindbrain are emulated first in the migrating neural crest cells and then later in the ganglia and branchial arches as the crest cells contribute to these tissues, provided molecular credence for the pre-programming model (Hunt et al., 1991a,b). Hence, under the neural crest pre-programming model, it was hypothesized that positional information encoded by the *Hox* genes was passively carried from the hindbrain to peripheral tissues and branchial arches by the migrating neural crest cells (Hunt et al., 1989 or 1990 or 1991).

Consequently, one of the predictions of the pre-programming model is that any molecular and/or cellular alteration to the hindbrain should alter the domains of *Hox* gene expression and ultimately lead to craniofacial abnormalities. The main vehicle for challenging the pre-programming hypothesis has been the avian embryo due to the ease of tissue manipulations in this species. The degree of autonomy and plasticity in cranial neural crest cells has been assessed in numerous rhombomere transplantation, rotation, and ablation experiments (Prince and Lumsden, 1994; Grapin-Botton et al., 1995; Hunt et al., 1995, 1998; Saldivar et al., 1996, 1997; Couly et al., 1998; Trainor and Krumlauf, 2000b). Overall these analysis implied that the spatial organization of cranial structures was determined by the neural crest and that the pattern was irreversibly set before the neural crest emigrates from the neural tube. The results however were far from conclusive. The experiments performed in avian embryos involved pairs of rhombomeres as a minimum, but generally much larger regions of the neural tube were being manipulated. The effects of inter-rhombomeric signaling and cell community may have masked the potential for cellular plasticity.

Recently significant advances in our understanding of craniofacial patterning have come via the development of new techniques for transposing small numbers of cells within the hindbrain of mouse embryos (Golding et al., 2000; Trainor and Krumlauf, 2000a) and single cells in zebrafish embryos (Schilling, 2001). In heterotopic transplantations of cells within mouse and zebrafish hindbrains, graft derived neural crest cells migrate into the nearest arch and demonstrate their plasticity by the complete downregulation of *Hox* gene expression in these cells (Trainor and Krumlauf, 2000a). Furthermore in zebrafish embryos, the transplanted cells differentiated and contributed to the pharyngeal cartilages appropriate to their new axial location (Schilling, 2001). These results argue that the axial character of cranial neural crest cells is neither fixed nor passively transferred from the hindbrain to the branchial arches and other peripheral tissues. Further support for the idea of neural crest cell plasticity comes from the demonstration that trunk neural crest cells can be transplanted to any anterior–posterior level in the trunk and will form structures appropriate to their new level (Le Douarin et al., 1975).

This neural crest plasticity correlates well with the cis-regulatory analysis of *Hox* genes, which have identified distinct elements responsible for regulating *Hox* gene expression in the hindbrain vs. the neural crest (Maconochie et al., 1999).

In the case of *Hoxa2* for example, its expression in r3 and r5 is directly regulated by *Krox20* and different elements control its expression in r2 and r4. Perhaps more significant is the fact that *Hoxa2* expression in second branchial arch neural crest cells derived from r3, r4, and r5 is regulated by an additional set of 4 elements, one of which binds to the transcription factor *AP2* (Nonchev et al., 1996b; Maconochie et al., 1999).

Together these results argue for a new model describing craniofacial development, one in which neural crest cells are considered developmentally plastic and that *Hox* gene expression is independently regulated in different tissues such as the hindbrain and neural crest. Neural crest cell development and patterning therefore is based upon a balance between the neural plate signals they receive during their formation and their response to the environmental signals and tissues with which they interact during their migration (Trainor and Krumlauf, 2001).

The question that remains, however, is how can these new findings for cranial neural crest plasticity and independent *Hox* gene regulation be reconciled with the landmark studies promoting neural crest pre-programming? First it is important to note that similar to the transplants of first arch neural crest cells, transplantations of frontonasal neural crest in place of second arch neural crest also generate duplicated first arch skeletal elements such as the quadrate and proximal region of Meckel's cartilage (Noden, 1983). The frontonasal crest does not normally give to these structures and as such this is a further example of neural crest plasticity. Consequently, it was noted that this result raised the issue of whether the same duplications will always occur when neural crest cells, irrespective of their origins, are transplanted in place of second arch neural crest (Noden, 1983). Second, in addition to forming duplicated first arch structures, the transplanted frontonasal and first arch neural crest also contributed to the normal development of the remaining second arch skeletal elements including the paraglossals and basihyoid, which make up part of the tongue skeleton. This again points towards neural crest plasticity. Thirdly, what is intriguing about the skeletal duplications is that they phenocopy the *Hox*a2 null mutant mouse in which the second arch structures are transformed into elements with a first arch identity.

These results raise the possibility that perhaps the landmark neural crest transplantations in effect created a conditional knockout of *Hox*a2 in the second branchial arch of the avian embryos. Recently, the isthmus was shown to be able to inhibit *Hoxa2* expression in rhombomere 1 via an FGF8 mediated signaling mechanism (Irving and Mason, 2002). Therefore, one plausible explanation, which could link together and explain the similar results obtained from the two distinct neural crest transplantations, is the possible inclusion of the isthmus in both the first arch and frontonasal neural crest grafts (Trainor et al., 2002a). The isthmus or junction between the midbrain and hindbrain is an obvious neuromeric landmark, which could have been used to delineate the anterior or posterior limits of the tissue to be transplanted. The landmark transplantations, which have shaped our thinking of craniofacial development for the past two decades, were performed in 1983 (Noden, 1983) and it was not until the mid 1990s that the isthmus and its organizing properties were discovered (Martinez et al., 1995; Crossley et al., 1996a; Wassarman et al., 1997;

Irving and Mason, 2000). The possibility that the isthmus could have led to the duplications has now been tested directly via posterior transplantations of the isthmus in place of r4 (Trainor et al., 2002a). The isthmus inhibits the expression of *Hoxa2* in second branchial arch neural crest cells and as expected these grafted chick embryos develop duplicated first arch skeletal structures including the quadrate and proximal portion of Meckel's cartilage similar to the classic transplantations and the *Hoxa2* null mutants. FGF8 soaked beads can only transiently block *Hoxa2* expression in second branchial arch neural crest cells indicating that *Fgf8* cannot recapitulate the entire effects of the isthmus and that other genes/factors must be involved.

Therefore rather than providing evidence for pre-patterning, the early neural crest transplantation experiments (Noden, 1983) highlight the effects of local signaling centers such as the isthmus on anterior–posterior patterning and regulation of *Hox* gene expression by FGFs.

18. Pharyngeal arch patterning: neural crest cells and evolutionary implications

A crucial issue in craniofacial development is to understand the mechanisms that regulate the size and shapes of the characteristic facial skeletal structures and branchial arch segmentation constitutes one of the first visible steps in this patterning process. The neural crest pre-programming model implied that branchial arch formation and patterning was dependent upon the neural crest cells. In contrast, the neural crest plasticity and independent gene regulation model described above implies that branchial arch patterning arises due to interactions between the arch components and the neural crest. This raises the question of what happens to the formation and patterning of the branchial arches in the absence of contributing neural crest cells. This issue has been investigated in chick embryos through rhombomere ablations (Veitch et al., 1999) and also in mouse embryos by the generation of *Hoxa1*/*Hoxb1* null mutants (Gavalas et al., 2001). In both experimental situations the branchial arches develop normally and are properly regionalized despite the absence of a neural crest cell contribution. The expression patterns of *Fgf8* in the anterior surface ectoderm, *Bmp7* and *Shh* in the posterior ectoderm and *Pax1* in the pharyngeal pouch endoderm were all normal and unchanged compared to wild type embryos. There was no evidence for enhanced cell death or reduction in proliferation in the arch epithelium, which implies that neural crest cells are not the source of indispensable branchial arch mitogenic or survival signals (Gavalas et al., 2001). Hence the branchial arches are not dependent upon the neural crest for their formation, or for their anterio–posterior and proximo-distal regionalization. This provides additional support for the neural crest plasticity and independent gene regulation model and is consistent with the evolutionary history of the branchial arches and neural crest cells. Pharyngeal segmentation is characteristic of the phylum chordata whereas neural crest cells are exclusively a craniate (vertebrates plus hagfish) characteristic implying that branchial arch segmentation occurred prior to the evolutionary origin of cranial neural crest cells (Schaeffer, 1987). Support for this idea comes from the observation of regionalized domains of *Pax* gene expression in Amphioxus (the nearest extant

vertebrate relative), which is indicative of pharyngeal segmentation. *Amphioxus* lack neural crest cells and therefore the mechanism for generating pharyngeal segmentation clearly predates the evolution of the neural crest cells (Holland and Garcia-Fernandez, 1996). Hence it is not surprising that the branchial arches do not rely upon the cranial neural crest for their initial formation and regional specification during vertebrate head development.

19. Pharyngeal arch patterning: roles for the mesoderm, endoderm, and ectoderm

Branchial arch formation and patterning can occur correctly, independently of a contribution from the neural crest and this implies that the branchial arches may rely on the paraxial mesoderm, endoderm and/or the surface ectoderm tissues for their patterning information.

19.1. The mesoderm

Fate mapping studies have shown that mesoderm and neural crest cells derived from the same axial level contribute to the same branchial arch during embryonic development (Noden, 1982, 1987, 1988; Trainor and Tam, 1995). The cranial mesoderm predominantly gives rise to the myogenic cores of each branchial arch, which are enveloped by migrating neural crest cells (Fig. 4B,C) (Noden, 1986b, 1987, 1988; Trainor et al., 1994; Trainor and Tam, 1995). Previously, the cranial mesoderm was thought not to play a patterning role during craniofacial development (Noden, 1983). However it has now been shown that the cranial mesoderm provides maintenance signals for regulating the identity of second branchial arch neural crest cells (Trainor and Krumlauf, 2000a) and that it may also play a role in patterning the pathways of neural crest cell migration (Fig. 4B,C) (Trainor et al., 2002b). When second arch neural crest cells are transplanted into the first arch, they downregulate their expression of *Hoxb1*. In contrast, if second arch neural crest cells are transplanted anteriorly in combination with second arch mesoderm, then *Hoxb1* expression is maintained in the grafted neural crest cells. The cranial mesoderm therefore provides maintenance signals that elaborate the programme of *Hox* expression, but the cranial mesoderm does not appear to initiate *Hox* gene expression in neural crest cells (Trainor and Krumlauf, 2000a). It is important to note however that *Hoxb1* is expressed in the cranial mesoderm at 7.5 dpc prior to its induction in the neuroepithelium. The effects of the mesoderm are consistent with the fact that the fate of the cranial mesoderm is primarily myogenic and the musculature is inextricably linked to neural crest derived skeletal and connective tissue patterning. Therefore one of the roles of the cranial mesoderm may be in maintaining an A–P register between these different primordial tissues, which is essential for subsequent craniofacial morphogenesis (Trainor and Tam, 1995).

19.2. The ectoderm

Similar to the neuroepithelium, it has been suggested that the ectoderm is regionalized into territories called ectomeres, which contribute specific regions of the branchial arches (Fig. 4B,C) (Couly and Le Douarin, 1990). Currently, there is no evidence to support the idea that an ectomere represents a functional developmental unit. In contrast however, there is evidence suggesting that the surface ectoderm plays a major role in the induction of odontogenesis during branchial arch development (Lumsden, 1988). The oral ectoderm of the first branchial arch directly regulates the patterning of the underlying neural crest mesenchyme into teeth and the ability to respond to these instructive or inducing signals is not confined to first arch neural crest cells (Tucker and Sharpe, 1999). *Fgf8*, which is expressed in the anterior surface ectoderm of the first arch, is essential for determining the polarity of the branchial arch and ectopic applications of FGF8 cause shifts in gene expression domains as well as re-patterning of the craniofacial primordia (Tucker et al., 1999). Not surprisingly then, in *Fgf8* null mutant mice, the branchial arches are severely abnormal (Trumpp et al., 1999). *Bmp4*, which is expressed in the ventral region of the first branchial arch ectoderm, appears to restrict the expression domain of *Fgf8* and consequently ectopic applications of BMP4 consistently reduce the size of the mandibular arch. Hence, the surface ectoderm plays important roles in patterning the branchial arch derivatives particularly through the BMP4 and FGF8 signaling mechanisms.

19.3. The endoderm

The neurogenic placodes (dorsolateral and epibranchial) form in characteristic positions in all vertebrates suggesting that conserved localized inductive interactions underlie their formation (Baker and Bronner-Fraser, 2001). The epibranchial placodes develop near the branchial clefts in close proximity to the cranial neural crest and the pharyngeal endoderm. Analysis of the nature of the signals, which underlie epibranchial placode formation, have found that the epibranchial placodes do not require cranial neural crest cells for their induction (Begbie et al., 1999). Rather, it is the pharyngeal endoderm that is the source of the BMP7 inducing signal. It has been suggested that neural crest cells will differentiate into cartilage only in the presence of pharyngeal endoderm and in amphibians, the endoderm has been shown to be responsible for promoting the formation of branchial arch components by directing neural crest cells towards a chondrogenic fate (Epperlein, 1974). Recently, the avian neural endoderm was tested in transplantation and ablation studies for its capacity to specify the facial skeleton (Couly et al., 2002). The experiments suggested that the endoderm instructs neural crest cells as to the size, shape, and position of all the skeletal elements whether they are cartilaginous or membranous bones. In addition bone orientation was shown to be influenced by the position of the endoderm relative to the embryonic axes. The nature of the signals arising from the endoderm are so far unknown. It is not clear whether the effects of the endoderm manipulations are direct and intrinsic to the tissue itself or whether the

effects of the manipulations indirectly alter local signaling centers or levels of FGF, Shh, or BMP which have been shown in other analysis to regulate the development of the characteristic craniofacial structures (Barlow and Francis-West, 1997; Hu and Helms, 1999; Trumpp et al., 1999; Lee et al., 2001).

20. Evolutionary significance of neural crest plasticity and its influence on craniofacial development

Craniofacial evolution is considered fundamental to the origin of vertebrates and in evolutionary terms the vertebrate head is a relatively new structure (Gans and Northcutt, 1983). The generation of animal diversity is believed to be based upon changes in the developmental processes that control morphology and comparative developmental biology indicates that these changes are regulatory, affecting the expression of developmental genes largely through changes in cis-regulatory elements (Averof and Patel, 1997; Sucena and Stern, 2000). Changes in cis-regulatory sequences are likely to have specific effects restricted to the expression of individual genes, while changes in regulatory proteins would be expected to affect the expression of multiple target genes, with potentially devastating effects. Accordingly, cis-regulatory sequences show rapid evolutionary turnover (Ludwig et al., 2000), whilst regulatory proteins are often highly conserved in primary sequence and biochemical function. For these reasons, changes in cis-regulatory elements are thought to play the larger role in morphological evolution (Carroll et al., 2001). A recent study utilizing cross-species transgenesis has provided some important insights into how the evolution of gene regulatory regions is related to the evolution of neural crest cells (Manzanares et al., 2000). The expression patterns of a series of reporter constructs containing the regulatory regions of the 3′ amphioxus *Hox* genes *AmphiHox1*, *AmphiHox2,* and *AmphiHox3* were analyzed in vertebrates. These *Hox* genes are normally involved in anterior–posterior patterning of the neural tube and certain populations of neural crest cells in vertebrates. Manzanares and colleagues (2001) observed that regulatory elements such as binding sites for the nuclear retinoic acid receptor were conserved between vertebrate and amphioxus genes (Manzanares et al., 2000). Remarkably, one amphioxus reporter construct directed expression to both neural crest and placodes in vertebrates. This result indicates that at least some of the regulatory elements necessary for directing *Hox* expression in neural crest cells had evolved before the neural crest precursors had developed the ability to migrate.

Evolutionary change in neural crest cells from which the majority of the craniofacial structures are derived could conceivably occur in two ways. Within the neural crest itself, there could be changes in the target genes of compartment specific selector proteins such as the *Hox* genes, or phenotype-determining factors such as *Snail*. The consequence of these changes could alter the cells repertoire of responses including the extent of proliferation, migration, and differentiation. This would probably have the most dramatic and long term effects on morphology due to its

impact in the earliest stages of neural crest development. Alternatively, molecular and cellular changes could occur in the periphery (i.e. after neural crest cells have completed their migration) in relation to the expression of specific organizer molecules such as FGFs, BMPs, Shh, Wnts, or retinoic acid which could effect or create new sites of neural crest deposition, condensation or differentiation in the branchial arches. It has recently been shown that local alteration of BMPs and retinoic acid has a dramatic effect on the number and identity of facial elements (Lee et al., 2001). The overall effects of alterations at this level would probably be much more subtle and less dramatic than changes of identities or developmental potential of the crest population within the hindbrain.

The observation described above that neural crest cells are plastic and that gene expression is independently regulated in different tissues provides a mechanism for how neural crest cells can be subtly modified or evolve in response to the environment through which they migrate independently of the neural tube. Although, the hindbrain exerts a profound influence in establishing the foundations of vertebrate head development, a rigid neural crest pre-patterning model in which the programme for head morphogenesis is set in the neural tube would offer very restricted opportunities for diversifying head structures (Trainor and Krumlauf, 2001). In contrast, the neural crest plasticity and independent gene regulation model could provide the flexibility and adaptability that facilitates diversity and we can speculate that it might be one reason for the successful radiation of vertebrates into new environments. This is because neural crest plasticity and independent gene regulation offers the potential for generating substantially distinct cranial phenotypes by subtle changes of the primordial pattern. Evidence potentially supporting this scenario can be found in the rapid morphological changes that have occurred in the beaks (a neural crest derived structure) of Galapagos Island finches over the past decade due to environmental fluctuations (Grant and Grant, 1993, 2002).

21. Evolution of the vertebrate jaw: perspectives from lampreys

In the evolutionary transition from chordates to vertebrates, sessile or slow moving filter feeders such as *amphioxus*, evolved into active moving creatures with high volume filter feeding and later predation (Northcutt and Gans, 1983). Jaw development was a critical event in vertebrate evolution facilitating the transition to a predatory lifestyle, but it remains a mystery, how this innovation came about. In the embryos of jawed vertebrates (gnathostomes) the jaw cartilage develops from the mandibular arch, where *Hox* genes are not expressed. If *Hox* genes are ectopically expressed in this region, jaw development is inhibited (Alexandre et al., 1996; Grammatopoulos et al., 2000; Pasqualetti et al., 2000; Couly et al., 2002; Creuzet et al., 2002).

The lamprey is a primitive jawless (agnathan) vertebrate fish and recently it has been shown that *HoxL6* gene is expressed in the mandibular arch of developing embryos and that it co-localizes with *Dlx*, a marker of lamprey neural crest cells

(Cohn, 2002). To date lampreys are the only vertebrates in which *Hox* genes are known to be expressed in the mandibular arch. This finding suggests that loss or suppression of *Hox* gene expression from the mandibular arch of gnathostomes may have facilitated the evolution of jaws. Given the inhibitory effects of *Hox* genes on jaw formation, lack of their expression in the first arch and the associated neural crest of early gnathostomes may permit ventral chondrification of the first arch crest and thus formation of ventral mandibular cartilages. These results raise the possibility that the ventral mandibular skeleton was added onto an evolutionarily ancient velar like cartilage after *Hox* expression was eliminated from the first pharyngeal arch (Langille and Hall, 1989).

Some recent work on epithelial–mesenchymal interactions in vertebrate jaw formation allows us to build on this view of vertebrate jaw evolution. The lamprey homologue of *Fgf8* is expressed in the peri-oral epidermis but appears not to be expressed in the neural tube at the same time (Shigetani et al., 2002). Although lampreys exhibit regionalized expression of *Otx* and *Emx* genes they do not appear to possess a true isthmus at least by virtue of the characteristic *Fgf8* expression usually found in this territory. Given the fact that *Hox* genes are expressed in the first arch of lampreys and that FGFs have the capacity to suppress *Hox* gene expression (Irving and Mason, 2000; Trainor et al., 2002a), it is tempting to speculate that evolution of the isthmus and new domains of *Fgf8* expression in the neural tube and mandibular arch led to the repression of mandibular *Hox* gene expression. Consequently this could have allowed endochondral and dermal bone formation to occur which ultimately facilitated vertebrate jaw development.

22. Conclusions: moving ahead

This chapter has highlighted the intimate involvement of *Hox* genes in hindbrain, neural crest cell and pharyngeal arch development where they exert a profound influence on craniofacial morphogenesis. The combined effects of neural crest cell plasticity and independent tissue regulation of *Hox* gene expression, underscore a mechanism for craniofacial evolution and the generation of diversity. Futures studies are aimed at uncovering more downstream targets of *Hox* genes, which will enable a better understanding of neural crest cell and pharyngeal arch differentiation. Consequently, this will provide further insights into craniofacial evolution and the transition from agnathans to gnathostomes.

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Role of *Otx* transcription factors in brain development

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Contents

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1. Introduction

The central nervous system (CNS) of vertebrates is a very complex structure derived from a multistep process involving sequential molecular and morphogenetic events that pattern the epiblast first and the neural plate later. During early gastrulation, the concerted and sequential action of both the anterior visceral endoderm (AVE) and the node and its derivatives (axial mesendoderm, AME, and anterior definitive endoderm, ADE) (Beddington and Robertson, 1999; Bachiller et al., 2000) drives the specification of the anterior neuroectoderm, which subsequently is subdivided in three main territories (forebrain, midbrain, and hindbrain) (Gallera, 1971; Storey et al., 1992; Ruiz i Altaba, 1994; Shimamura and Rubenstein, 1997; Rubenstein and Beachy, 1998).

Anatomical and histological studies postulate the existence of genetic fate determinants, which subdivide these large neural regions into progressively smaller longitudinal and transverse domains (Vaage, 1969; Altman and Bayer, 1988; Figdor and Stern, 1993; Rubenstein et al., 1994). Some of the patterning events along the antero-posterior (A/P) axis require the presence of specific cell populations (e.g. the anterior neural ridge, ANR, and the zona limitans intrathalamica ZLI) and transverse rings of neuroepithelia (e.g. the isthmic organizer, IsO) that possess inductive and boundary properties (Marin and Puelles, 1994; Crossley et al., 1996; Houart et al., 1998; Rubenstein et al., 1998; Ruiz i Altaba, 1998).

In vertebrates, several genes controlling developmental programmes underlying brain morphogenesis have been isolated and their role studied in detail. Most of them are the vertebrate homologs of *Drosophila* genes encoding signaling molecules or transcription factors (Lemaire and Kodjabachian, 1996; Tam and Behringer, 1997; Rubenstein et al., 1998). Among these, the *orthodenticle* group is strictly defined by the *Drosophila orthodenticle (otd)* and the vertebrate *Otx1* and *Otx2* genes, which contain a *bicoid*-like homeodomain (Finkelstein and Boncinelli, 1994; Simeone, 1998). However, other genes that might be more distantly related to *otd*/*Otx* genes have been also identified (Muccielli et al., 1996; Szeto et al., 1996; Chen et al., 1997; Furukawa et al., 1997).

Expression pattern analysis of *Otx* genes had suggested that these transcription factors might play an important role during brain morphogenesis in vertebrates. A systematic genetic approach using transgenic mice is revealing that they contribute to the molecular mechanisms underlying all of the major events (induction, maintenance, regionalization, corticogenesis, and axon connectivity) necessary to build a normal brain.

2. Distinct signaling centers are required for early anterior patterning

2.1. Spemann and Mangold organizer

Classical experiments by Spemann and Mangold (1924) identified a specific group of cells in the dorsal lip of the blastopore of the amphibian gastrula, the organizer, which was sufficient to induce a complete secondary axis when grafted into the ventral region of a host embryo. Histological analysis revealed that in this ectopic axis, the notochord was derived from the grafted tissue, but the neural tube was composed mostly of host cells, thus demonstrating that the dorsal blastopore lip contained an activity capable of changing the fate of the surrounding tissues. Spemann also showed that the inducing activities of the organizer were stage dependent. Transplantation of the early gastrula organizer induced complete ectopic axis whilst grafts of equivalent tissues of a late gastrula organizer induced partial secondary axes lacking the head region (Spemann, 1931). Based on the cell fate and inductive properties of the grafted tissue, homologous organizers have been described in all vertebrates: the embryonic shield in zebrafish, Hensen's node in chick, and the node in mouse. As in Amphibia, the zebrafish and chick organizers contained head and trunk inductive activities that together can induce a complete ectopic axis, which can be visualized both morphologically and by the expression of specific regional markers (Waddington, 1933; Storey et al., 1992; Zoltewicz and Gerhart, 1997; Saude et al., 2000). In mouse, transplantation of the node has been conducted using donor tissue isolated from gastrulating embryos at different stages. Early- and full-length streak nodes were able to induce secondary neural axes, but the fore- and midbrain regions were absent (Beddington, 1994; Tam and Steiner, 1999). In contrast, when donor tissue was obtained from mid-streak embryos, *Otx2* expression was induced in the ectopic neural axis (Kinder et al., 2001). Similarly, heterotopic transplantation of the mouse or rabbit node has shown that the mammalian organizer can induce a complete ectopic neural axis in chick embryos (Knöetgen et al., 2000). These studies suggest that the mammalian node might resemble other vertebrate organizers in its neural inducing properties when assayed in favorable conditions. Nevertheless, the fact that experimental and genetic ablation of the node fails to abrogate formation of anterior neural structures, suggests that tissues other than the node must be involved in anterior neural patterning in the mouse (Davidson et al., 1999; Klingensmith et al., 1999; Episkopou et al., 2001).

2.2. A new signaling center, the anterior visceral endoderm (AVE) is necessary for early neural patterning

Much evidence has now accumulated that in mammalian development a separate signaling center, distinct from the classical organizer, is required prior to and during early gastrulation for normal anterior neural induction (Beddington and Robertson, 1999). This signaling center, the anterior visceral endoderm (AVE) is composed of a group of cells destined to populate only the visceral yolk sac. Cell lineage and gene expression studies have shown that the antecedents of the AVE are located at the distal tip of the 5.0 days post coitum (dpc) mouse embryo and are fated to move anteriorly, underlying the prospective anterior neural plate, by the onset of gastrulation at 6.5 dpc (Fig. 1). This anterior displacement appears to be part of a global anteriorward rotation of the visceral endoderm (Weber et al., 1999). However, it has recently been shown that AVE cells acquire a distinct morphology during this movement, suggesting that they may detach from the epithelial sheet and move in an anteriorward direction (Kimura et al., 2001). During gastrulation, the node derived definitive endoderm intercalates in the visceral endoderm layer, displacing most of it towards its final location in the extraembryonic region (Lawson and Pedersen, 1987; Tam and Beddington, 1992; Thomas and Beddington, 1996). By early head-fold stages, the anterior definitive endoderm (ADE), fated to form the foregut and the liver, and the axial mesendoderm (AME), which gives rise to the prechordal plate and notochord, replace most of the AVE. Chimeric analysis has shown that some visceral endodermal cells are not displaced proximally, but remain in definitive endoderm derivatives forming part of the foregut lining (Narita et al., 1997; Rhinn et al., 1998). Therefore, the AVE, ADE, and AME are underlying the anterior epiblast at a time when these cells are thought to acquire anterior neural character.

Pioneering evidence for a role of the AVE in anterior neural patterning has been provided from elegant ablation experiments in mouse. It was shown that removal of the anterior region of the visceral endoderm during the earlier stages of gastrulation either prevented or severely impaired the expression of the forebrain marker *Hesx1* in the anterior neural ectoderm, without affecting the expression of the hindbrain marker *Gbx2* (Thomas and Beddington, 1996). The idea that the AVE might be an important signaling center for anterior neural patterning was further supported by the finding that several transcription factors, such as *Hex*, *Otx2*, *Lim1*, *Hnf3β*, *Gsc*, *Hesx1* and signaling molecules, such as *nodal*, *Cerrl*, *Dkk1*, and *Lefty1*, were found to be expressed specifically in the AVE, in many cases prior to the appearance of the primitive streak or any mesoderm (Beddington and Robertson, 1999). Interestingly, many of these genes are later expressed in the node and its derivatives. The fact that mutants for many of these genes showed anterior neural defects opened the exciting possibility that impaired AVE function was underlying the brain abnormalities. Indeed, detailed studies showed that the expression of several AVE markers was abnormal in mutant embryos analyzed at the onset of gastrulation (Ang and Rossant, 1994; Rhinn et al., 1998; Shawlot et al., 1998; Acampora et al., 1998b; Klingensmith et al., 1999; Perea-Gomez et al., 2001).

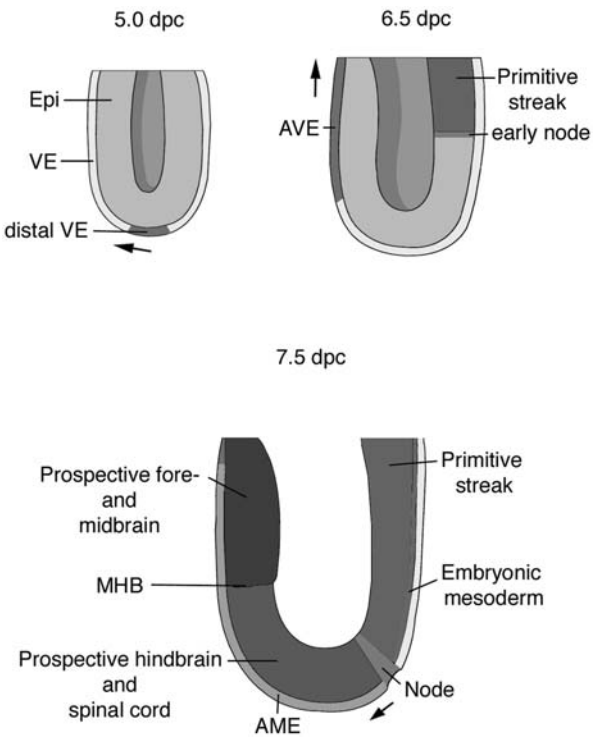

Fig. 1. Schematic representation of mouse embryos from 5.0 to 7.5 dpc. The anterior visceral endoderm (AVE, purple) precursors are located at the distal tip of the conceptus at 5.0 dpc. As the embryo develops, these distal visceral endoderm (VE) cells move to assume their antero-proximal position at the onset of gastrulation at 6.5 dpc. This movement of the VE is probably accompanied by the rotation of the proximal epiblast towards the posterior region where the primitive streak will form (green). Cells located in the distal tip of the primitive streak (early node, brown) are fated to form the node subsequently in development. During gastrulation, the primitive streak elongates and two new germ layers are formed: the mesoderm (red) and the definitive endoderm. The mesoderm is intercalated between the epiblast and the endoderm in the embryonic and extraembryonic regions of the embryo. The definitive endoderm, which derives from the early node, moves anteriorly merging with and displacing the VE towards the extraembryonic region. In the anterior portion of the embryo the AVE is displaced by anterior definitive endoderm (ADE), which is fated to form the liver. Midline ADE, prechordal and notochordal plates form the axial mesendoderm (AME), which underlie the medial aspects of the neural plate. On the overlying ectoderm, the neural plate is divided into two regions: the prospective forebrain and midbrain (dark blue); and the prospective hindbrain and spinal cord (light blue). Anterior is to the left. (See Color Insert.)

The requirement of the AVE for normal brain formation was definitely confirmed from chimeric studies for genes such as *nodal*, *Otx2*, *Lim1*, and *Hnf3β*. The specific absence of each of these genes in the visceral endoderm led to the induction of a neural axis lacking the most anterior values, fore- and midbrain, whilst restoration of their functions only in the visceral endoderm was sufficient to rescue the initial induction of fore- and midbrain neural markers expression at headfold stages (Varlet et al., 1997; Dufort et al., 1998; Rhinn et al., 1998; Acampora et al., 1998b; Shawlot et al., 1999). The rescue of the anterior position neural plate was

accompanied by a normalization of pre- and gastrulation movements that were defective in the null mutants, i.e. the rotation of AVE precursors from distal to anterior position at the onset of gastrulation, and later the proximal displacement of AVE by the ADE and AME. Nevertheless, this raised the crucial question whether the rescue of the anterior neural plate induction in these chimeras was direct effect of having a normal AVE during pre- and early streak stages, or it was an indirect consequence of the normalization of morphogenetic movements that bring the ADE and AME to underlie the prospective anterior neural plate.

2.3. AVE initiates neural anterior patterning

The analysis of the *Cripto* and *Fgf8* null mutants provided insights into this problem, because these mutants fail to form a normal primitive streak, ADE, AME or a recognizable node, but yet the AVE is properly specified (Ding et al., 1998; Sun et al., 1999). This leads to the induction only of fore- and midbrain markers in the epiblast overlying the AVE, suggesting that the ADE or AME are not required for the induction of the anterior neural plate. It is interesting to note that expression of early node markers such as *T*, *Lim1*, *Gsc*, and *Hnf3β* was detected proximally in these mutants. Although, a possible interpretation for the phenotype of these mutants is that the AVE might be sufficient for anterior neural induction, other genetic and embryological evidence suggest that this may not be the case.

Mouse embryos deficient for *Wnt3* or *β-catenin* show a normal specification of the AVE, evidenced by normal expression of *Cerrl* and *Lim1*, but the epiblast fails to acquire neural character, demonstrating that the AVE is not sufficient to impart neural character on its own (Liu et al., 1999b; Huelsken et al., 2000). In these mutants, expression of early node markers was not detected, suggesting the absence of node specification. Together with the *Fgf8* and *Cripto* mutants, these mouse models suggest that the induction of anterior neural markers require the synergistic actions of the AVE and the early node. Similar conclusion has been obtained from elegant transplantation experiments in mouse (Tam and Steiner, 1999).

Grafting experiments have provided contradictory results on the neural inducing capabilities of the mouse AVE in anterior patterning. On one hand, homotopic grafting experiments in mouse have shown that the AVE is not able to induce neural character to naive ectoderm (Tam and Steiner, 1999). Likewise, the AVE equivalent in chick, the hypoblast, is unable to induce definitive neural or forebrain markers in naive epiblast, but has the ability to induce transiently the expression of *Sox3* and *Otx2* (Foley et al., 2000). On the other hand, heterotopic transplantations of mouse and rabbit AVE into chick embryos have evidenced the anterior neural inducing activities of the mammalian AVE (Knöetgen et al., 1999, 2000). Taking together the genetic and embryological data, it seems likely that AVE derived signals are required for the initiation of anterior neural patterning, prior to and at early stages of gastrulation, but subsequently in development other signals are also necessary for the maintenance and embellishment of the anterior neural character (Thomas and Beddington, 1996).

2.4. Node derivatives maintain and reinforce the anterior neural character

Once the initial patterning of the neural plate is set up by the activities of the AVE and early node, this has to be maintained and refined subsequently in development by the ADE and AME. Tissue recombination experiments have shown that positive signals emanating from the anterior mesendoderm are required for the neuroectodermal stabilization of *Otx2* expression whilst negative signals from posterior mesendoderm can actively repress *Otx2* expression in the explants (Ang et al., 1994). Likewise, anterior mesendoderm induces the expression of *Engrailed* genes in explant cultures (Ang and Rossant, 1993). Removal of anterior midline tissue including both the AME and ventral neuroectoderm from late-streak mouse embryos leads to fore- and midbrain defects (Camus et al., 2000). Chimeric analysis has also provided genetic evidence for an essential role of the AME and/or ADE in brain formation. These studies have demonstrated the necessity of *Lim1*, *Otx2*, and *FoxH1* in these tissues to maintain the fore- and midbrain identities (Rhinn et al., 1998; Shawlot et al., 1999; Hoodless et al., 2001). Recently, a specific requirement for the ADE in forebrain development has been revealed from embryological and genetic studies. Removal of the ADE in chick embryos leads to a significant reduction of forebrain tissue, without affecting expression of anterior axial mesoderm markers such as *Shh*, *chordin*, and *Bmp7* (Withington et al., 2001). Mouse mutants for the homeobox transcription factor *Hex*, which is expressed in the AVE and ADE, showed forebrain defects owing to a requirement of *Hex* in the ADE (Martinez Barbera et al., 2000). Recently, it has been shown that reciprocal interactions between the ADE and the AME are required for the maintenance of forebrain identity (Hallonet et al., 2002).

Taking into consideration embryological and genetic evidence, the most plausible model for the generation of the mammalian brain suggests that the anterior visceral endoderm (AVE) and the early node are required for the initial induction of the rostral identity, and subsequently this identity is maintained and elaborated by signals from the ADE and AME. Other signaling centers within the neuroectoderm itself will be also required for further growth and differentiation of the brain (see below).

3. *Otx2* in early anterior neural patterning

Among all genes involved in the early steps of anterior neural induction and brain regionalization, the bicoid class homeobox gene *Otx2* has been shown to play a pivotal role in these processes. *Otx2* is expressed in the mouse embryo throughout the entire epiblast and visceral endoderm prior the onset of gastrulation, but later its expression is downregulated from the posterior pole of the embryo, where the primitive streak will form, and it is maintained only in anterior epiblast and AVE at the onset of gastrulation (Simeone et al., 1993; Ang et al., 1994). As gastrulation proceeds *Otx2* transcripts are detected in the node derivatives, the ADE and rostral portion of the AME, as well as in the anterior neural plate. During brain

regionalization, *Otx2* transcripts are localized in the fore- and midbrain territories with a sharp boundary at the mid-hindbrain border where the isthmic organizer (IsO) will subsequently form. Therefore, *Otx2* is expressed in relevant tissues for anterior patterning (AVE, ADE, AME, and anterior neural ectoderm, ANE), where it plays a crucial role in anterior neural induction and maintenance of anterior character.

3.1. *Otx2 is required for normal anterior neural induction and gastrulation*

Otx2 null mutants show pre- and gastrulation defects and severe abnormalities in anterior neural patterning (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996). Prior to the onset of gastrulation, $Otx2^{-/-}$ mutants fail to anteriorize the precedents of the AVE into their final antero-proximal position at 6.5 dpc, as evidenced by the distal expression of AVE markers such as *Cerrl*, *Hex*, *Hesx1*, and *Lim1* (Acampora et al., 1998b; Kimura et al., 2001; Perea-Gomez et al., 2001). Primitive streak markers, such as *T*, *Fgf8*, and *Cripto* are not restricted to the posterior proximal epiblast as in wild-type embryos, but they are ectopically expressed in a ring around the entire proximal epiblast at 6.5 dpc (Fig. 2D). During gastrulation (6.5–7.5 dpc), formation of the primitive streak, node and node derivatives, such as ADE and AME are severely impaired in $Otx2^{-/-}$ mutants. At 8.5 dpc, mutant embryos are delayed and lack neural tissue rostral to the rhombomere 3 (Fig. 2E). Analysis of early neural markers during gastrulation demonstrated that the anterior defects had an early conception owing to a failure of induction of fore- and midbrain at streak stages. Expression of the fore- and midbrain markers *Pax2*, *Six3*, and *Hesx1* was abolished in the anterior neural plate of $Otx2^{-/-}$ mutants at 7.5 dpc, whilst more posterior regions of the neural axis were normally induced as evidenced by expression of the hindbrain and spinal cord markers *Gbx2* and *Hoxb1*.

Chimeric studies were conducted to better understand the reasons of the anterior defects and to address when and where *Otx2* was required during mouse development. The possibilities were that *Otx2* might be required primarily either in the visceral endoderm, or in the epiblast and its derivatives (ADE, AME, and ANE), or in all of these tissues. Chimeras were generated in which only the epiblast was wild-type for the *Otx2* function, whilst the visceral endoderm was of $Otx2^{-/-}$ genotype ($Otx2^{+/+} => Otx2^{-/-}$) and viceversa ($Otx2^{-/-} => Otx2^{+/+}$). These experiments are based on the developmental bias of embryonic stem (ES) cells when injected into host blastocysts, which colonize the embryo proper (epiblast derivatives) but very rarely the visceral endoderm or extraembryonic ectoderm (Beddington and Robertson, 1989). Chimeric embryos composed predominantly of $Otx2^{+/+}$ epiblast cells developing within $Otx2^{-/-}$ visceral endoderm ($Otx2^{+/+} => Otx2^{-/-}$) gave rise to chimeras showing the same neural defects observed in the $Otx2^{-/-}$ mutants. In contrast, chimeras obtained from injection of $Otx2^{-/-}$ ES cells into wild-type blastocysts ($Otx2^{-/-} => Otx2^{+/+}$) exhibited a normal induction of the fore- and midbrain regions of the neural plate at 7.5 dpc (Rhinn et al., 1998). As *Otx2* is not expressed in extraembryonic ectoderm, these experiments

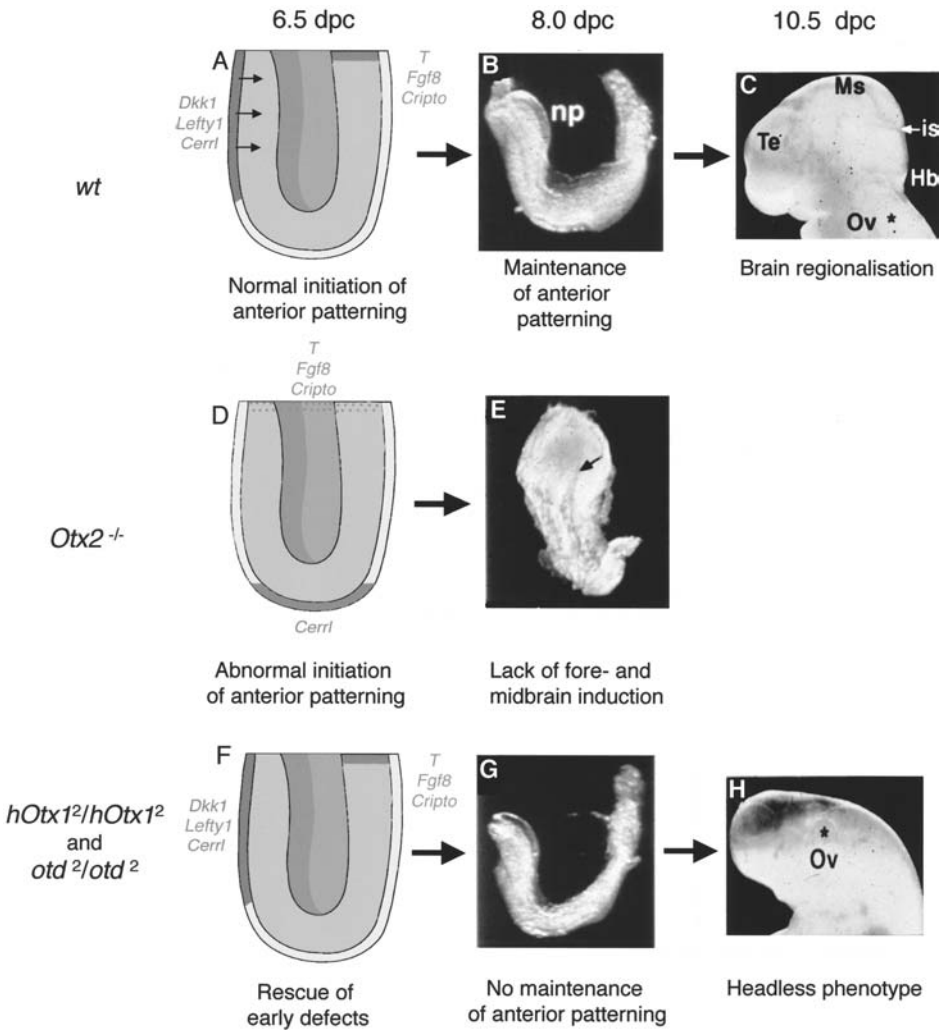

Fig. 2. Molecular and morphological defects in $Otx2^{-/-}$, $hOtx1^{2}/hOtx1^{2}$ and otd^{2}/otd^{2} mutant embryos at different stages of development. In wild-type embryos at the onset of gastrulation (6.5 dpc), genes such as *Dkk1*, *Lefty1*, and *Cerrl* are expressed in the AVE whilst *T*, *Fgf8*, and *Cripto* are expressed in the primitive streak. One of the functions of the AVE is to protect the overlying epiblast from posteriorizing signals by secreting BMP, Wnt, and nodal antagonists. This is required for normal neural induction of fore- and midbrain territories. This anterior neural character is maintained subsequently by the activities of the ADE and AME (B). Finally, the brain is divided into broad regions: fore-, mid- and hindbrain (C). In $Otx2^{-/-}$ mutants, pre-gastrulation movements are impaired and the AVE is located at the distal tip of the mutant embryo at 6.5 dpc (D). Furthermore, the activity of the AVE is also severely affected as no *Dkk1* or *Lefty1* expression is detected. As a consequence, the primitive streak markers *T*, *Fgf8*, and *Cripto* are expressed ectopically in a ring around the proximal epiblast (D). This leads to a failure in the initial induction of the fore- and midbrain territories in $Otx2^{-/-}$ mutants (E). In contrast, in $hOtx1^{2}/hOtx1^{2}$ and otd^{2}/otd^{2} mutants, the early requirement of *Otx2* is compensated for, and a normal induction of the anterior neural plate takes place (F). However, this anterior character is not maintained subsequently (G) what results in a headless phenotype at 10.5 dpc (H). The arrow in (E) points to the rostral limit of the neuroectoderm in an $Otx2^{-/-}$ embryo. Hb, hindbrain; is, isthmus; Ms, mesencephalon; np, neural plate; Ov, otic vescicle; Te, telencephalon. Anterior is to the left. Colors as in Fig. 1. (See Color Insert.)

provided irrefutable evidence that *Otx2* is first absolutely required in the visceral endoderm, but not in the epiblast, for normal gastrulation and anterior neural induction. Nevertheless, as it will be discussed below, $Otx2^{-/-} => Otx2^{+/+}$ chimeras showed a headless phenotype owing to a further requirement of *Otx2* function in ADE, AME and/or ANE.

Similar conclusions to those obtained from the *Otx2* chimeras were drawn from the analysis of another mouse model where *Otx2* gene was replaced by the human *Otx1* cDNA ($hOtx1^2$) (Acampora et al., 1998b). In homozygous mutant embryos for the *hOtx1* allele ($hOtx1^2/hOtx1^2$ embryos) hOTX1 protein was detectable only in the visceral endoderm, but not in the epiblast or its derivatives (ADE, AME, and ANE). Interestingly, expression of hOTX1 in the visceral endoderm was sufficient to rescue the early neural and gastrulation defects observed in $Otx2^{-/-}$ mutants at 7.5 dpc, but $hOtx1^2/hOtx1^2$ mutants invariably showed a headless phenotype at 9.5 dpc as hOTX1 protein was absent in the epiblast derivatives (Fig. 2F–H). Together, this genetic evidence demonstrated that *Otx2* plays an early crucial role in the visceral endoderm for the induction of fore- and midbrain territories prior to and during gastrulation, and later it is required for the maintenance of the anterior neural character.

Recently, the function of *Otx2* in the AVE has been investigated in more detail. Using cell labeling techniques, it has been confirmed, as previously suggested (Acampora et al., 1995), that the AVE antecedents fail to move anteriorly by the onset of gastrulation in the $Otx2^{-/-}$ mutants (Perea-Gomez et al., 2001). This is concomitant with the ectopic expression of the primitive streak and mesodermal markers *Cripto*, *Fgf8*, *Lefty2*, *Mesp1*, and *T* in a ring around the proximal epiblast (Acampora et al., 1998b; Perea-Gomez et al., 2001). Interestingly, the distally located AVE fails to express the *Wnt* and *nodal* antagonists *Dkk1* and *Lefty1*, although other AVE markers such as *Lim1*, *Hesx1*, and *Cerrl* are expressed in $Otx2^{-/-}$ mutants. As *Wnt* and *nodal* signaling are involved in primitive streak formation (Lu et al., 2001), the absence of *Dkk1* and *lefty1* is likely the reason for the persistent expression of primitive streak and mesodermal markers in the antero-proximal epiblast in 6.5 dpc $Otx2^{-/-}$ mutants. Fate map studies have shown that at the onset of gastrulation, antero-proximal epiblast is destined to populate the anterior neural and non-neural ectoderm in wild-type embryos, but in $Otx2^{-/-}$ embryos there is a tendency of these cells to give rise to mesoderm (Perea-Gomez et al., 2001). This support the idea that one of the functions of *Otx2* in the visceral endoderm and specifically in the AVE is to protect the antero-proximal epiblast from posteriorizing signals involved in the formation of the primitive streak. The failure of AVE antecedents to move anteriorly, and the lack of *Dkk1* and *Lefty1* in the AVE might leave the anterior epiblast exposed to the action of posteriorizing factors. In fact, tissue recombination experiments have shown that the AVE is able to repress the posterior markers *T* and *Cripto*, and that *Otx2* is specifically required in the AVE for this repression to occur (Kimura et al., 2001). This supports the model that the AVE and specifically an *Otx2* positive AVE, is required to restrict expression of posterior genes involved in mesoderm induction, thereby allowing the anterior epiblast to remain receptive to later anterior neural

induction and patterning. Compelling evidence supporting this model has been obtained from the analysis of the *nodal* and *Smad2* single, as well as the *Lim1*; *Hnf3β* double mutants (Waldrip et al., 1998; Perea-Gomez et al., 1999).

The change of fate of the anterior epiblast might also explain an observation in *Otx2* deficient embryos where the wild-type *Otx2* locus was replaced by the *lacZ* gene (Acampora et al., 1995). In these null embryos, *lacZ* expression was activated in the AVE, but not in the overlying anterior epiblast at 6.5 dpc. This is the expected result if the anterior epiblast acquires a posterior fate in $Otx2^{-/-}$ mutants, as *Otx2* is not expressed in posterior epiblast. Therefore, *Otx2* is required in the visceral endoderm for normal anterior displacement of AVE precursors, and specifically in the AVE for activation of the *Wnt* and *nodal* antagonists, *Dkk1* and *Lefty1*, respectively.

3.2. Role of Otx2 in maintenance of fore- and midbrain identities

As mentioned above, although $Otx2^{-/-} => Otx2^{+/+}$ chimeras and $hOtx1^{2}/hOtx1^{2}$ homozygous embryos recovered the early induction of fore- and midbrain territories of the neural plate at 7.5 dpc, they exhibited a headless phenotype by 9.5 dpc. Therefore, *Otx2* is required early for normal initiation of antero-posterior patterning of the neural plate and later its function is also necessary for proper maintenance and refinement of the anterior character (Acampora et al., 1998b; Rhinn et al., 1998). Indeed, *Otx2* is expressed in the ADE and AME, which are both source of signals involved in neural patterning, as well as in the ANE, where it could be required for providing competence to respond to those signals.

Morphologically, gastrulating $hOtx1^{2}/hOtx1^{2}$ mutants looked normal and expression of several ADE and AME markers *Cerrl*, *Lim1*, *Gsc*, *T*, *Hesx1*, and *Noggin* was undistinguishable from wild-type embryos (Acampora et al., 1998b). However, as hOTX1 protein was undetectable in the ADE, AME, and ANE, by Western blot or immunohistochemical analysis, this analysis did not clarify in which of these tissues *Otx2* was required for maintenace properties. Similarly, the *Otx2* chimeric analyis could not address this issue as it was not possible to generate chimeras where only one of these tissues was of $Otx2^{-/-}$ genotype (Beddington and Robertson 1989; Rhinn et al., 1998).

Tissue recombination experiments using anterior mesendoderm and anterior ectoderm isolated from *Otx2* mutants carrying a hypomorphic allele have suggested a specific function of *Otx2* in the anterior ectoderm for providing competence to respond to signals emanating from the anterior mesendoderm and anterior neural ridge (ANR) (Tian et al., 2002). Likewise, recombination of wild-type anterior mesendoderm and $Otx2^{-/-}$ ectoderm failed to induce neural markers in the latter, thus suggesting a lack of competence of $Otx2^{-/-}$ ectoderm to respond to the signal emanated from the anterior mesendoderm (Rhinn et al., 1998).

The analysis of some mouse models carrying *Otx2* hypomorphic alleles suggests a differential requirement of OTX2 protein levels in the AVE, anterior mesendoderm, and ANE (Pilo Boyl et al., 2001; Tian et al., 2002). These mouse models show a headless phenotype by midgestation, which appears to be due to low levels of

OTX2 protein within the anterior neuroectoderm, as the early induction and regionalization of the fore- and midbrain is normal. Moreover, the anterior mesendoderm is able to induce the neural markers *Nkx2.1* and *Otx2* in tissue recombination experiments (Tian et al., 2002). Assuming that the reduction of OTX2 protein is comparable in all the tissues of the mutants carrying these hypomorphic alleles, this may suggest that lower level of OTX2 protein are required within the AVE and anterior mesendoderm and higher within the anterior neuroectoderm for normal anterior patterning. Alternatively, it is conceivable that OTX2 protein might accumulate at higher levels in the AVE and anterior mesendoderm compared with anterior neuroectoderm due to specific cell type differences in RNA transcription, processing, translation, and degradation of *Otx2* RNA and/or protein (see below).

In conclusion, although genetic and embryological evidence suggests that *Otx2* is required within the ANE for the maintenance of fore- and midbrain regions, its function in the ADE and AME remains unclear. A definitive answer for the role of *Otx2* in these tissues awaits further experiments, such as the conditional inactivation of *Otx2* or transplantation experiments using $hOtx1^{2}/hOtx1^{2}$ anterior mesendoderm as donor tissue.

4. Brain patterning depends on a critical *Otx* gene dosage

The function of *Otx2* within the anterior neuroectoderm has been the focus of numerous studies and the amount of data available is compelling. It is now clear that *Otx* genes are absolutely indispensable in the anterior neural ectoderm for maintenance of fore- and midbrain regions and for normal specification of signaling centers, such the IsO at the mid-hindbrain boundary, and the anterior neural ridge at the rostral most limit of the neural plate. At a molecular level, it seems likely that the overall function of *Otx* genes within the anterior neuroectoderm, either directly or indirectly, is to antagonize posteriorizing determinants, such as *Gbx2* and *Fgf8*, and to provide territorial competence to the fore- and midbrain regions to respond to local signals.

4.1. Signaling centers in the anterior neuroectoderm: the isthmic organizer (IsO) and the anterior neural ridge (ANR)

Patterning of the vertebrate neural plate is dependent upon signals produced by discrete organizing centers. In mouse, signals from the anterior visceral endoderm (AVE) and the node and its derivatives are responsible for the initial induction and early maintenance of anterior patterning (Beddington and Robertson, 1999; Stern, 2001). Subsequently, maintenance and refinement of regionally restricted identities is believed to occur through the formation of compartments where positional identity is maintained by polyclonal cell population with restricted cell lineages (Lumsden, 1990; Figdor and Stern, 1993; Lumsden and Krumlauf, 1996). Local signaling centers with polarizing and inductive properties develop

within the broadly regionalized neuroectoderm in genetically defined positions and operate to refine local identities (Meinhardt, 1983; Rubenstein et al., 1998; Joyner et al., 2000; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). Two signaling centers have been so far identified and correspond to the anterior neural ridge (ANR), at the junction between the most anterior neural plate and the non-neural ectoderm (Shimamura and Rubenstein, 1997; Houart et al., 1998) and the isthmic organizer (IsO), which develops within the neural plate at the mid-hindbrain boundary (MHB) (Martinez et al., 1991). Among other signaling molecules, both centers express the *Fgf8* gene.

In mouse, embryological and genetic evidence suggests that the ANR and *Fgf8* expression in this domain are important for forebrain development. Ablation of the ANR, and tissue recombination experiments in mouse and rat suggest that this signaling center is required for the induction and/or maintenance of *Bf1*, a gene required for proper development of forebrain and for the specification of dopaminergic neurons in the rostral brain. FGF8 can substitute for the ANR in both these respects (Shimamura and Rubenstein, 1997; Ye et al., 1998), and mouse embryos carrying a hypomorphic *Fgf8* allele display variable forebrain reduction, including small telencephalic vescicles (Meyers et al., 1998).

A remarkable amount of data has been collected on the morphogenetic properties of the IsO and molecules involved in its development. Organizing property of the IsO was originally discovered in transplantation experiments. When transplanted into the caudal forebrain or rostral hindbrain of chick embryos, the MHB tissue is able to induce ectopic midbrain or rostral hindbrain structures (Alvarado-Mallart et al., 1990; Martinez et al., 1991; Puelles et al., 1996). Midbrain and cerebellum-inducing activity that characterizes the IsO has hitherto been demonstrated only for FGF8 (Crossley et al., 1996; Martinez et al., 1999). FGF8-soaked beads implanted into the caudal diencephalon, are able to induce ectopic midbrain and cerebellum structures by modifying the fate of the host tissue surrounding the bead and to activate mid-rostral hindbrain gene expression. Therefore, the FGF8 molecule is capable of inducing forebrain restricted (ANR) or mid-rostral hindbrain specific (IsO) gene expression, suggesting the existence of a differential territorial competence in responding to the same signal.

In mouse, by the end of gastrulation, *Otx2* is expressed along the presumptive fore- and midbrain region, with a sharp posterior border adjacent to the anterior border of the *Gbx2* expression domain, which, in turn, defines the prospective anterior hindbrain (Wassarmann et al., 1997). Subsequently, at somitogenesis, the transcription factors *En1*, *Pax2*, *Pax5* and *Pax8*, and the signaling molecules *Wnt1* and *Fgf8* are transcribed in broad domains across the *Otx2*/*Gbx2* border. Later in development, their expression domains sharpen and refine around the MHB. Specifically, *Wnt1* and *Fgf8* are expressed in two narrow rings within the *Otx2* and *Gbx2* expression domains, respectively, thus defining the anterior and posterior border of the MHB, whilst *En1*, *Pax2*, *Pax5*, and *Pax8* are expressed in a wider domain comprising the MHB as well as the caudal midbrain and rostral hindbrain (Joyner et al., 2000; Simeone, 2000; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001).

Transplantation and FGF8-soaked bead experiments as well as genetic studies in mouse and zebrafish have provided insights into the function and interactions of these molecules in IsO development. Altogether, these previous studies have indicated that maintenance of IsO activity and transduction of its inducing properties require a positive loop involving *Fgf8*, *Wnt1*, *En1*, and *Pax* genes, whilst positioning of the IsO is defined by negative interactions between *Otx2* and *Gbx2* (Joyner et al., 2000; Simeone, 2000; Garda et al., 2001; Liu and Joyner, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001).

The analysis of different *Otx2* mouse models has provided insights into the mechanisms underlying the formation of the important signaling centers in the brain (ANR and IsO), as well as those responsible for the differential competence of the fore- and midbrain territories to respond to FGF8 signaling.

4.2. Analysis of Otx1 and Otx2 compound mutants

Original evidence suggesting the crucial role of adequate levels of OTX proteins in anterior neuroectoderm for normal fore- and midbrain development were obtained from the analysis of mouse models carrying different dosages of *Otx1* and *Otx2* genes (Acampora et al., 1997). It was shown that mice carrying a single functional *Otx2* allele in an *Otx1* null background ($Otx1^{-/-}$; $Otx2^{+/-}$), gastrulated normally but the posterior diencephalon and midbrain were transformed into an expanded cerebellum and pons. In these mutants, *Otx2* repression in the fore- and midbrain regions was paralleled by the co-ordinated anterior displacement of isthmic organizer markers such as *Fgf8*, *Wnt1*, *Pax2*, and *Gbx2*. Low levels of OTX proteins in these compound mutants led primarily to an anterior expansion of *Fgf8* expression domain at 8.5 dpc which triggered the repression of *Otx2* expression and anterior expansion of other IsO markers such as *Wnt1*, *Pax2*, and *Gbx2*. The outcome of this re-patterning process is that the isthmic region is repositioned at the level of the posterior diencephalon of $Otx2^{+/-}$; $Otx1^{-/-}$ mutant embryos at 10.5 dpc. In fact, FGF8b soaked beads implanted in the midbrain of chick embryos lead to repression of *Otx2* and ectopic generation of an isthmic region (Martinez et al., 1999). Moreover, misexpression of FGF8b in the midbrain of transgenic mouse embryos caused repression of *Otx2* expression in the midbrain and led to a transformation of the midbrain into hindbrain-like tissue (Liu et al., 1999a). In mouse embryos double heterozygotes for *Otx1* and *Otx2* ($Otx2^{+/-}$; $Otx1^{+/-}$), a similar but less severe repatterning process of the midbrain was observed, which was influenced by the genetic background (Suda et al., 1997; Martinez Barbera et al., 2001). These results suggest that an adequate *Otx* gene dosage is required to maintain *Fgf8* expression at the mid-hindbrain boundary.

4.3. Gbx2: Otx2 antagonizing functions

The relevance of *Gbx2* in driving this anterior to posterior transformation was evidenced by a midbrain-restricted repression of *Otx2* expression in transgenic

embryos ectopically expressing *Gbx2* under the *Wnt1* promoter (Millet et al., 1999). Conversely, mouse embryos lacking *Gbx2* showed an early and permanent expansion of *Otx2* expression domain into the rostral hindbrain (Wassarmann et al., 1997; Millet et al., 1999). Likewise, rostral hindbrain was transformed into posterior midbrain in transgenic embryos ectopically expressing *Otx2* under the *En1* promoter (Broccoli et al., 1999). All together, these analyses demonstrate that the antagonizing activities of OTX2 and GBX2 are required for positioning of the *Fgf8* expression domain and the IsO itself at the MHB in the interface between the *Otx2* and *Gbx2* expression domains (Fig. 3).

As mentioned previously, $hOtx1^{2}/hOtx1^{2}$ mutant embryos, in which the *Otx2* gene was replaced by the human *Otx1* cDNA, recovered the early gastrulation defects observed in $Otx2^{-/-}$ mutants and exhibited a normal initiation of anterior neural patterning. However, as no hOTX1 protein was detectable within the anterior neural ectoderm and anterior mesendoderm, $hOtx1^{2}/hOtx1^{2}$ mutants lacked the fore- and midbrain regions by 8.5 dpc (Acampora et al., 1998b) (Fig. 3). The lack of anterior neural structures was consequence of an early repatterning process which was observable at late head-fold stages by an anteriorization of the *Gbx2* expression domain a few hours prior to the onset of *Fgf8* expression at the mid-hindbrain boundary (Martinez Barbera et al., 2001).

Otx2 is not only important for positioning of the IsO, but also controls the differential competence of anterior neuroectoderm to respond to local signals emanating from the IsO and the ANR. Mouse embryos double deficient for *Otx2* and *Gbx2* ($hOtx1^{2}/hOtx1^{2}$; $Gbx2^{-/-}$) have been generated recently (Li and Joyner, 2001; Martinez Barbera et al., 2001). In these embryos, the regional markers *Wnt1*, *Fgf8*, *En*, *Pax2*, *Gbx2*, and *hOtx1* are broadly co-expressed in the anterior neuroectoderm, suggesting that *Otx2* and *Gbx* are both required for early segregation of fore-, mid-, and rostral hindbrain identities (Fig. 3). The finding that *Fgf8* is broadly co-expressed confirms the idea that neither *Otx2* nor *Gbx2* are required for *Fgf8* activation, but rather their antagonizing activities are necessary for restricting the *Fgf8* expression domain at the MHB. In spite of the abundant *Fgf8* expression, the anterior neuroectoderm failed to express the specific fore- and midbrain markers *Bf1* and *Atx* respectively, and no morphologically distinguishable fore- or midbrain structures were formed. In contrast, mouse embryos carrying a hypomorphic *Otx2* allele in a $Gbx2^{-/-}$ mutant background, showed an overall improvement of the regionalization of the anterior neuroectoderm and fore- and midbrain development (Martinez Barbera et al., 2001).

In conclusion, these mouse models support the idea that: (i) a minimum threshold level of OTX proteins are required within the anterior neuroectoderm to repress either directly or indirectly, the hindbrain markers *Fgf8* and *Gbx2*; (ii) the antagonizing activities of *Otx2* and *Gbx2* are required for positioning the *Fgf8* expression domain at the mid-hindbrain boundary in the interface between *Otx2* and *Gbx2* expression domains (Joyner et al., 2000; Simeone et al., 2000; Wurst and Bally-Cuif, 2001); (iii) *Otx2* is required for providing territorial competence to the anterior neuroectoderm to respond to signals emanating from the IsO and the ANR.

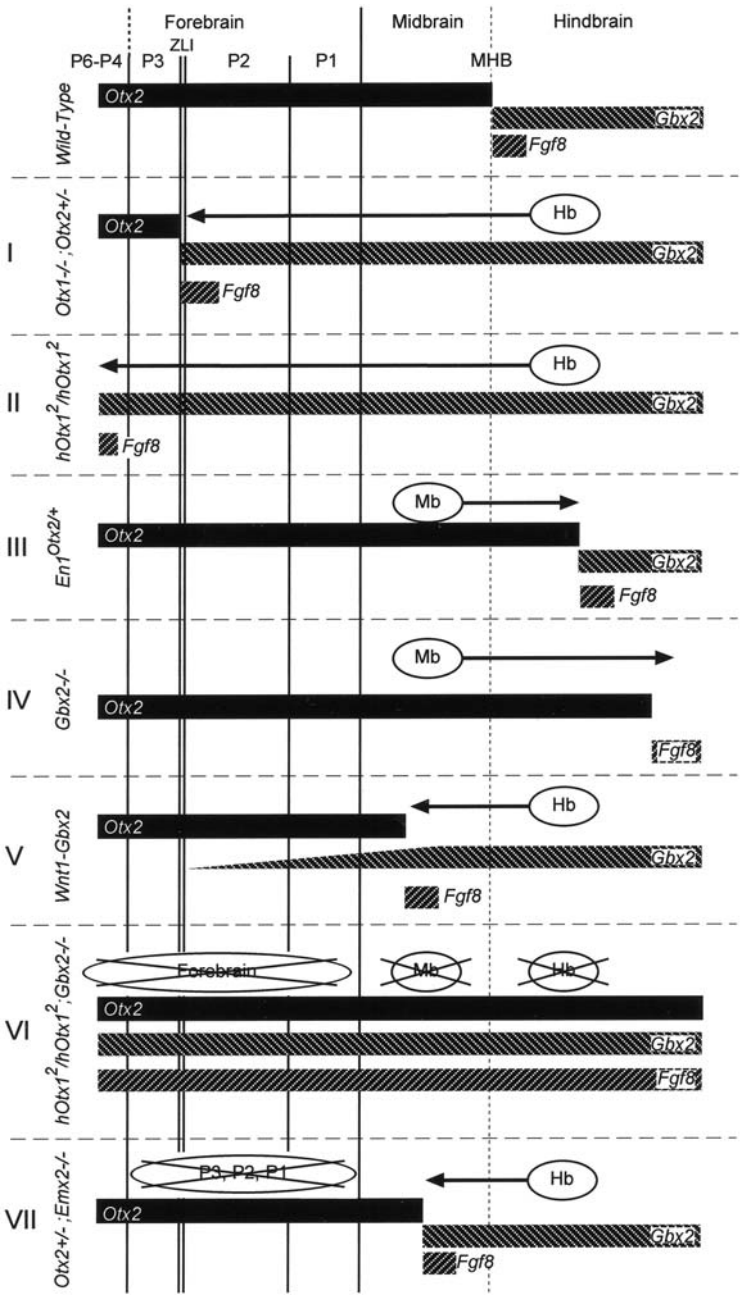

Fig. 3. Schematic representation of the expression pattern of *Otx2*, *Gbx2*, and *Fgf8* in single (II, IV) and double mutants (I, VI, VII). Two additional mutants ectopically expressing *Otx2* under the *En1* transcriptional control (III) or *Gbx2* under the *Wnt1* transcriptional control (V) are also shown. In general, reduction of OTX proteins or *Gbx2* ectopic expression throughout the midbrain result in anterior

5. Multiple roles of the *Otx1* gene in the developing and adult mouse

5.1. *Otx1* expression in early embryogenesis

Otx1 starts to be expressed at early stages (2–5 somite stage, 8.2–8.5 dpc) in the developing mouse embryo throughout the presumptive forebrain and midbrain neuroepithelium (Simeone et al., 1992). From these stages onwards its expression largely overlaps with that of *Otx2*, but while the expression of the latter disappears from the dorsal telencephalon since 10.5 dpc (Simeone et al., 1993), *Otx1* expression is maintained uniformly across the ventricular zone (VZ) of the cortical anlage from the onset of corticogenesis up to mid- to late gestation stages (Simeone et al., 1993; Frantz et al., 1994).

Otx1 is also expressed at early stages in precursor structures of sense organs corresponding to the olfactory placode, otic and optic vescicles (Simeone et al., 1993). Later on, *Otx1* is transcribed in the olfactory epithelium, the saccule, the cochlea, and the lateral semicircular canal of the inner ear as well as in the iris, the ciliary process in the eye and the lachrymal gland primordia (Simeone et al., 1993). From the birthday onwards, *Otx1* is also expressed at a relatively low level in the anterior lobe of the pituitary gland (Acampora et al., 1998c).

5.2. *Otx1* expression during corticogenesis

The cerebral cortex develops according to molecular strategies that determine the fate of precursor cells linked to specific neuronal phenotypes. Two main processes have been identified so far: laminar determination, by which committed cells migrate to their appropriate layer, and cortical areas formation, by which cortical neurons interact to create functionally distinct regions. During corticogenesis, postmitotic neurons migrate along radial glial cells (Rakic, 1972), through the overlying intermediate zone (IZ), and to the cortical plate (CP), which will later create the typical layered organization of the adult cortex. The layers are generated in an inside-out pattern, in which cells of the deepest layers (6 and 5) are born first in the VZ, and those of the upper layers (4, 3, and 2) progressively later (Rakic, 1974). *Otx1* represents a molecular correlate of deep layer neurogenesis and its expression is confined to neurons of layers 5 and 6 in the adult cortex (Frantz et al., 1994).

At mid-late gestation, much transcription of *Otx1* occurs only in ventricular cells, which at these stages are precursors of deep layer neurons. By the time upper layer neurons are generated, *Otx1* expression decreases in the VZ and becomes progressively prominent in the cortical plate, which consists of postmigratory

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expansion of rostral hindbrain at the expense of midbrain (I, V) or midbrain and forebrain (II). In contrast, reduction of GBX2 protein or ectopic expression of *Otx2* throughout the rostral hindbrain result in a posterior expansion of the midbrain at the expense of the hindbrain (III, IV). Mutants lacking both GBX2 and OTX2 fail to segregate early forebrain, midbrain, and rostral hindbrain territory (VI). $Otx2^{+/-}$; $Emx2^{-/-}$ mutants lack prosomeres 1,2,3 and exhibit a moderate expansion of the hindbrain. Hb, Hindbrain; Mb, Midbrain; P1-6, prosomeres 1 to 6; ZLI, zona limitans intrathalamica.

neurons of layer 5 and 6. *Otx1* is absent in later differentiated neurons of upper layers 1–4 (Frantz et al., 1994).

Thus, the progressive downregulation of *Otx1* in the ventricular cells suggests that *Otx1* may confer deep-layer identity to young neurons. Heterochronic transplantation experiments have demonstrated that the broad differentiative potentials of the early progenitors (McConnell and Kaznowski, 1991) become progressively restricted over time (Frantz and McConnell, 1996).

Indeed, *Otx1* expression is heterogeneous across the regions of the adult cortex, suggesting that it might also be involved in the forming of the cortical areas. Its expression in layer 5 is more prominent in the posterior and lateral cortex but absent in the frontal, insular, and orbital cortices, while in layer 6 it is more uniform throughout the neocortex (Frantz et al., 1994).

Mouse mutant models have been generated and analyzed (Suda et al., 1996; Acampora et al., 1996, 1998a, 1998c; Morsli et al., 1999; Weimann et al., 1999; Sancini et al., 2001) to gain insight into the different roles that *Otx1* plays during brain, cortex, and sense organ development (Table 1).

5.3. *Otx1 is required for correct brain development*

Heterozygous ($Otx1^{+/-}$) mice are healthy and their intercross generates homozygous mice ($Otx1^{-/-}$) in the expected Mendelian ratio. However, about 30% of the mutants die before the first postnatal month, and appear smaller in size.

$Otx1^{-/-}$ adult brains are reduced in weight and size and, at the anatomo-histological inspection, show reduction in thickness of the dorsal telencephalic cortex, a dorsally displaced sulcus rhinalis and shrunken hippocampus with a divaricated dentate gyrus. The cortex is particularly affected at the level of the temporal and perirhinal areas, where a 40% reduction in cell number is detected. Furthermore, in these same areas, cortical layer organization is less evident (Acampora et al., 1996), although the expression of layer-specific molecular markers demonstrates that the laminar identities are preserved (Weimann et al., 1999).

The origin of the overall reduction of the $Otx1^{-/-}$ brains has been investigated through experiments aiming to determine possible changes of the normal number of apoptotic or proliferating cells within the neuroepithelium of the developing telencephalon. No differences in apoptosis were observed by comparing wild-type and *Otx1* mutant embryos. By contrast, Bromodeoxyuridine (BrdU) labeling experiments revealed a reduction of proliferating cells (by about 25%) in the dorsal telencephalic neuroepithelium of 9.75 dpc $Otx1^{-/-}$ embryos (Acampora et al., 1998a). A defective proliferation of neuronal progenitors at these early stages may thus be responsible for the adult phenotype of the *Otx1* mutant mice.

5.4. $Otx1^{-/-}$ *mutant mice exhibit an epileptic phenotype*

$Otx1^{-/-}$ mice exhibit both spontaneous high speed turning behavior and epileptic behavior (Acampora et al., 1996). The latter consists of the combination of: (i) focal

Table 1
Major phenotypes observed in $Otx1^{-/-}$; $hOtx2^{1}/hOtx2^{1}$ and otd^{1}/otd^{1} mutant mice

Major phenotypes	$Otx1^{-/-}$	$hOtx2^{1}$/ $hOtx2^{1}$	otd^{1}/otd^{1}
Dorsal telencephalon			
Cell proliferation rate at E9.75	reduced by 25%	full recovery	full recovery
at E13.5 and E15.5	reduced by 10%	full recovery	full recovery
Cerebral Cortex			
Cell number	reduced	full recovery	full recovery
Layer organisation	altered	full recovery	full recovery
Laminar fate[a]	normal	N/A	N/A
Temporal cortex	reduced by 40%	full recovery	full recovery
Perirhinal cortex	reduced by 40%	full recovery	full recovery
Hippocampus	shrunken	full recovery	full recovery
Mesencephalon			
Size of colliculi	enlarged	normal in 30% intermediate in 45%	normal in 15% intermediate in 50%
Identities of colliculi[a]	normal	N/A	N/A
Cerebellum	abnormal foliation	recovery in 50%	recovery in 10%
Axonal projection from layer 5 neurons of visual cortex[a]	defective refinement of exuberant projections	N/A	N/A
Behavior			
Turning behavior	high-speed	moderate-speed	moderate-speed
Epileptic seizures	present	absent	absent
Pituitary gland	impaired	normal	normal
Ear			
Lateral semicircular duct	absent	absent	absent
Eye			
Iris	reduced	recovery in 80%	recovery in 80%
Ciliary process	absent	present in 70%	present in 80%
Lachrymal and Harderian gland	absent	present in 75%	present in 34%

N/A = not analyzed.
[a]Data from Weimann et al. (1999).

seizures characterized by automatisms (head bobbing and teeth chattering) and electroencefalographic (EEG) recording of spikes in hippocampus; (ii) generalized seizures characterized by convulsions and high voltage synchronized EEG activity in hippocampus and cortex. Occasionally, convulsions are followed by status epilepticus and exitus.

Recently, the epileptogenic mechanisms accounting for these seizures have been further investigated by means of electrophysiological recordings (current clump intracellular recordings) made from layer 5 pyramidal neurons in somatosensory cortical slices (Sancini et al., 2001). This analysis shows that γ-aminobutyric acid (GABA)-mediated inhibitory post-synaptic potentials (IPSP), as compared to

control pyramidal neurons, are more pronounced in the mutants where they seem to be involved in the synchronization of the excitatory activity from the earliest postnatal period. On the other hand, multisynaptic excitatory post-synaptic potentials (EPSP) are significantly more expressed in the mutants than in controls, also at the end of the first postnatal month. These results suggest that the excessive excitatory amino acid-mediated synaptic driving, without a well-developed GABA counteraction, may lead to a hyperexcitable condition that is responsible for the epileptic manifestations occurring in *Otx1*$^{-/-}$ mice.

5.5. *Otx1 controls cortical connectivity to subcortical targets*

Recent analysis of axonal projections in *Otx1*$^{-/-}$ mutants has shed new light on the role of *Otx1* during brain development (Weimann et al., 1999). In several brain regions, connections usually develop by a biphasic mechanism in which an excess of early formed axon projections is finely pruned by elimination of inappropriate axon terminals. Layer 5 neurons in the visual cortex provide a good example of exuberance in connectivity, establishing, among others (e.g. to corpus callosum), connections to a number of subcortical targets such as the pons, superior and inferior colliculi, and spinal cord. During early postnatal life, they selectively eliminate connections from the inferior colliculus and spinal cord (Stanfield et al., 1982; Stanfield and O'Leary, 1985), but the molecular mechanisms underlying this remodeling are poorly understood.

Otx1 is strongly expressed in a subset of layer 5 neurons that form subcortical but not cortical connections (Weimann et al., 1999) and in layer 6 neurons, many of which forming thalamic projections. Analysis of *Otx1*$^{-/-}$ mutants reveals significant defects, specifically in the patterning of subcortical projections. In fact, while callosal and thalamic projections appear normal in the *Otx1*$^{-/-}$ mutants, there are additional extensive innervations of both the inferior colliculus and the spinal cord that have been erroneously maintained. This phenotype suggests that *Otx1* function is required for the last step of subcortical axon development, in which exuberant connections undergo extensive refinement with the elimination of axon projections from inappropriate targets (Weimann et al., 1999). This is supported by the fact that OTX1 is retained in the cytoplasm of progenitor cells and undergoes nuclear translocation during the first week of postnatal life, a time that corresponds to the onset of axon remodeling by layer 5 neurons (Zhang et al., 2002).

5.6. *Otx1 transiently controls GH, FSH, and LH in the pituitary*

Otx1 is postnatally transcribed and translated in the pituitary gland. Cell culture experiments indicate that *Otx1* may activate transcription of the growth hormone (GH), follicle-stimulating hormone (β-FSH), luteinizing hormone (β-LH), and α-glycoprotein subunit (α-GSU) genes. Analysis of *Otx1* null mice (Acampora et al., 1998c) indicates that, at the prepubescent stage, they exhibit transient dwarfism and hypogonadism due to low levels of pituitary GH, FSH, and LH hormones, which, in turn, dramatically affect downstream molecular and organ targets. Nevertheless,

Otx1$^{-/-}$ mice gradually recover from most of these abnormalities, showing normal levels of pituitary hormones with restored growth and gonadal function at 4 months of age. Expression patterns of related hypothalamic genes such as the growth hormone releasing hormone (GRH), gonadotropin releasing hormone (GnRH), and their pituitary receptors (GRHR and GnRHR) suggest that, in *Otx1*$^{-/-}$ mice, hypothalamic and pituitary cells of the somatotropic and gonadotropic lineages appear unaltered and that it is the ability to synthesize GH, FSH, and LH, rather than the number of cells producing these hormones, to be affected (Acampora et al., 1998c).

An intriguing aspect of our observation is the fact that transcription factors of the *Ptx* and *Otx* subfamilies recognize similar DNA target sequences (Simeone et al., 1993; Lamonerie et al., 1996; Szeto et al., 1996; Tremblay et al., 1998), and that *Ptx1* and *Ptx2* are expressed in most pituitary lineages, in particular, in somatotrophic and gonadotrophic cells (Tremblay et al., 1998). *Ptx1* is the most highly expressed of these genes, followed by *Ptx2* and then *Otx1* (Tremblay et al., 1998). Yet, the *Otx1* knock-out has a dramatic effect only during the prepuberal period. The unique activity of *Otx1* during this period might reflect a specific interaction of *Otx1*, but not of the related *Ptx* factor(s), with a transcription co-regulator in the somatotrophic and gonadotrophic cells. Taken together with previous reports, our observations support the existence of complex regulatory mechanisms defining combinatorial cell- and stage-specific interactions between transcription factors belonging to the same or to different gene families for the establishment/maintenance of pituitary function.

A novel feature of this phenotype is the fact that most of the impaired functions are recovered by the adult stage. Indeed, after the prepubescent stage, *Otx1* mutant mice begin to gradually recover from their abnormalities, showing at 4 months of age normal levels of GH, FSH, and LH which are paralleled by a restored normal body weight, differentiation and size of both testis and ovary, as confirmed also by their sexual fertility, and by normal levels of downstream molecular targets such as testosterone and insulin growth factor 1 (IGF1). Although we are unable to explain the mechanism underlying this recovery, this observation might represent a possible example of temporal-restricted competence in hormonal regulation of specific cell-lineages by the *Otx1* transcription factor. This recovery appears similar to the "catch-up growth" (Boersma and Wit, 1997) described in children with delayed growth and puberty, also called "constitutional delay in growth and adolescence," CDGA (Horner et al., 1978).

5.7. *Otx1 is necessary for correct sense organ development*

Otx1$^{-/-}$ mutants show inner ear and eye abnormalities that are consistent with *Otx1* expression pattern (Acampora et al., 1996). *Otx1* is expressed in the lateral canal and ampulla as well as in a part of the utricle, in the saccule and in the cochlea. Interestingly, *Otx2* is co-expressed with *Otx1* in the saccule and cochlea but not in the components of the pars superior. Lack of *Otx1* results in the absence of the lateral semicircular canal and lateral ampulla, in abnormal utriculosaccular and

cochleosaccular ducts and in a poorly defined hook (the proximal part) of the cochlea (Acampora et al., 1996; Morsli et al., 1999). Defects in the shape of the saccule and cochlea are variable in *Otx1*$^{-/-}$ mice and are much more severe in *Otx1*$^{-/-}$; *Otx2*$^{+/-}$ background. In *Otx1*$^{-/-}$ and *Otx1*$^{-/-}$; *Otx2*$^{+/-}$ mutants the lateral crista is absent and the maculae of the utricle and saccule are partially fused (Morsli et al., 1999).

In the eye and annexed structures *Otx1* transcripts are restricted to the iris, ciliary process, and ectodermal cells migrating from the eyelid and included in the mesenchymal component of the lachrymal glands. These ectodermal cells are believed to induce differentiation of the mesenchymal cells into a glandular exocrine cell-type. In *Otx1*$^{-/-}$ mice the ciliary processes are absent, the iris is thinner and the lachrymal and Harderian glands do not develop, failing the differentiation to a glandular cell-type. Interestingly, the ectodermal cells embedded within the mesenchymal components are not identified in *Otx1*$^{-/-}$ mice, thus indicating that failure in development of the glands is a consequence of the impaired migration of the ectodermal cells from the eyelid to the mesenchyme of the lachrymal primordium that in turn is not induced to differentiate into the exocrine glandular phenotype (Acampora et al., 1996). This phenotype is more pronounced, in *Otx1*$^{-/-}$; *Otx2*$^{+/-}$ mice and in 30% of double heterozygous *Otx1*$^{+/-}$; *Otx2*$^{+/-}$, with early gross eye malformations, including lens defects. The optic vesicle is unable to fold properly and the reticular pigmented epithelium is not established, generating an ectopic neural retina. This evidence proves that a minimal dosage of *Otx* genes is required for the proper formation of the eye (Martinez Morales et al., 2001).

6. *Otx* conserved functions throughout evolution

6.1. Cloning of Otx-related genes: from Cnidarians to Mammals

Otx-related genes have been isolated from a wide range of organisms (Fig. 4). From the *Drosophila* member, founder of the *Otx* family, vertebrate homologs have been firstly identified by cross-homology or degenerated PCR and then many other descendents followed, starting in the evolutionary scale from as early as Cnidarians. Most of the species, up to protochordates seem to have only one member of this family, with few exceptions where duplication might be considered an event which occurred in independent lineages (Li et al., 1996; Umesono et al., 1999).

Cnidarians are primitive metazoans with a defined body plan and radial symmetry. In these organisms the *Otx* function is associated to muscle contraction or to cell movements involved in the formation of new axes, rather than in the formation of the head (Muller et al., 1999; Smith et al., 1999). Rising in the evolutionary scale, *Otx* has been found in animals with primitive bilateral symmetry such as planarians (Stornaiuolo et al., 1998; Umesono et al., 1999). In the planarian *Dugesia tigrina*, *Otx* expression has been found in regeneration blastemas after transverse sectioning, with an asymmetric pattern of transcripts more abundant in head regenerating tissues (Stornaiuolo et al., 1998). The expression of the *Otx* genes

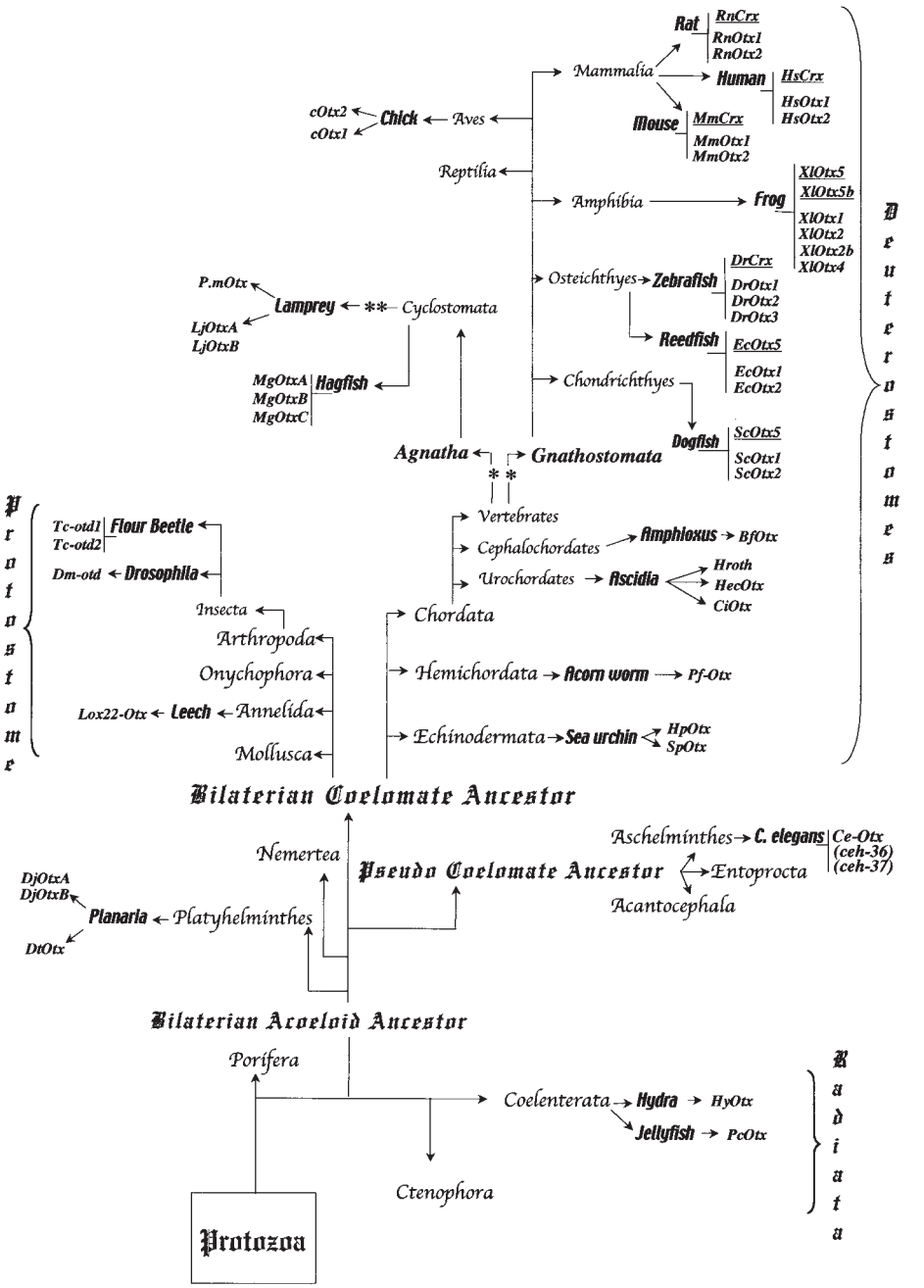

Fig. 4. A simplified phylogenetic tree of the Metazoa indicating the major phyla and species where *Otx*-related genes have been isolated. The asterisks point to the two possible positions of the first *Otx* duplication from a single ancestral gene. The presence of only one *Otx*-like gene in protochordates suggests that the *Otx* duplication observed in the flour beetle *Tribolium castaneum* and in the planaria *Dugesia japonica* are likely independent events occurred in these organisms. Members of the recently established *Crx* gene family are underlined.

in these early metazoans reveals some features in common with chordate *Otx* genes. Although not directly correlated with a defined anterior structure, the primitive function seems to deal with patterning body axis and making tissues competent to respond to anteriorizing signals (Smith et al., 1999), at least in budding and regeneration processes which involve cell movements.

Three *Otx*-related genes have been identified in the nematode *C. elegans* (Ruvkun and Hobert, 1998; Galliot et al., 1999). However, only one of them can be confidentially aligned to a true *Otx* prototype.

The first animal with an *Otx* expression clearly associated to anterior patterning belongs to annelids (the leech *Helobdella triserialis*) (Bruce and Shankland, 1998). This study is particularly relevant in evolutionary terms, since it supports the idea of the origin of bilaterians from radial ancestors (Brusca and Brusca, 1990). The passage from radials to bilaterians might have occurred through specification and subsequent expansion of a trunk precursor cell-population from one side of the radial ancestor. The leech *Otx* expression, which is radially organized around its mouth may represent a reminiscence of the *Otx* expression in a radial ancestor, thus suggesting that, if not recruited to specify trunk structures, relegation of head genes to head domain occurred in bilaterians as a consequence of their evolutionary origin.

Otx expression in insects will be discussed more in detail in a separate section.

The adult pentaradial symmetry shown by sea urchin seems to contradict the general head-associated *Otx* expression rule. It is generally held that the radial symmetry in echinoderms is a shared derived character (synapomorphy) because of their embryonic and larval bilateral symmetry (Lowe and Wray, 1997). Thus the highly divergent and non-head specific *Otx* expression in sea urchin can be justified as a consequence of the highly modified body plan of this phylum.

Comparative studies have demonstrated the existence of *Otx*-related genes in hemichordates (Harada et al., 2000) and in all chordates (Simeone et al., 1992, 1993; Frantz et al., 1994; Li et al., 1994; Mori et al., 1994; Bally-Cuif et al., 1995; Blitz and Cho, 1995; Mercier et al., 1995; Pannese et al., 1995; Kablar et al., 1996; Germot et al., 2001) including urochordates (Wada et al., 1996; Hinman and Degnan, 2000; Hudson and Lemaire, 2001), cephalochordates (Williams and Holland, 1996), and agnathan vertebrates (Ueki et al., 1998; Tomsa and Langeland, 1999; Germot et al., 2001), where they are expressed in the rostralmost CNS, independently of the complexity acquired by this area during evolution. The critical evolutionary position of *lampreys* will be discussed below, mainly with regard to *Otx* gene duplication.

In urochordates and cephalochordates, only one *Otx* gene has been identified so far that may be related to *Otx2* (Wada et al., 1996; Williams and Holland, 1998). Indeed, in addition to similarities in amino acid sequence and rostral expression, they are both expressed during gastrulation in endoderm cells, which suggests that their oldest and primary role might be to mediate signals required to specify anterior neuroectoderm. Restriction of an early widespread expression of *Otx2*-like genes to the anterior CNS is a remarkable feature of all vertebrates, which can be already observed in Ascidians and Amphioxus. This led to hypothesize homology between

fore–midbrain territory of vertebrates to the less-evolved sensory vescicle and cerebral vescicle of Ascidians and Amphioxus, respectively (Williams and Holland, 1998). Some authors have also extended this homology further backwards in evolution. As previously mentioned, it has been proposed that a primitive *Otx* function could be associated with muscle contraction and cell movement rather than with anterior patterning (Muller et al., 1999; Smith et al., 1999). In Drosophila, *otd* expression is not restricted to the head but is also extended to the ventral midline. An early role of the ascidian *Otx2*-related gene *Hroth* has been suggested in the inhibition of notochord and muscle cell fate or in the interference with notochord cell movements (Wada and Saiga, 1999). Similarly, data both in frog (Blitz and Cho, 1995; Pannese et al., 1995; Morgan et al., 1999) and mouse (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996; Zakin et al., 2000; Perea-Gomez et al., 2001) suggest a possible involvement of *Otx2* in controlling the cell movements occurring during gastrulation. Thus, the role of *Otx* has been modified and adapted during evolution through intermediate steps leading to the present condition, but some of its primitive functions might have been kept notwithstanding they are not very obvious.

Conservation of the expression domains among *Otx2*-like orthologs from low chordates (Wada et al., 1996; Williams and Holland, 1996; Ueki et al., 1998) to vertebrates (Acampora and Simeone, 1999 and references there in) is consistent with the low rate of divergence of their alignable sequences.

The duplication event generating the *Otx1* branch from the ancestor *Otx2*-like gene in gnathostome vertebrates cannot be dated precisely and will be discussed below. However, comparative sequence analysis clearly indicates that *Otx1*-like genes evolve more rapidly, as also shown by a further duplication event occurred in both *Xenopus* (Kablar et al., 1996) and *Zebrafish* (Mori et al., 1994), and by a ratio of sequence divergence higher than *Otx2*-like genes (Williams and Holland, 1998). These data can be reinforced by the profound changes in the expression domains of different vertebrate *Otx1*-like genes (Simeone et al., 1993; Mori et al., 1994), which underlie rapid evolution of regulatory sequences as well.

Similarly, another rapidly evolving gene family, clearly originated by a recent *Otx* duplication, has to be considered the *Crx* family (Fig. 4). Members have been originally found only in mammals, where null mutant mice and mutational analysis in humans has shown their involvement in the differentiation of retinal photoreceptors and in the control of circadian entrainment (Freund et al., 1997; Furukawa et al., 1999). These roles are consistent with their extremely specialized eye- and epiphysis-restricted expression. Recently, *Xlotx5/5b* genes in *Xenopus*, *ScOtx5* in the dogfish, *Scyliorhinus caniculata* and *DrCrx* in zebrafish have been cloned and their expression analyzed (Kuroda et al., 2000; Vignali et al., 2000; Liu et al., 2001; Sauka-Spengler et al., 2001). The emerging phylogenetic reconstruction allowed to assign them *bona fide* to the *Crx* gene family. Hence, the specific sensory functions of the *Otx5/Crx* genes, as compared to the vertebrate *Otx* genes, have been probably fixed during evolution before the splitting of chondrichthyan and osteichthyan lineages. Interestingly, both *Xenopus* and dogfish homologs show only a transient or weak mRNA expression in the

neuroectoderm which is reminiscent of the wider *Otx*-pattern in all vertebrates. Since expression of *Crx* is completely lost in mammalian neuroectoderm, the condition of *Xenopus* and dogfish can represent an interesting intermediate evolutionary step where it will be interesting to test whether their neuroectodermal expression is paralleled by protein expression.

In sum, *Otx* expression profiles are consistent with functions in different aspects of anterior patterning. This has been largely confirmed by cross-phylum experiments (see below), in which different members of the family have been swapped in mutant background and were found to be equivalent. According to a recently proposed phylogenetic tree (Adoutte et al., 1999), the establishment of an anterior patterning of the central nervous system is strictly associated to the acquisition of bilateral symmetry. *Otx* genes have been identified in all of the three phyla of bilaterians in which this tree has been divided, supporting this link. The presence of an *Otx*-related gene in cnidarians, a radial symmetry species, might represent an exception, but in this case functional data should be awaited.

6.2. *Otx1 and Otx2 functional equivalence*

When comparing *Otx1* and *Otx2* amino acid sequence and their expression patterns, the correspondent mutant phenotypes are compatible with two possibilities: (i) the functional properties of *Otx1* and *Otx2* may be very similar and it is their different temporal and spatial transcriptional control to be responsible for the highly divergent phenotypes of $Otx1^{-/-}$ and $Otx2^{-/-}$ mice; or (ii) OTX1 and OTX2 proteins may display unique functional properties specified by their limited amino acid diversity. In order to distinguish between these two possibilities, mice models that replace *Otx1* with the human *Otx2* (*hOtx2*) cDNA and vice versa were generated.

Replacement of *Otx1* with the human *Otx2* (*hOtx2*) cDNA ($hOtx2^{1}/hOtx2^{1}$), despite a reduced expression of the transgenic allele, allows the mutants to recover from both epilepsy and corticogenic abnormalities caused by the absence of *Otx1*. This rescue was shown to depend on the capability of the hOTX2 protein to restore a normal cell proliferation activity in the dorsal neuroepithelium of the presumptive telencephalon, a region of the brain from where it normally disappears early in development (Acampora et al., 1999a).

$hOtx2^{1}/hOtx2^{1}$ mice also show a significant improvement of mesencephalon, eye and lachrymal gland defects. Some of the $Otx1^{-/-}$ inner ear abnormalities are also rescued in the regions where the *Otx* genes are normally co-expressed, but not the absence of the lateral semicircular canal (see below) (Table 1).

As previously mentioned, homozygous mutant mice in which *Otx2* has been replaced with the human *Otx1* (*hOtx1*) cDNA ($hOtx1^{2}/hOtx1^{2}$) recover the early induction of the anterior neural plate and proper gastrulation but fail to maintain fore-midbrain identities, displaying a headless phenotype from 9.0 dpc onwards (Acampora et al., 1998b). A combined in situ and immunohistochemical analysis has revealed that despite RNA was detected in both VE and epiblast, the hOTX1

protein was synthesized only in the VE, where it was sufficient to rescue VE-restricted *Otx2* functions.

However, these studies could not address the question of whether OTX1 is functionally equivalent to OTX2 also in the embryonic neuroectoderm and ame, due to the lack of protein in these tissues. In a similar mouse model carrying a different construct, Suda and colleagues have provided some evidence that OTX1 is only partially equivalent to OTX2 in the epiblast derivatives (Suda et al., 1999). These data do not seem to be conclusive, since the amount of OTX1 expressed in the neuroectoderm was not directly compared to that of OTX2 in these mice. This is an important issue, since depletion of OTX gene products below a critical threshold is always accompanied by a mis-specification of fore- and midbrain regions (Suda et al., 1997; Acampora et al., 1997, 1998b; Broccoli et al., 1999; Millet et al., 1999; Simeone, 2000). Indeed, preliminary data from a third mouse model recently generated in our lab indicate that an *Otx1* cDNA can fully rescue absence of *Otx2* when provided with complete *Otx2* UTR sequences (D. A. and A. S., unpublished).

In sum, these mouse models support an extended functional equivalence between OTX1 and OTX2 proteins and provide evidence that the apparently in contrast phenotypes of the $Otx1^{-/-}$ and $Otx2^{-/-}$ mutants, indeed stem from differences in their spatio-temporal expression patterns rather than in amino acid sequences.

As all the rules have exceptions, *Otx* genes have their own too. Their overall functional equivalence is not applicable to the lateral semicircular canal of the inner ear, that in $hOtx2^{1}/hOtx2^{1}$ mice is never restored (Morsli et al., 1999) as well as in mice in which *Otx1* is replaced with the *Drosophila otd* gene (Acampora et al., 1998a). These findings, discussed below in evolutionary terms, suggest that the ability to specify the lateral semicircular canal of the inner ear may be, indeed, an *Otx1*-specific property (Acampora and Simeone, 1999).

6.3. *Head evolution, lessons from Drosophila*

Cloning of several master genes conserved throughout evolution and their comparative expression analysis has been largely used as a means to identify homologous body regions and molecular mechanisms among animals of different phyla (Abouheif et al., 1997). The most sensational example of such a functional conservation is represented by the *Pax-6*/*eyeless* gene, which in both *Drosophila* and vertebrates controls eye development (Callaerts et al., 1997).

Until few years ago, it was generally assumed that the CNS of protostomes (gastroneuralia) and chordates (notoneuralia) had evolved independently (Garstang, 1928; Lacalli, 1994). This was mainly due to the opposite position of the nerve cord along their respective dorso-ventral (D/V) axis. One theory, the so-called "auricularia hypothesis," postulated the homology between the outer ectoderm of the insect embryo and the chordate CNS, based on both anatomical studies made in echinoderms (auricularia larvae) and urochordates, and on comparative expression of the HOX genes. On the other hand, almost two centuries ago, Geoffroy Saint-Hilaire had instead postulated the homology between protostome ventral nerve cord and vertebrate dorsal nerve cord. Over the last decade a number

of debated theories have alternated, supporting one or the other possibility (Arendt and Nübler-Jung, 1994; Lacalli, 1995; Peterson, 1995).

Recently, the combination of morphological, embryological, and molecular evidence seems to definitely favor a common phylogenetic origin of the CNS. New hypotheses have been formulated about how and when the inversion might have taken place, thanks to studies also involving species that occupy intermediate positions in evolution, such as enteropneusts and echinoderms (Nielsen, 1999; Gerhart, 2000). Comparative morphological and topographical analysis of several embryonic landmarks has indicated the ontogenetic movement of the vertebrate mouth as an important clue to support a common phylogenetic origin of protostome and chordate CNS (Nielsen 1999).

With the advent of an increasing number of molecular probes, it has been shown that the overall arrangement of neuroblasts in three longitudinal columns on either side of the midline in both insect and vertebrate CNS (Doe and Goodman, 1985; Chitnis et al., 1995) is paralleled by conserved topographical expression (although with inverted D/V polarity) of pairs of homologous genes (*NK-2*/*NK-2.2*; *ind*/*Gsh* and *Msh*/*Msx*) (Arendt and Nübler-Jung, 1996; D'Alessio and Frasch, 1996; Weiss et al., 1998).

Similarly, and even more convincing was the functional equivalence shown by *Drosophila short gastrulation* and *decapentaplegic* genes, respectively with *Xenopus chordin* and *Bmp4*, (De Robertis and Sasai, 1996) despite their expression is inverted with respect to their D/V axis. Altogether these data support the existence of a homologous mechanism of D/V patterning in a common ancestor of arthropods and vertebrates and reinforce the hypothesis of an inversion of the D/V axis during evolution (De Robertis and Sasai, 1996; Arendt and Nübler-Jung, 1999).

The concept of homology can be easily extended to the A/P axis, where the insect/vertebrate master genes belonging to the conserved families of HOM/HOX and *otd*/*Otx* genes control, respectively, the development of nerve cord/posterior brain and anterior brain. The parallel continues and strengthens when considering that the rule of the conservation of gross expression patterns also applies to the respect of histological landmarks such as the boundary of metameric units or neuromeres (Reichert and Simeone, 1999) and, as for the HOX genes, also to the intriguing phenomenon of the "spatial co-linearity" (Lumsden and Krumlauf, 1996).

Also for the A/P axis, functional experiments have substantiated the equivalence of HOM/HOX genes suggested by the comparative expression data (Malicki et al., 1990; Zhao et al., 1993; Bachiller et al., 1994).

As for the rostralmost part of the CNS, given the enormous complexity of the brain of the vertebrates as compared to that of a fly, the equivalence of the *otd*/*Otx* genes was not taken for granted at all.

6.4. The Otx/otd functional equivalence as a means to support a common origin of the CNS in bilaterians

The embryonic *Drosophila* brain is composed of two supraesophageal ganglia, each subdivided into three neuromeres. The anterior ganglion is subdivided into

protocerebral, deutocerebral, and tritocerebral neuromeres. The gap gene *otd* is mainly expressed in the anteriormost (protocerebral) neuromere, which is almost entirely deleted in *otd* null embryos (Finkelstein et al., 1990; Finkelstein and Perrimon, 1990; Cohen and Jurgens, 1991; Hirth et al., 1995; Younossi-Hartenstein et al., 1997). This phenotype is due to failure of expression of the *otd* downstream gene *lethal of scute*, one of the proneural genes regulating the above-mentioned three-column arrangement of neuroblasts. Other defects are also observed in the ventral nerve cord and in non-neural structures. Flies that are homozygotes for *Ocelliless* (*oc*), a different *otd* allele, are viable and lack the ocelli (light-sensing organs) and associated sensory bristles of the vertex (Finkelstein et al., 1990). Moreover, in cephalic development, different levels of OTD protein are required for the formation of specific subdomains of the adult head (Royet and Finkelstein, 1995). Despite the overall morphological differences, however, expression pattern and mutant phenotypes of *Drosophila otd* and mouse *Otx* genes can be easily paralleled (see previous sections). Nevertheless, the phylogenetic distance between insect and mammals as well as the poor homology shared by OTD and OTX proteins made the hypothesis of a functional equivalence a real challenge.

To gain insight into the possibility that *otd* and *Otx* genes might share conserved genetic functions during CNS development, we expressed the human *Otx1* and *Otx2* genes in *Drosophila otd* null mutant flies and, as a reciprocal approach, the *Drosophila* cDNA in *Otx1* and *Otx2* null mutant mice. Heat-shock induced expression of hOTX1 and hOTX2 proteins rescues the CNS defects of *otd* null mutant embryos (Leuzinger et al., 1998) as well as cephalic defects of the *ocelliless* mutations, both at the morphological and molecular level (Nagao et al., 1998). Efficacy of the mammalian proteins was also confirmed by their overexpression in a wild-type background, which leads to induction of ectopic neural structures in the fly (Leuzinger et al., 1998). In similar experiments it has been recently shown that the Ascidian *Otx* ortholog is also able to rescue the defects of *Drosophila otd* mutants (Adachi et al., 2001).

On the other hand, when a full-coding *Drosophila otd* cDNA is introduced into a disrupted *Otx1* locus by homologous recombination many abnormalities of the $Otx1^{-/-}$ mice are rescued, regardless of a lower level of OTD (about 30%) as compared to the endogenous OTX1 level (Acampora et al., 1998a). Homozygous knock-in *otd* mice (otd^{1}/otd^{1}) show no significant perinatal death (with respect to a 30% death of $Otx1^{-/-}$ newborns) and, most importantly, they have neither the abnormal behavior nor EEG characteristics observed in the *Otx1* null mutants.

In otd^{1}/otd^{1} adult mice, brain size as well as the thickness and cell number of the temporal and perirhinal cortices, both reduced in $Otx1^{-/-}$ mice, are very similar to wild-type (Table 1). As for the $hOtx1^{2}/hOtx1^{2}$ mutants, the rescue is likely to be ascribed at least in part, to a restored normal proliferating activity of the dorsal telencephalic neuroepithelium at 9.75 dpc (Acampora et al., 1998a). In addition and similarly to its mechanism of action in *Drosophila*, *otd* is also able to rescue the brain patterning abnormalities of the $Otx1-/-$; $Otx2^{+/-}$ double mutants (Acampora et al., 1997) in a dose-dependent manner (Acampora et al., 1998a). The extent of this rescue progressively decreases along the A/P axis, being the posterior mesencephalon

still severely affected with respect to a normal telencephalon and to ameliorated diencephalic and anterior mesencephalic structures. All of these data strongly indicate that regionalization of the brain requires levels of OTX proteins that increase along the A/P axis and are particularly critical at the MHB (Acampora et al., 1999b). A partial rescue is also observed in some sensory and sensory-associated structures such as iris, ciliary processes and Harderian glands.

On the contrary, the lateral semicircular canal of the inner ear (last to be established in evolution) (Fritzsch et al., 1986; Torres and Giraldez, 1998) is never restored in otd^{1}/otd^{1} mice, suggesting that, either it requires higher levels of protein or that specification of this structure is dependent upon an *Otx1*-newly established function. This seems indeed to be the case since, as previously mentioned, in $hOtx2^{1}/hOtx2^{1}$ mice *Otx2* is able to rescue some ear defects in the regions where both *Otx* genes are transcribed but not in the lateral semicircular canal, where only *Otx1* is normally expressed (Acampora et al., 1999a; Morsli et al., 1999).

More recently, we have generated two mouse models in which it is the *Otx2* gene to be substituted by a *Drosophila otd* cDNA, either flanked (otd^{2FL}) or not flanked (otd^{2}) by *Otx2* 5′ and 3′ UTR sequences (Acampora et al., 2001). The otd^{2} model was generated by using the same targeting strategy as in the replacement of *Otx2* with *lacZ* (Acampora et al., 1995) or with the *hOtx1* cDNA (Acampora et al., 1998b). Also in this case, the *otd* mRNA is detected in both AVE and epiblast, whereas the protein only in the VE. The OTD protein, exactly as the hOTX1 protein does, is able to take over all of the *Otx2* functions in the AVE, thus recovering both gastrulation defects and absence of an early anterior neural plate due to lack of *Otx2*. Later on, as in the $hOtx1^{2}/hOtx1^{2}$ mutants, however, otd^{2}/otd^{2} embryos fail to maintain the anteriormost identities of the brain and become headless. These results, as far as limited to the AVE, provide a further proof of functional equivalence, shared by *otd*/*Otx* genes, via the activation of the same basic genetic pathway(s).

The second mouse model (otd^{2FL}) shows that, when provided with *Otx2* complete 5′ and 3′ UTR sequences, the *Drosophila otd* coding sequences is able to rescue the defects due to absence of *Otx2* also in the neuroectoderm (maintenance of anterior patterning). Two considerations are to be made. First, the extent of this rescue, that is not complete with full penetrance, is likely to be dependent on the reduced levels of OTD in the epiblast derivatives, rather than on its specific biochemical properties. In fact a control mouse model, differing from otd^{2FL} only by the presence of the *Otx2* coding region in place of that of *otd* ($Otx2^{2c}$), shows exactly the same variety and penetrance of phenotypes of otd^{2FL} homozygous mice (Acampora et al., 2001). The persistence of the neo-cassette and the lack of introns in these mutant loci are likely responsible for the reduced transgene expression. However, it is still possible that a level of OTD expression comparable to the endogenous OTX2 may not be able to compensate for some peculiar functions of the replaced gene. Second, the molecular basis of the OTD-mediated rescue, that is, its expression in the neuroectoderm, underlies two important functions controlled by *Otx2* UTRs: the nucleo-cytoplasmic export and the epiblast-restricted translation of the *Otx2* mRNA. This control is missing in the

neuroectoderm of both the *hOtx1*^{2} and *otd*^{2} mutant embryos, in which both accumulation of nuclear RNA and impaired translation, monitored as the capability of the mRNA to form efficient polyribosome complexes, have been observed (Acampora et al., 2001).

Interspecies rescues are not unique to *Otx* genes. Interestingly, substitution of a different key gene involved in brain patterning processes with its *Drosophila* ortholog has led to very similar conclusions (Hanks et al., 1998). In fact, *Drosophila engrailed* (*en*) was able to substitute for mouse *Engrailed 1* (*En1*) functions in mid- and hind-brain regions. However, as *otd* (and *Otx2*) for the inner ear defects of the *Otx1* mutants, *en* could not recover the defects of limb development. Therefore, despite a higher degree of homology between EN proteins with respect to OTD/OTX proteins, this rescue reinforces the idea that some of the biochemical properties of highly conserved regulatory genes may be conserved across the two phyla whereas new functions have been acquired by the vertebrate genes during evolution.

The finding that OTD and OTX proteins are able to drive cephalic development through the activation of genetic pathways conserved between the two taxa, reinforces the idea that insect and chordate CNS are indeed homologous structures that originated from a common ancestor and controlled by a common basic genetic program of development (Sharman and Brand, 1998; Acampora and Simeone, 1999; Reichert and Simeone, 1999).

6.5. *Molecular basis of the OTX equivalence: conserved targets or convergent pathways?*

OTD and OTX proteins are highly conserved only in the homeodomain, which represents about one tenth of the whole OTD protein and one-sixth and one-fifth of OTX1 and OTX2 proteins, respectively (Simeone et al., 1993).

Outside the homeodomain, homology is restricted to a few very short sequences. *Drosophila* OTD protein lacks the so-called "OTX tail" (Freund et al., 1997), a conserved motif of about 20 amino acids, which is present in single copy in echinoderm, Ascidian, and Amphioxus *Otx*-related genes, whilst it is tandemly duplicated in all vertebrate OTX proteins at the COOH terminus (Williams and Holland, 1998). OTD also lacks the WSP domain, a hexapeptide clearly conserved from deuterostomes. Thus, while the homeodomains of all the *Otx* orthologs are extremely well conserved and not phylogenetically informative, sequences outside are difficult to align, and in most of the protostomes they appear to be derived. An exception is the flour beetle *Tribolium* (Li et al., 1996), which has kept only the WSP motif. Special mentioning deserves the striking case of the *Otx* ortholog from the jellyfish *Podocoryne carnea* (Muller et al., 1999), which contains both the WSP motif and a rudimentary single tail domain. Its overall structure, more similar to the vertebrate- than to other cnidarian- or protostome-type, clearly indicates that the OTX-prototype was already established in organisms with radial symmetry, where the function could not be associated to head development as previously discussed. The homeodomain-restricted homology shared by OTX

proteins of distantly related species underlines the importance that this DNA binding motif might have in accomplishing OTX functions but, on the other hand it raises the question of which are the target genes common to all these species. In addition, what is the role of the sequences outside the homeodomain? One possibility is that once the basic function of OTX is guaranteed by the homeodomain and possibly by a transcriptional activation/repression domain, the remaining sequences have less selective constraints and are free to diverge to acquire new and specific functions, possibly by gaining new protein–protein interactions. Alternatively, it is the overall tertiary structure of the OTX protein to be conserved, regardless of the differences indicated by the alignments of the primary sequence. In both cases, however, whether the downstream targets or the morphogenetic pathways controlled by *otd*/*Otx* genes are the same or are different but functionally equivalent remains an open question. In this context, a crucial point is to define whether the functional equivalence is only an operative definition or it underlines the presence of different pathways each one specific for each member of the *otd*/*Otx* gene family, which can result in the accomplishment of the same final morphogenetic program. In other words, it should be assessed whether *otd*/*Otx* genes act through the same target(s) or rather operate through different pathways converging on the same final result. The answer to this question will clarify whether or not the equivalence among *otd*/*Otx* members does exist also at the molecular level. Based on the fact that a) OTD and OTX proteins exhibit a remarkable amino acid divergence and that b) OTX2 displays an impressive codogenic conservation not only between individuals of the same species (almost no intra-species polymorphism) but also between different mammalian and/or vertebrate species, we can hypothesize that the profound difference within the coding sequence of OTD and OTX proteins would be reflected on changes of their molecular properties. These changes however, might be equally related both to the selection of the target and to the modulation of the expression of the same target. An additional level of complexity can be certainly provided by gene duplication events. Once duplicated, one copy of a gene retains its original functions, thus ensuring species survival, while the other copy is free to try new routes that might guarantee new selective advantages. This might indeed be the case for developmental key-genes like the members of the *Otx*, *Emx*, *Dlx*, *En*, *Wnt*, *Pax* (and others) families, which are important transcription factors directly or indirectly morphogenetically interconnected. Together, these considerations lead us to suspect that functional equivalence might be only an operative definition employed to justify the recovery of phenotypic impairments observed in the absence of the replaced gene. This, for example, is the case of the head-less phenotype, which is recovered in the *Otx2* replacement by the *otd* gene.

Indeed, the brain vescicle of protochordates has been deeply and suddenly modified in a much more complex brain that has been maintained in its basic topography until mammals. This morphogenetic event might have coincided with duplication, recruitment, and stabilization of conserved genetic functions into new cell-types that in turn, have refined/modified or created new versions of pre-existing developmental pathways possibly by increased combinations of new molecular interactions (Holland, 1999; Acampora et al., 2001). Indeed, it might be that

conserved functions such as those encoded by OTD/OTX proteins were able to perform new roles even while retaining an evolutionary functional equivalence because they have acquired the ability to be expressed in new cell-types. Based on this hypothesis, it is expected that drastic evolutionary events should act on the regulatory control (transcription and translation) of *Otx*-related genes rather than on their coding sequences and the functional studies previously mentioned support this possibility.

In this context, these molecular events, by controlling OTX2 protein level, may have contributed to the increase of the rostral neuroectodermal territory, to the establishment of the MHB and positioning of the IsO.

As previously discussed, exceptions such as the peculiar *Otx1*-associated specification of the lateral semicircular canal highlight the role that newly established domains or even scattered amino acid modifications of a protein (i.e. creation or elimination of a phosphorylation site) might have in evolution.

6.6. *Otx duplication and other routes of evolution*

Changes in regulatory control of gene expression cannot solely explain the molecular basis of interspecies morphological diversity. Recent experiments have indicated a possible fascinating mechanism of limbs evolution through changes in protein domains. By studying the Ubx function in onychophorans (limbs on all segments) and insects (limbs on thoracic segments only), it was shown that the Ubx (Hox-like) protein was able to act either as an activator (onychophorans) or as a repressor (insects) on the master gene *distalless*, which controls limb development. Ubx in these species differs by the presence of an alanine-rich peptide in the *Drosophila* protein, which is able to confer repression properties to the insect protein. Repression of *distalless* in the fly abdomen may be the mechanism to explain how insects have lost the abdominal limbs as compared to other evolutionary related species (Levine, 2002).

Modification in protein functions despite being risky, can be a quick way to achieve substantial changes of morphology. This may be true especially for those organisms that require a rapid adaptation to new environmental situations. Rising the scale, massive gene duplication events have provided a safer way to evolve, likely offering to nature more substrates to gain new selective advantages.

This might indeed be the case for the role played by *Otx1* in the development of some sensory structures. Lack of the horizontal semicircular canal in the inner ear (as well as the utriculo-saccular duct and the eye ciliary processes) of *Otx1* null mutants mice has been hypothesized to represent a form of atavism (Acampora et al., 1996), an issue that has raised the questions of when the ancestral *Otx* gene has duplicated and if the advent of an *Otx1*-like gene perfectly coincides with the establishment of the new inner ear structure(s). Gain of the lateral semicircular canal is clearly associated to the passage, from vertebrate agnathes to gnathostomes. In fact, living jawless vertebrates have only two (vertical) semicircular canals in the inner ear. Despite two *Otx*-like genes have been identified in lampreys and three in

the hagfish, none of them is clearly related to *Otx1*. However, it cannot be excluded that the *Otx* genes evolved by duplication in a common ancestor of agnates and gnathostomes (Fig. 4) and that one of the unclassified cyclostome *Otx* represents a still evolutionary unstable version of an *Otx1*-like gene (Ueki et al., 1998; Germot et al., 2001).

When considering the evolution of the inner ear in a more general scenario, it is interesting to note that for other features it has been easier to reconstruct the phylogenetic origin of modern structures from those belonging to primitive vertebrates. This has been largely possible thanks to the help of jawless fossils (i.e. ostracoderms) which have provided intermediate steps of evolution necessary to reduce the big gap existing between agnathes and gnathostomes when considering living species only. Despite this, a third canal in the inner ear is only formed in jawed vertebrates, both fossils (i.e. placoderms) and extants. The absence of any structures precursor of the lateral semicircular canal in all jawless vertebrate (also fossils) and its likely function for predation might indicate that its establishment was not compatible with attempts leading to imperfect functioning (Mazan et al., 2000).

The sudden appearance of structures like this can be traced back to a gene duplication event followed by diversification of both its expression domains and functional properties. *Otx1* seems to perfectly fit with this theory. In fact, so far *Otx1*-homologs have been cloned only in jawless vertebrates, thus making of this gene a molecular correlate of the lateral semicircular canal development (and possibly of other structures). Neither of the two *Otx*-related genes in lamprey is clearly homologous to the mammalian *Otx1* and none of them is expressed in otic vesicles in mammals. Thus *Otx* duplication might have been accompanied both by gain (and/or modification) of expression territories and by changes of their coding sequences, as indicated by lack of rescue of the lateral semicircular canal observed when *Otx1* is replaced by *Otx2*. OTX1 and OTX2 mainly differ by the insertion of two alanine-rich and histidine-rich peptides present in the OTX1 protein, a feature that sounds similar to the Ubx protein (see above). It is certainly tempting to speculate that one or both of these domains might be responsible for conferring new biochemical properties, some of which might be specifically required for inner ear development. Besides, our unpublished data indicate that one of these OTX1 domains may confer in vitro transcriptional repressor properties.

6.7. *Target genes*

The extensive genetic analysis performed on *Otx* genes in mouse has shed light on the partners and possible targets these genes might be interacting with. In the $Otx2^{-/-}$ embryos, a large percentage of mutants lose expression of the *goosecoid* gene (Acampora et al., 1995), while all of them fail to express the *Lefty1*, *Fgf*-15, and *Dkk1* genes (Zakin et al., 2000; Perea-Gomez et al., 2001). Other genetic evidence is provided by the cascade of interactions occurring at the MHB. *Gbx2*, *Fgf8* and

others are likely candidates to be negatively or positively regulated by *Otx* genes, also in dose-dependent manner (Acampora et al., 1997; Martinez Barbera et al., 2001). Chimera experiments have indicated that *Wnt*, *Rpx*, *R-cadherin*, and *ephrin-A2* genes can be cell-autonomously regulated by *Otx2* (Rhinn et al., 1999). Other experiments both in *Xenopus* and mouse identified as putative *Otx2* targets the *XclpH3* gene and a tropomyosin gene in mouse (Morgan *et al*., 1999; Zakin et al., 2000). These two genes encode similar actin and myosin binding proteins not expressed in muscle. The former (a calponin isoform) could regulate the exclusion of the *Otx2*- expressing cells from convergent extension movement, as previously mentioned, since it can act by preventing the sliding of actin filaments over a myosin substrate. The latter has been identified in SAGE libraries constructed from wild type and $Otx2^{-/-}$ early streak embryos (Zakin et al., 2000) and it turns out that this gene is homologous to the sea urchin *Spec2A* gene which is regulated by the orthologous *SpOtx* (Mao et al., 1994). Involvement of *Otx2* in controlling cell movement and aggregation is also confirmed by overexpression experiments carried out in zebrafish (Bellipanni et al., 2000). Despite the number of potential targets of the *Otx* genes is rapidly increasing, to date for not many of these candidates there is a proof of a direct binding to their regulatory sequences. One of these is *clock*, a gene involved in the circadian rhythm (Green et al., 2001). On the other hand, in vitro studies have indicated that *Otx* genes might co-operate with other transcription factors such as *HNF-3β* and *Lim1* via protein–protein interactions (Nakano et al. 2000). In vivo dissection of functional subdomains of the OTX proteins, either by deletion or swapping, might help to address this issue more directly. Other information will soon be available thanks to the study of compound mutants, which may highlight functions masked by gene redundancy or by cell-type-restricted genetic co-operation. Finally, a different approach has been undertaken recently in *Drosophila*. It takes advantage from the DNA microarray technology, which allowed Reichert and colleagues to identify a huge number of putative *OTD*/*OTX* target genes. The screening, based on overexpression of *Drosophila otd* or human *Otx2* in transgenic flies, has revealed that one-third ($n = 93$) of the genes differentially expressed following heat-shock induced *otd* overexpression were also common to *Otx2* induction. Among these, genes belonging to the ''transcription factors'' and ''enzyme'' categories were found to be more abundantly represented, but surprisingly also in this screening genes involved in binding to actin or microtubules have been identified (Montalta-He et al., 2002). In sum, *Otx* genes seem to regulate an unexpectedly large number of both master and structural genes. Despite at first glance they may appear unrelated, in most of the cases it is still possible to assign them to common genetic pathways, which are consistent with the *Otx* functions.

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