REVIEW ARTICLE

Developmental functions of the Distal-less/Dlx homeobox genes

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Accepted 5 July 2002

SUMMARY

Distal-less is the earliest known gene specifically expressed in developing insect limbs; its expression is maintained throughout limb development. The homeodomain transcription factor encoded by Distal-less is required for the elaboration of proximodistal pattern elements in Drosophila limbs and can initiate proximodistal axis formation when expressed ectopically. Distal-less homologs, the Dlx genes, are expressed in developing appendages in at least six phyla, including chordates, consistent with requirements for Dlx function in normal appendage development across the animal kingdom. Recent work implicates the Dlx genes of vertebrates in a variety of other developmental processes ranging from neurogenesis to hematopoiesis. We review what is known about the invertebrate and vertebrate Dll/Dlx genes and their varied roles during development. We propose revising the vertebrate nomenclature to reflect phylogenetic relationships among the Dlx genes.

Key words: Distal-less, Dlx, extradenticle, homothorax, Meis, Msx, Pbx, Antenna, Audition, Brain, Branchial arch, Dentition, Ear, Hematopoiesis, Interneuron, Leg, Limb, Neural crest, Neural tube, Olfaction, Placode, Telencephalon, Tooth, Tricho-dento-osseous syndrome (TDO), Split hand/split foot malformation (SHFM), GABAergic, Otic

DROSOPHILA DISTAL-LESS

Distal-less function

Distal-less (Dll), as its name suggests, is required for distal limb development. Drosophila mutants lacking Dll function die as embryos because they lack the rudimentary larval limbs (Cohen and Jurgens, 1989). Viable combinations of Dll alleles of increasing severity can be used to generate a phenotypic series in which weak allelic combinations lead to fusions of the distal leg segments or tarsi (Fig. 1); intermediate combinations result in loss of the tarsal segments; and stronger combinations cause loss of both the tarsi and a medial leg segment, the tibia (Cohen et al., 1989; Dong et al., 2000; Sunkel and Whittle, 1987). Mitotic clonal analysis was used to generate clones of cells null for Dll in the distal leg. Small Dll null clones in the distal leg delaminate from the disc epithelium and form vesicles within the leg (Gorfinkiel et al., 1997; Wu and Cohen, 1999). In addition, when the behavior of cells in the Dll null clones was observed during the larval stages, they were found to segregate from distal imaginal disc epithelium and to migrate towards the presumptive proximal cells (Wu and Cohen, 1999). These results confirm that Dll is required for the specification of distal leg pattern elements, and indicate that Dll regulates the expression of as yet unknown molecules required for differential affinities between proximal and distal cells.

The Drosophila antenna is a second appendage in which Dll is required for development of the proximodistal (PD) axis. However, in the antenna, Dll has a second function, that of specifying antennal identity (Fig. 1) (Cohen et al., 1989; Dong et al., 2000; Sato, 1984; Sunkel and Whittle, 1987). This is of particular interest because the antenna is both the ear and nose of the fly, and vertebrate Dlx genes have been implicated in both ear (Acampora et al., 1999; Depew et al., 1999; Solomon and Fritz, 2002) (reviewed by Kraus and Lufkin, 1999) and nose development (Acampora et al., 1999; Akinenko et al., 1994; Depew et al., 1999; Quint et al., 2000; Robinson et al., 1991; Yang et al., 1998; Zhao et al., 2002). It remains to be seen whether Dll and the Dlx genes have similar roles during the development of these organs. Other limb-derived structures in which Dll is required include the mouthparts (Cohen and Jurgens, 1989) and the analia (Gorfinkiel et al., 1999). It is noteworthy that vertebrate Dlx genes have a prominent role in the development of the mandible and maxilla (Depew et al., 2002). Dll is not required for formation of the PD axis of the wing.

In addition to functioning during adult appendage development, the Drosophila Dll gene is required for the formation of parts of the peripheral nervous system. In Dll-null animals, the larval antennal, maxillary and labial sense organs do not form, nor do the mechanosensory vestigial larval legs called ‘Keilin’s organs’. Mutations in vertebrate Dlx genes...
appear to affect the development of peripheral nervous system as well (Qui et al., 1995). WhetherDll is required in the central nervous system of the fly is unknown, although it is expressed in both the optic lobe of the brain (Fig. 2) (Kaphingst and Kunes, 1994) and in the glial cells of the ventral nerve cord (J. B. Skeath and G. P., unpublished). The vertebrate Dlx genes have major roles in forebrain development (Anderson et al., 1997a; Anderson et al., 1997b; Marin et al., 2000; Pleasure et al., 2000) (K. Yun, S. J. J. Fischman, M. Hrabe de Angelis, G. Weinmaster and J. L. R. R., unpublished).

**Dll expression and its regulation**

The initiation of Dll expression in the embryonic leg primordia (imaginal discs) represents the first specific marker of *Drosophila* leg formation (Cohen, 1990; Goto and Hayashi, 1997). At this stage, Dll is expressed in both presumptive proximal and distal cells of the adult leg as well as in cells that will give rise to the rudimentary larval leg or Keilin’s organ (Fig. 3) (Campbell and Tomlinson, 1998; Weigmann and Cohen, 1999). Dll expression is dynamic and subsequently becomes restricted to presumptive Keilin’s organ and distal leg cells (Cohen, 1990; Goto and Hayashi, 1997; Weigmann and Cohen, 1999). The regulation of Dll expression also is dynamic. For example, Dll activation in the embryo requires activity of the Wnt family member, Wingless (Wg) (Cohen, 1990), and is repressed both by a bone morphogenetic protein (BMP) homolog, Decapentaplegic (Dpp) (Goto and Hayashi, 1997) and by the epidermal growth factor (EGF) signaling pathway (Raz and Shilo, 1993). By contrast, maintenance and refinement of the Dll expression pattern through the larval stages requires cooperative positive inputs from both Dpp and Wg, as well autoregulatory inputs from Dll itself (Diaz-Benjumea et al., 1994; Goto and Hayashi, 1997; Lecuit and Cohen, 1997; Vachon et al., 1992).

In *Drosophila*, Dll expression and limb formation are repressed in the abdomen by products of two Hox genes, *Ultrabithorax* (Ubx) and *abdominal A* (abdA) (Vachon et al., 1992). In more primitive insects such as beetles, only the more posteriorly expressed AbdA can repress Dll (Lewis et al., 2000), while in other arthropods such as myriapods and crustaceans, neither Ubx nor AbdA represses Dll (Averof and Patel, 1997; Grenier et al., 1997; Panganiban et al., 1995). Thus, the repression of Dll by Hox genes apparently was acquired progressively within the arthropod lineage.

**Dll targets**

Several genes lie genetically downstream of Dll in the developing leg, and represent candidate targets for direct regulation by Dll. These include bric a brac (bab) (Campbell and Tomlinson, 1998; Gorftinkiel et al., 1997), spineless (ss) (Duncan et al., 1998), aristaeless (al) (Campbell and Tomlinson, 1998), BarH1/BarH2 (Kojima et al., 2000), Dwnt5 (Eisenberg et al., 1992) and disconnected (disco) (Cohen et al., 1991). The expression of all six is lost in Dll mutants. As might be expected for target genes, their phenotypes represent subsets of the Dll phenotypes. bab-null animals, for example, exhibit tarsal segment fusions (Gott et al., 1993) like those of weak Dll hypomorphic combinations (Cohen et al., 1989; Dong et al., 2000; Sunkel and Whittle, 1987). A seventh target of Dll regulation in the leg is the gene encoding the Notch ligand Serrate (Ser), which is repressed, rather than activated, by Dll in the tarsus (Rauskolb, 2001). The Ser regulation is of particular interest for two reasons. First, although there is strong genetic evidence that Dll represses Ser, this repression must be conditional, because Ser is expressed in rings within the Dll domain. It has been proposed that other Dll targets such as ss and/or bab could override Dll repression (Rauskolb, 2001). However, it could be that Ss and/or Bab convert Dll from a Ser repressor to a Ser activator. A second reason that Ser is an intriguing target is that Dlx genes also have been found to downregulate Notch signaling in the vertebrate nervous system (K. Yun, S. J. J. Fischman, M. Hrabe de Angelis, G. Weinmaster and J. L. R. R., unpublished). Thus, this repression represents a potentially conserved genetic function between Dll and Dlx genes.

Three other genes have been identified as potential targets of Dll activation specifically in the developing antenna. These are spalt (sal) (Dong et al., 2000), dachshund (dac) (Dong et al., 2001) and aortal (ato) (Dong et al., 2002). Intriguingly, all

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**Fig. 1.** Expression and phenotypes of *Dll* in the *Drosophila* antenna and leg. (A) Wild-type adult *Drosophila* antenna. The arista (ar) vibrates in response to sound, putting torque on the third antennal segment (a3), which then rotates. A large chordotonal organ, the Johnston’s organ, inside the second antennal segment (a2) processes and transmits auditory information via the antennal nerve through the first antennal segment (a1) to the brain. The antenna also serves as a major olfactory organ. a3 is covered with olfactory sensilla. (B) *Dll* expression, visualized via use of a β-galactosidase-encoding enhancer trap, in a late pupal antenna. *Dll* is expressed from distal a2 through the arista. (C) A weak combination of hypomorphic *Dll* alleles results in antenna toward leg transformations. Distal a3 and the arista. (D) Wild-type adult *Drosophila* leg. The proximal-most coxa (cx) and distal-most claws (cl) are indicated. tr, trochanter; fe, femur; ti, tibia; t1-t5, first to fifth tarsal segments. (E) *Dll* expression, visualized via use of a β-galactosidase-encoding enhancer trap, in a late pupal leg. *Dll* is expressed in the distal trochanter, weakly in the tibia, and in the tarsal segments. (F) A weak combination of hypomorphic *Dll* alleles results in truncation of distal leg structures.
three of these genes have vertebrate homologs that are expressed in either the limb (sal/SALL1 and dac/Dac) or ear (sal/SALL1 and ato/Math1/Zath1) (Bermingham et al., 1999; Buck et al., 2001; Caubit et al., 1999; Davis et al., 1999; Davis et al., 2001a; Davis et al., 2001b; Hammond et al., 1998; Kohlhase et al., 1998). Thus, it is possible that these genes are evolutionarily conserved Dll/Dlx targets.

### THE VERTEBRATE DLX GENES

#### Dlx gene organization

Dlx genes are found in all chordate phyla. There are six known Dlx genes each in mice and humans (Nakamura et al., 1996; Porteus et al., 1991; Price et al., 1991; Robinson and Mahon, 1994; Robinson et al., 1991; Scherer et al., 1995; Simeone et al., 1994; Stock et al., 1996; Weiss et al., 1994). The mouse and human Dlx genes are found in three convergently transcribed pairs. Each pair is linked to a Hox cluster. For example, in mice and humans, Dlx1 and Dlx2 are linked to Hoxd; Dlx3 and Dlx4 (the latter also known as Dlx7 and Dlx8, see Table 1) are linked to Hoxb; and Dlx5 and Dlx6 are linked to Hoxa (McGuinness et al., 1996; Nakamura et al., 1996; Ozcelik et al., 1992; Simeone et al., 1994; Stock et al., 1996). The intergenic regions of each pair contain some of the enhancer elements (see below). The primitive chordate amphioxus has a single Dlx gene (Holland et al., 1996), whereas the somewhat more advanced tunicates possess at least three Dlx genes (Caracciolo et al., 2000; Gregorio et al., 1995). Lampreys have at least four Dlx genes (Myojin et al., 2001; Neidert et al., 2001). It has been proposed that adjacent duplication of an ancestral Dlx gene, followed by two rounds of genome duplication and a subsequent loss of the Dlx pair linked to Hox could account for the present complement of Dlx genes.

#### Table 1. Suggested nomenclature revisions for some of the vertebrate Dlx genes

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These changes, agreed to by the respective nomenclature committees, make it possible to identify direct orthologs between species by their names. DLX1, Dlx1 and dlx1a are thus direct orthologs. All uppercase (e.g. DLX) refers to human genes. Combined upper and lowercases (e.g. Dlx) refers to mouse genes. All lowercase (e.g. dlx) refers to zebrafish genes. The ‘a’ or ‘b’ at the end of each zebrafish gene name indicates Hox linkage. For example, DLX1 and Dlx1 are linked to the human and mouse Hoxd clusters, respectively, whereas dlx1a is linked to zebrafish hoxa.


mammalian Dlx genes (Ellies et al., 1997b; Neidert et al., 2001).

This scenario is supported by analyses of Dlx protein and nucleotide sequences that indicate there are two general types of Dlx-coding regions: Dlx2, Dlx3 and Dlx5; and Dlx1, Dlx4 and Dlx6 (Ellies et al., 1997b; Stock et al., 1996). Whether there are functional differences between the two groups is not yet known. However, mouse Dlx1 and Dlx2, mouse Dlx5 and Dlx6, and zebrafish dlx3 and dlx4 are partially redundant (Qiu et al., 1997; Robledo et al., 2002; Solomon and Fritz, 2002), suggesting that some key functions are shared between the two types of Dlx-coding regions, even though the encoded protein sequences outside of the homeodomains are fairly divergent. Drosophila and amphioxus Dll are most closely related to Dlx1 (Holland et al., 1996; Stock et al., 1996). Dlx1 may thus be the founding member of the vertebrate Dlx family.

In zebrafish, which have seven Hox clusters (Amores et al., 1998); it is thought that there has been at least partial duplication of the genome beyond that which occurred in the mammalian lineage (Robinson-Rechavi et al., 2001). Consistent with this, there are eight known Dlx genes (Akimenko et al., 1994; Ekker et al., 1992; Ellies et al., 1997b; Stock et al., 1996). Six of these, like their mammalian orthologs, are found in three convergently transcribed pairs. The remaining two are apparently not linked to other Dlx genes. The three convergently transcribed pairs and one of the single Dlx genes are linked to Hox clusters and are likely to have arisen by duplication of genomic segments that included the Hox clusters (Ekker et al., 1992; Ellies et al., 1997b; Stock et al., 1996). Table 1 contains a list of the known DII and Dlx genes, and suggested revisions to the nomenclature that would make it possible to determine from their names which Dlx genes are orthologous to one another.

Each vertebrate Dlx gene has a common exon-intron organization: three exons and two introns (Ellies et al., 1997b; Liu et al., 1997; McGuinness et al., 1996; Price et al., 1991). Each exon contains some coding sequence; the homeobox is split between exons 2 and 3. The Drosophila Dll gene has an intron at the identical location within the homeobox (Ellies et al., 1997b; Vachon et al., 1992). Several of the Dlx genes produce multiple transcripts either due to alternative transcription initiation (e.g. Dlx1) (McGuinness et al., 1996) or due to alternative splicing (e.g. Dlx4, previously Dlx7, and Dlx5) (Liu et al., 1997; Nakamura et al., 1996; Yang et al., 1998). In the case of Dlx5, these encode proteins both with and without the homeodomain and nuclear localization signal (Liu et al., 1997; Yang et al., 1998). Dlx5 protein can be detected in the cytoplasm of some cells in the forebrain (Eisenstat et al., 1999).

**Dlx expression**

Pairs of the murine Dlx genes exhibit similar patterns of expression that are generally conserved in their non-mammalian counterparts (Zerucha et al., 2000) [see elsewhere for exceptions (Quint et al., 2000; Robledo et al., 2002; Solomon and Fritz, 2002)]. To simplify this review, we have focussed our descriptions on the expression of the mouse Dlx genes. During midgestational stages, all six mouse Dlx genes are primarily expressed in ectodermal derivatives: the nervous system and the surface ectoderm. Four of the genes, Dlx1, Dlx2, Dlx5 and Dlx6, are expressed in the central nervous system (Bulfone et al., 1993; Dolle et al., 1992; Eisenstat et al., 1999; Liu et al., 1997; Price et al., 1991; Robinson et al., 1991; Simeone et al., 1994; Yang et al., 1998). Within the neural tube, their expression appears to be highly restricted to the forebrain, where they are expressed in two domains: one diencephalic and one telencephalic. These two domains are also present in chickens, frogs, turtles, zebrafish and lampreys (Fernandez et al., 1998; Myojin et al., 2001; Neidert et al., 2001; Puelles et al., 2000; Zerucha and Ekker, 2000) (L. Puelles, personal communication).
communication). Where it has been studied, their expression follows a temporal sequence: \(Dlx2, Dlx1\) and \(Dlx5\), then \(Dlx6\) (Fig. 4) (Eisenstat et al., 1999; Liu et al., 1997; Zerucha et al., 2000). The general trend is for \(Dlx2\) to be expressed in subsets of ventricular zone neuroepithelial cells. \(Dlx1, Dlx2\) and \(Dlx5\) are expressed together in most subventricular zone cells, while \(Dlx5\) and \(Dlx6\) are expressed in many of the postmitotic differentiating neurons (Eisenstat et al., 1999; Liu et al., 1997). \(Dlx2\) and \(Dlx1\) are also expressed in a more restricted subset of postmitotic neurons. This temporal sequence suggested that a regulatory cascade might exist among the Dlx genes themselves. Analysis of the \(Dlx1/Dlx2\) double mutant confirms that this is the case (see below).

All of the Dlx genes, except zebrafish \(dlx2b\), are expressed in ectomesenchymal cells derived from the cranial neural crest (Akimenko et al., 1994; Bulfone et al., 1993; Davideau et al., 1999; Dolle et al., 1992; Myoin et al., 2001; Neidert et al., 2001; Qiu et al., 1997; Robinson and Mahon, 1994; Simeone et al., 1994; Yang et al., 1998). The migratory neural crest cells populate the branchial arches, which in turn give rise to much of the facial skeleton and connective tissue (Depew et al., 2002). Within the branchial arches, the Dlx genes are expressed in nested patterns along the proximodistal axis. In proximal regions, only \(Dlx1\) and \(Dlx2\) are expressed, in intermediate regions \(Dlx1, Dlx2, Dlx5\) and \(Dlx6\) are expressed, whereas in distal regions all six Dlx genes are expressed. In addition, they exhibit a temporal sequence of expression that is reminiscent of that observed in the forebrain. The overlapping expression patterns suggest that there exist both redundant and distinct functions of the Dlx genes in morphogenesis of the visceral skeleton. This has been confirmed by analysis of the \(Dlx1, Dlx2, Dlx1/Dlx2, Dlx2/Dlx5\) and \(Dlx5\) mutants (Acampora et al., 1999; Depew et al., 1999; Qiu et al., 1997; Qiu et al., 1995) (M. Depew and J. L. R. R., unpublished). Some of the Dlx genes also are expressed in and required for development of other neural crest-derived cells, including the peripheral and enteric nervous systems (Depew et al., 1999; Dolle et al., 1992; Qiu et al., 1995). Indeed, based on expression in the primitive chordate amphioxus, it has been proposed that an ancient function of \(Dlx/Dlx1\) was in specifying or patterning the neural crest (Holland et al., 1996).

In addition to expression in the developing brain and in neural crest derivatives, such as the branchial arches, the Dlx genes are expressed in discrete domains in both neural and non-neural components of the surface ectoderm. For example, during gastrulation expression of \(Dlx3\) (Akimenko et al., 1994; Feledy et al., 1999a; Pera and Kessel, 1999) and \(Dlx5\) (Yang et al., 1998) is observed around the lateral parts of the neural plate. \(Dlx3\) expression in the anterior neural ridge probably correlates to later expression in the olfactory placode. Several of the Dlx genes (\(Dlx2, Dlx3, Dlx5\) and \(Dlx6\)) are also expressed in the otic placode, and later regionally expressed in the otic vesicle (Depew et al., 1999; Ekker et al., 1992; Liu et al., 1997; Quint et al., 2000; Robinson and Mahon, 1994; Zhao et al., 1994). By contrast, in zebrafish, \(dlx3b\) and \(dlx4b\) may be the predominant Dlx genes of the otic placode and vesicle (Solomon and Fritz, 2002). In the developing retina, \(Dlx1\) and \(Dlx2\) are expressed in neuronal precursors and in subsets of neurons (Dolle et al., 1992; Eisenstat et al., 1999) (Eisenstat and J. L. R. R., unpublished). Chick \(Dlx3\) is expressed in optic cup and neural retina (Dhawan et al., 1997). \(Dlx3\) also is broadly expressed in the non-neural ectoderm (Morasso et al., 1996), whereas most Dlx genes are expressed in restricted surface ectodermal domains of outgrowths from the body (appendages) such as the apical ectodermal ridge of the limb bud (Beauchemin and Savard, 1992; Bulfone et al., 1993; Dolle et al., 1992; Ferrari et al., 1995; Morasso et al., 1995; Zhao et al., 1994), genital eminence (Porteus et al., 1994) and branchial arches (Bulfone et al., 1993; Porteus et al., 1994; Qiu et al., 1997; Robinson and Mahon, 1994; Weiss et al., 1994; Zhao et al., 1994).

At later developmental stages, Dlx gene expression is found in differentiating skeletal tissues. The Dlx genes are expressed in both ectodermal and mesenchymal compartments of developing teeth (Depew et al., 2002; Thomas et al., 1995; Zhao et al., 2000). In particular, \(Dlx5\) and \(Dlx6\) are broadly expressed in mesodermally as well as neural crest-derived skeletal tissues (Acampora et al., 1999; Chen et al., 1996; Depew et al., 1999; Ferrari et al., 1995; Ryoo et al., 1997; Simeone et al., 1994; Xu et al., 2001; Yang et al., 1998; Zhao et al., 1994). \(Dlx4\) (previously \(Dlx7\)) also is expressed in other mesodermally derived tissues (hematopoietic cells), where its function is implicated in proliferation and survival (Shimamoto et al., 1997; Shimamoto et al., 2000).

**Trans and cis regulation of Dlx genes**

Efforts to identify the substances, signal transduction pathways, and cis-elements that regulate Dlx expression are just beginning. Gain-of-function experiments demonstrate that sonic hedgehog (Shh) can induce Dlx expression in the forebrain (Gaiano et al., 1999), while mice lacking Shh have greatly reduced levels of \(Dlx2\) expression in the forebrain (Y. Ohkubo, K. Yun and J. L. R. R., unpublished). Bone morphogenetic protein 2 (BMP2) can induce \(Dlx2\) expression in chondrocytes (Xu et al., 2001); bone morphogenetic protein 4 (BMP4) can induce \(Dlx5\) expression in osteoblasts (Miyama et al., 1999), \(Dlx1\) and \(Dlx2\) expression in dental mesenchyme (Beil and Maas, 1998) and \(Dlx3\) in embryonic ectoderm (Feledy et al., 1999a). However, there is evidence in embryonic ectoderm that BMP induction of \(Dlx3\) is not an immediate-early response, as blocking BMP signaling using dominant-negative BMP receptors reduces, without eliminating, \(Dlx3\) expression (Feledy et al., 1999a). Fibroblast growth factors (FGFs) can maintain or induce Dlx expression. Treatment with FGF2 maintains \(Dlx3\) expression in axolotl limb ectoderm following a manipulation (denervation) that ordinarily reduces \(Dlx3\) expression (Mullen et al., 1996) and can induce \(Dlx5\) expression in the developing inner ear (Ladher et al., 2000). FGF8 induces \(Dlx1\) and \(Dlx2\) expression in murine dental mesenchyme (Beil and Maas, 1998) and \(Dlx1\) expression in the chicken mandibular and hyoid branchial arches (Shigetani et al., 2002). Nonetheless, mice with greatly reduced FGF8 expression continue to express \(Dlx1, Dlx2\) and \(Dlx5\) in the branchial arches (Trumpp et al., 1999). Given the large number of FGF family members, and their overlapping patterns of expression, there may be compensatory mechanisms for maintaining Dlx expression in the absence of any single Fgf gene. Retinoids have been implicated in Dlx repression. For example, administration of retinoic acid to zebrafish embryos prior to or during cranial neural crest migration reduces Dlx.
gene expression in ectomesenchymal cells (Ellies et al., 1997a).

Several cis-acting elements of the Dlx genes have been characterized. The largely coincident expression of members of each Dlx pair suggests regulation via shared enhancers. Indeed, the intergenic region of the zebrafish dlx5a (previously dlx4) and dlb6a (previously dlx6) contains enhancer elements sufficient to drive correct forebrain expression (Zerucha et al., 2000). The nucleotide sequence and function of this region are highly conserved between zebrafish and mouse. Thus, a transgene encoding the zebrafish intergenic region drives lacZ-reporter expression in mice that closely resembles that of mouse Dlx5. Furthermore, this enhancer appears to be regulated by Dlx1 and Dlx2 in vivo and in vitro (Stühmer et al., 2002a; Zerucha et al., 2000). The intergenic regions of mouse Dlx1 and Dlx2 and Dlx3 and Dlx4 (previously Dlx7) also appear to contain shared cis elements (M. Ekker, personal communication) (Sumiyama et al., 2002). During zebrafish ear development, dlb3b (previously dlb3) is required for the activation of dlb5a (previously dlb4) (Mendonsa and Riley, 1999). Transcriptional enhancers upstream of mouse Dlx3 (Park and Morasso, 1999) and of its Xenopus ortholog (Xdll2) (Morasso et al., 1995) that drive ectodermal expression of a reporter gene in transgenic mice also have been identified. Sequences 5’ of Dlx2 regulate expression in the surface ectoderm of the branchial arches and limbs (Thomas et al., 2000); these elements are responsive to FGFs and BMPs.

A few other transcription factors also have been implicated in regulating Dlx expression. For example, Msx1 is required to maintain Dlx2 (but not Dlx1) expression in the branchial arch mesenchyme (Bei and Maas, 1998). In zebrafish, ectopic expression of Fez1 (forebrain specific zinc finger) can induce Dlx expression (Yang et al., 2001).

Dlx gene function

The roles of the Dlx genes in vertebrate development have primarily been ascertained through the analysis of loss-of-function mutations in mice and humans, but some gain-of-function information is available. While mice that are homozygous for mutations in individual Dlx genes die during embryogenesis, the tissues expressing these genes generally lack obvious phenotypes when other Dlx genes are normally co-expressed in these regions (Acampora et al., 1999; Anderson et al., 1997a; Anderson et al., 1997b; Depew et al., 1999; Qiu et al., 1997; Qiu et al., 1995). Phenotypes in these tissues are often detectable once mice are generated that lack at least two Dlx genes. This is the case when both mutated genes belong to the same pair, e.g. Dlx1 and Dlx2 (Qiu et al., 1997) or Dlx5 and Dlx6 (Robledo et al., 2002), and also when they are in different pairs, e.g. Dlx2 and Dlx5 (M. Depew and J. L. R. R., unpublished). Similar genetic redundancy also has been observed between the dlb3/dlb4 pair in zebrafish (Solomon and Fritz, 2002) (M. Westerfield, personal communication). Thus, members of the Dlx family appear to have both unique and redundant functions. Examples of these phenomena will be discussed below. The degree of functional compensation between the Dlx genes, particularly in the CNS, will probably impede forward genetic approaches to isolating Dlx mutants, thereby making reverse genetic approaches particularly important in elucidating Dlx gene functions.

Dlx genes control development of ectodermal tissues derived from lateral border of the neural plate

The primitive ectoderm gives rise to a neural plate encircled by surface ectoderm/epidermis. At the border of the neural plate and epidermis lie cells that give rise to placodes, neural crest and the dorsal midline of the neural tube. Several of the Dlx genes are required for development of these tissues. For example, Dlx5 mutants have defects in all of the structures derived from the border cells. Development of the olfactory and otic placodes is abnormal in these animals. Dlx5 mutants exhibit defects in the morphogenesis of the olfactory pit and associated skeletal elements, as well as in differentiation of the olfactory epithelium (Depew et al., 1999) (J. Long, M. Depew and J. L. R. R., unpublished). These mutants also exhibit regionally restricted defects in their inner ear (derivatives of the otic placode), particularly in the semicircular canals (Acampora et al., 1999; Depew et al., 1999). The ectomesenchymal derivatives of the cranial neural crest follow abnormal skeletal morphogenetic programs in Dlx1, Dlx2 and Dlx5 mutants; these are described in more detail below. Finally, the caudal parts of the cranial neural crest contribute to the enteric nervous system, which appears to be abnormal in the Dlx1, Dlx2, and Dlx1/2 mutants (Qiu et al., 1997; Qiu et al., 1995).

Expression of Dlx3 in the surface ectoderm has been implicated in epidermal development. Humans with a four-base deletion in the coding region of DLX3 have a disorder known as tricho-dento-osseous (TDO) syndrome (Price et al., 1998a; Price et al., 1998b) that affects morphogenesis of epidermal derivatives (hair) and other ectodermal derivatives (teeth and craniofacial skeleton) (Lichtenstein et al., 1972). The deletion lies just downstream of the DLX3 homeobox and is predicted to result in a truncated protein with an altered C terminus that can still bind to DNA (Price et al., 1998a). Interestingly, the TDO mutation results in a dominant, although incompletely penetrant, phenotype (Wright et al., 1997), while mice with a total loss-of-function mutation in Dlx3 exhibit a recessive phenotype (Morasso et al., 1999). This suggests that either the mouse and human Dlx3 genes do not have identical functions or the mutant Dlx3 protein produced in individuals with TDO is acting as a dominant-negative. Dlx3-null mouse embryos die in midgestation because of a deficiency in the vascularization of the placenta (Morasso et al., 1999). Dlx3 is expressed in ectodermal components of the developing placenta (ectoplacental cone, chorionic plate and labyrinthine trophoblast) that appear to be present in Dlx3-null mice but are unable to support the ingrowth of the vasculature. The role of Dlx3 in epidermal development also has been studied via overexpression in epidermal basal cells in transgenic mice. This manipulation disrupts skin differentiation by prematurely inducing maturation of the basal cells (Morasso et al., 1996).

The Dlx genes control differentiation of a subset of GABAergic neurons of the basal ganglia and cerebral cortex

Within the forebrain, the expression of the Dlx genes coincides with the location of virtually all neurons that use γ-amino butyric acid (GABA) as their neurotransmitter. This suggests
that the Dlx genes may have a general role in the development of this cell type (Anderson et al., 1997a; Anderson et al., 1997b; Stühmer et al., 2002a; Stühmer et al., 2002b). Indeed, ectopic expression of Dlx2 or Dlx5 in cortical neurons using either retroviral vectors or electroporation, induces expression of the GABAergic phenotype (Anderson et al., 1999; Stühmer et al., 2002a). Consistent with a role for Dlx proteins in the differentiation of GABAergic neurons, Dlx proteins also can activate transcription from a glutamic acid decarboxylase enhancer (B. Condie, personal communication). Glutamic acid decarboxylase synthesizes GABA from glutamic acid. As Dlx1/Dlx2 mutants still express GABA in the subcortical telencephalon (Anderson et al., 1997b), other genes also are involved in the control of GABAergic neuronal development. Candidates include the Mash1 (Ascl1 – Mouse Genome Informatics) bHLH transcription factor, whose expression appears to be upstream of the Dlx genes (Fode et al., 2000).

Dlx1, Dlx2, Dlx5 and Dlx6 are expressed in overlapping sets of cells in the developing forebrain, suggesting potential redundant functions (Bulfone et al., 1993; Eisenstat et al., 1999; Liu et al., 1997). Indeed, while Dlx single mutants have subtle defects in forebrain development (e.g. Dlx2 mutants have reduced numbers of dopaminergic neurons in the olfactory bulb) (Acampora et al., 1999; Anderson et al., 1997b; Depew et al., 1999; Eisenstat et al., 1999; Qiu et al., 1995), the Dlx1/Dlx2 double mutants exhibit a major block in neurogenesis within the subcortical telencephalon (Anderson et al., 1997b; Marin et al., 2000). In the Dlx1/Dlx2 double mutants, the first wave of neurogenesis (from approximately embryonic days 10-12) appears to be undisturbed, whereas differentiation of later born neurons is largely aborted. This leads to abnormalities in the subventricular zone, the region that contains the secondary proliferative population (spp) of neuroblasts. While the primary proliferative population (ppp; which is in the ventricular zone) appears normal, several lines of evidence demonstrate that the spp fails to mature. For example, the mutant spp continues to express Lhx2 (Anderson et al., 1997b), a homeodomain gene whose function is associated with the proliferative properties of the ppp (Porter et al., 1997). In addition, the mutant spp expresses high levels of Notch1 and its ligand Delta1, which are features of the ppp (K. Yun, S. J. J. Fischman, M. Hrade de Angelis, G. Weinmaster and J. L. R. R., unpublished). Indeed, an increase in Notch signaling may participate in blocking differentiation in the Dlx1/Dlx2 double mutants, as there is increased expression of Hes5 (K. Yun, S. J. J. Fischman, M. Hrade de Angelis, G. Weinmaster and J. L. R. R., unpublished). Hes5 encodes a bHLH transcription factor induced by Notch signaling, and is capable of repressing differentiation (reviewed by Kageyama and Ohtsuka, 1999). The failure of the mutant spp to mature also is reflected by the lack of Dlx5, Dlx6 and Otx6/SCIP (Pou3f1 – Mouse Genome Informatics) expression (spp markers), and a block in the radial migration of these cells to the postmitotic zone (mantle) (Anderson et al., 1997b). Thus, the mutant spp partially differentiates, expressing some neuronal markers (e.g. MAP2 and glutamic acid decarboxylase) and forms periventricular neuronal ectopia (Anderson et al., 1997b; Marin et al., 2000).

This block in differentiation not only reduces the production of basal ganglia late-born projection neurons (GABAergic neurons that project to distant targets), it also blocks the development of several types of GABAergic, dopaminergic and cholinergic interneurons (Anderson et al., 1997a; Anderson et al., 2001; Marin et al., 2000; Pleasure et al., 2000; Qiu et al., 1995). A number of studies now suggest that most telencephalic inhibitory interneurons are derived from progenitors in the subcortical telencephalon. Thus, in the Dlx1/Dlx2 double mutant, there is a massive reduction in the GABAergic interneurons of the cerebral cortex (hippocampal complex, isocortex, olfactory cortex and olfactory bulb (Anderson et al., 1997a; Anderson et al., 1999; Anderson et al., 2001; Bulfone et al., 1998; Marin and Rubenstein, 2001a; Pleasure et al., 2000). This is due to the lack of tangentially migrating immature interneurons from the subcortical telencephalon into the cerebral cortex (Anderson et al., 1997a). There are at least two principal subcortical telencephalic sources of these tangentially migrating interneurons. One is in a region that includes the lateral ganglionic eminence (LGE) and the septum. This region appears to produce interneurons that migrate rostromediodorsally to populate the olfactory bulb and perhaps the cerebral cortex (Anderson et al., 1999; de Carlos et al., 1996; Luskin and Boone, 1994; Meyer et al., 1998; Wichterle et al., 1999). The other is from the medial ganglionic eminence (MGE) that produces interneurons that contribute to the striatum and cerebral cortex through a laterodorsal migration (Anderson et al., 1997a; Anderson et al., 2001; Lavdas et al., 1999; Marin et al., 2000; Marin and Rubenstein, 2001a; Marin and Rubenstein, 2001b; Marin et al., 2001; Parravelas, 2000; Parravelas et al., 2000; Pleasure et al., 2000; Tamamaki et al., 1999; Wichterle et al., 1999; Wichterle et al., 2001). Dlx1/Dlx2 double mutants have defects in both migrations, and thus have reduced numbers of striatal (GABAergic and cholinergic), olfactory bulb (GABAergic and dopaminergic) and cortical (GABAergic) interneurons (Anderson et al., 1997b; Bulfone et al., 1998; Marin et al., 2000). Ongoing studies are aimed at elucidating the signals that regulate these long-distance migrations from the subcortical telencephalon to the striatum, olfactory bulb and cortex (reviewed by Marin and Rubenstein, 2001b). For example, neuropilin/semaphorin signaling is implicated in sorting migrating subcortical telencephalic interneurons to distinct target tissues (Marin and Rubenstein, 2001a).

The adult telencephalon contains neural stem cells that are capable of generating olfactory bulb GABAergic neurons (Alvarez-Buylla et al., 2002). The least differentiated cells progenitors are Dlx-gene negative; as these cells mature, they express the Dlx2 (A. Alvarez-Buylla, personal communication). This finding in the adult telencephalon mirrors the fact that during embryogenesis, Dlx expression begins as progenitors migrate from the ventricular zone to the subventricular zone in the subcortical telencephalon (Eisenstat et al., 1999). In vitro primary cell culture analysis also supports this model (He et al., 2001).

Thus, Dlx function is tightly linked to the development of neurons derived from the basal telencephalon that produce GABA, acetylcholine and dopamine (reviewed by Marin and Rubenstein, 2001a; Marin and Rubenstein, 2001b). We therefore hypothesize that hypomorphic or regionally restricted defects in Dlx function might not be lethal, and might alter the function of forebrain GABAergic, cholinergic and dopaminergic neurons. For example, reduced numbers or function of cortical GABAergic neurons could lead to
hyperactivity states, such as seizures, or could result in defects in the functions of local cortical circuits. Likewise, GABAergic dysfunction in the basal ganglia could disrupt the learning and/or deployment of complex motor and cognitive behaviors. \(Dlx\) dysfunction in the diencephalon could disrupt the operation of the hypothalamic-pituitary circuitry and of the thalamus, through \(Dlx\) expression in the reticular nucleus. It is intriguing that two chromosomal regions that are associated with Autism on chromosomes 2q and 7q, contain the \(Dlx1/2\) and \(Dlx5/6\) loci, respectively (IMGSAC, 2001; http://www.well.ox.ac.uk/~maestrin/iat.html). Although there are many genes within the implicated regions, these results underscore the potential roles of these genes in human neuropsychiatric disorders. Thus, future studies aimed at studying neurological function in \(Dlx\) mutant mice have added importance.

The \(Dlx\) genes control patterning of the branchial arch skeleton

The \(Dlx\) genes have a nested pattern of expression in the ectomesenchyme of the branchial arches (Fig. 5) (Acampora et al., 1999; Bullone et al., 1993; Depew et al., 1999; Dolle et al., 1992; Porteus et al., 1994; Qiu et al., 1997; Robinson and Mahon, 1994; Weiss et al., 1994; Zhao et al., 1994). The branchial arches are populated in part by migratory cells from the hindbrain neural crest and subsequently contribute to a variety of head structures including the craniofacial skeleton and the dental mesenchyme (Depew et al., 2002). \(Dlx1\) and \(Dlx2\) are expressed along much of the proximodistal axis of the arches, while other \(Dlx\) genes are expressed more distally. Because mutations of \(Dlx1\) and \(Dlx2\) affect only proximal regions, it has been proposed that there is some functional redundancy among the other \(Dlx\) genes expressed in the distal first and second arches [\(Dlx3, Dlx4\) (previously \(Dlx7\), \(Dlx5\) and \(Dlx6\)] (Qiu et al., 1997; Qiu et al., 1995). Furthermore, it is postulated that \(Dlx3, Dlx4, Dlx5\) and \(Dlx6\) have distinct roles in patterning distal regions of these arches (M. Depew and J. L. R. R., unpublished). Both hypotheses are supported by analysis of \(Dlx5\) mutants (Acampora et al., 1999; Depew et al., 1999), as well as the analysis of compound \(Dlx\) mutants (e.g. \(Dlx2\) and \(Dlx5\); \(Dlx5\) and \(Dlx6\)) (M. Depew, T. Lufkin and J. L. R. R., unpublished). These studies reveal two interesting similarities between the vertebrate \(Dlx\) genes and invertebrate \(Dll\). First, as in the fly limb, \(Dlx\) dose plays a role in controlling the length of the first branchial arch (M. Depew and J. L. R. R., unpublished). Second, as in the fly antenna, \(Dlx\) genes appear to specify the identity of components of the first arch (Qiu et al., 1995) (M. Depew and J. L. R. R., unpublished). For example, in \(Dlx5/6^{-/-}\) mutants, there is a homeotic transformation of the mandibular skeleton into a maxillary skeleton (M. Depew, T. Lufkin and J. L. R. R., unpublished).

\(Dlx\) mutations cause severe craniofacial deformities, including cleft palate, and dysmorphic middle ear and jawbones (reviewed by Depew et al., 2002). These results have implications for human craniofacial disorders. In addition, defects in the \(Dlx2\) mutants are reminiscent of skeletal morphologies of non-mammalian vertebrates (Qiu et al., 1995), although whether they are atavistic has been debated (Smith and Schneider, 1998). Later expression of the \(Dlx\) genes in the ectomesenchymal and surface ectoderm (Thomas et al., 1995; Zhao et al., 2000) contributes to tooth development. For example, \(Dlx1/Dlx2\) double mutants lack upper molars (Qiu et al., 1997; Thomas et al., 1997).

**\(Dlx\) functions in bone and cartilage formation**

\(Dlx5\) and \(Dlx6\) expression in developing bone was first described by Simeone et al. (Simeone et al., 1994). \(Dlx5\) expression is found in the perichondrium, periosteum and in osteoblasts of developing endochondral bones (Acampora et al., 1999; Zhao et al., 1994). \(Dlx5\) also is expressed in differentiating dermal (intramembranous) bones (Depew et al., 1999). \(Dlx5\) mutants exhibit a defect in the structure of the endosteal component of long bone diaphyses and have a reduction in the periosteal lamina (Acampora et al., 1999). In addition, there appears to be a delay in the maturation of

**Fig. 5.** Expression of three type A \(Dlx\) genes (\(Dlx2, Dlx5\) and \(Dlx3\); A-C) and one type B \(Dlx\) gene (\(Dlx1\); D) in E10.5 mouse embryos shown by whole-mount in situ hybridization. The top pictures show a lateral view of the entire embryo (A-D); the bottom pictures show frontal views of dissected branchial arches. Expression of the \(Dlx\) genes is absent from the medial-most regions (these regions are under the control of the \(Msx\) and other genes). (E) Schematic lateral view of an E10.5 mouse embryo, highlighting craniofacial primordia that are under the control of the \(Dlx\) genes: the jaw, otic and olfactory apparatus (the branchial arches, otic vesicle and olfactory placode, respectively). The color wheel in the bottom right corner defines colors that correspond to the expression of type A \(Dlx\) genes: the jaw, otic and olfactory apparatus (the branchial arches, otic vesicle and olfactory placode/pit, respectively).

- **A**: Axial view of E9.5 mouse embryo, showing lateral recess of the brain, nasal pits and branchial arches, with expression of the \(Dlx5\) gene. (Qiu et al., 1997; Qiu et al., 1995).
- **B,C**: Lateral view of E10.5 mouse embryos, highlighting effects of \(Dlx5/6^{-/-}\) (previously \(Dlx3/4^{-/-}\)) mutants on the branchial arches (M. Depew, T. Lufkin and J. L. R. R., unpublished).
- **D**: Lateral view of an E10.5 mouse embryo, showing effects of \(Dlx1, Dlx2, Dlx3, Dlx5, Dlx6\) mutants on the branchial arches (M. Depew, T. Lufkin and J. L. R. R., unpublished).
- **E**: Schematic view of an E10.5 mouse embryo, showing craniofacial primordia that are under the control of the \(Dlx\) genes: the jaw, otic and olfactory apparatus (the branchial arches, otic vesicle and olfactory placode/pit, respectively). The color wheel in the bottom right corner defines colors that correspond to the expression of type A \(Dlx\) genes: the jaw, otic and olfactory apparatus (the branchial arches, otic vesicle and olfactory placode/pit, respectively).
specific dermal bones (Depew et al., 1999). The mechanisms underlying these histological defects are unknown, although there is tissue culture evidence that the Dlx genes can regulate skeletal development through controlling the expression of two collagen genes and osteocalcin (Dodge et al., 1996; Ryoo et al., 1997; Xu et al., 2001).

Dlx functions in limb development

In invertebrates, Dlx function is best understood in the development of appendages, particularly of the limbs. As noted above, all of the vertebrate Dlx genes are expressed in the apical ectodermal ridge of the limb bud, which regulates the patterned outgrowth of the limb. As in the CNS, limb development in the Dlx1, Dlx2 and Dlx5 single mutants appears to be largely normal (Acampora et al., 1999; Depew et al., 1999; Qiu et al., 1997; Qiu et al., 1995). However, compound Dlx mutants have limb defects. While the Dlx1/Dlx2 mutant limb is usually normal, Dlx2/Dlx5 (M. Depew and J. L. R. R., unpublished) and Dlx5/Dlx6 (Robledo et al., 2002) mutants have severe malformations of the distal limb. The Dlx5/Dlx6 mutants have split distal limb defects, similar to ectrodactyly syndromes seen in humans. In this regard, it is interesting that one of the ectrodactyly syndromes, Split Hand/Split Foot Malformation (SHFM), can be caused by mutations in a locus, SHFM1, which is closely linked to the human Dlx5 and Dlx6 genes (Crackower et al., 1996). SHFM is a disorder that can increase in severity in successive generations. Based on this, and the fact that Dlx6 encodes CAG repeats, it has recently been proposed that SHFM might constitute a type of polyglutamine repeat disorder (Ferro et al., 2001). Also intriguing, given the known roles of Dll in Drosophila auditory system development and of the vertebrate Dlx genes in both brain and ear development is that individuals with SHFM exhibit mental retardation and hearing loss.

Fig. 6. Alignments of various Dll and Dlx subdomains. (A) Dll/Dlx homodomain alignments. The consensus represents amino acids conserved among all family members. Dots in the consensus represent non-conserved residues. (B) Extended homodomain alignments of Dll and Dlx proteins. Underlined residues are conserved among two or more Dlx subgroup members or between Dll and Dlx. The consensus represent residues conserved in two or more subgroup members. Dots in the individual amino acid sequences represent gaps introduced to enhance the alignment. Dots in the consensus represent non-conserved residues. Asterisks denote human sequences. (C) Dlx2, Dlx3 and Dlx5 contain DllA domains. This domain originally was defined for various vertebrate Dlx3 proteins (Akimenko et al., 1994; Robinson and Mahon, 1994). Underlined residues are conserved between at least two Dlx proteins. The consensus represents residues conserved in two or more Dlx proteins. Dots represent non-conserved residues. (D) Sequences surrounding the conserved C-terminal tryptophan residues are also partially conserved. Underlined residues are conserved among two or more Dlx subgroup members or between Dll and Dlx. The consensus represent residues conserved in two or more subgroup members. (E) A conserved motif is found upstream of the Dll homeodomain and downstream of the Dlx2 homeodomain. (F) An spp motif is found upstream of the first conserved tryptophan. Underlined residues are conserved among two or more family members. Bold text is used to highlight the abundant serines and prolines.
known function. However, it lies within a larger region of similarity (Fig. 6B-F). For example, all Dll and Dlx proteins possess at least two tryptophan residues that are C terminal to the homeodomain. The first typically is followed by an aspartic acid, while the second is followed by a tyrosine (Fig. 6D). Tryptophan residues embedded in a hexapeptide motif upstream of the homeodomain mediate interactions of Hox proteins with PBC-family co-factors such as Pbx and Extradenticle (Exd) (Chang et al., 1995; Neuteboom et al., 1995; Phelan et al., 1995). A tryptophan residue in a myogenic basic helix-loop-helix (bHLH) transcription factor recently was found to mediate interaction with a PBC family member (Knoepfler et al., 1999). For both Hox and bHLH proteins, the tryptophan interacts specifically with the three-amino-acid-loop-extension (TALE) between helices one and two of the PBC homeodomains (Passner et al., 1999; Piper et al., 1999). Dll and Dlx proteins lack tryptophans upstream of their homeodomains, but it is possible that one of the downstream tryptophans mediates a similar interaction.

The Dll and Dlx proteins are proline-rich both upstream and downstream of their homeodomains. Proline-rich domains have been implicated in a variety of functions, including oligomerization (Xiao et al., 2000) and transcriptional activation (Mermod et al., 1989; Tanaka and Herr, 1990). Indeed, the N-terminal proline-rich sequences of Dlx5 function as an activation domain when fused to the yeast Gal4 DNA-binding domain (Masuda et al., 2001) while the proline-rich N and C termini of Dlx3 cooperate in transcriptional activation in the context of the Dlx3 homeodomain (Feledy et al., 1999b).

In other transcription factors, proline-rich activation domains contact components of the basal transcriptional machinery such as p300 and TFIIID (de Caestecker et al., 2000; Tanese et al., 1991). It therefore is likely that the proline-rich sequences in Dll and the Dlx proteins play roles in transcriptional activation, possibly by recruiting basal transcriptional machinery.

In addition to their homeodomains, tryptophan residues and proline-rich domains, there are several other conserved features of DII/Dlx proteins. For example, there is substantial conservation in the amino acids flanking the homeodomain, particularly within the Dlx1,4,6 and Dlx2,3,5 subgroups (Fig. 6B). Dlx2, Dlx3 and Dlx5 also share a 'DIIA' domain upstream of the homeodomain (Fig. 6C). This domain, first noted by Robinson et al., in Dlx3 proteins from various species (Akimenko et al., 1994; Robinson and Mahon, 1994), has no known function. However, it lies within a larger region implicated in transcriptional activation (Feledy et al., 1999b) and thus is likely to mediate interactions with other proteins. In addition, an unusual motif containing multiple prolines and a cysteine is found upstream of the Dlx2 homeodomain and downstream of the Drosophila Dll homeodomain (Fig. 6E). This motif also has no known function.

Only one type of post-translational modification to a Dll or Dlx protein has been described. That is the phosphorylation of residues within the Dlx3 homeodomain by protein kinase C (PKC) (Park et al., 2001). This phosphorylation, which reduces the DNA binding ability of the homeodomain, is thought to occur normally in vivo in developing keratinocytes and to be regulated by Ca2+ (Park et al., 2001).

Many homeodomain proteins have little DNA-binding specificity on their own and often act in conjunction with other transcription factors that augment both their DNA binding affinity and specificity (reviewed by Mann and Affolter, 1998; Mann and Chan, 1996). Until recently, there were no known co-factors for Dll or any of the Dlx proteins. However, Dlx2 and Dlx5 can complex with the mesodermal homeodomain proteins Msx1 and Msx2 in a tissue culture assay, thereby preventing both Msx DNA binding and Msx transcriptional activation of a reporter gene. This interaction occurs via the Dlx and Msx homeodomains (Zhang et al., 1997). Yeast two-hybrid screens also have identified other potential Dlx interacting molecules including the GRIP1 PDZ protein (Yu et al., 2001) and a protein call Dlxin 1 that has homology to both necladin- and melanoma-associated antigens (MAGEs) (Masuda et al., 2001). In Drosophila, based on cooperative genetic interactions, it has been proposed that the TALE homeodomain protein, Homothorax (Hth) and its PBC class homeodomain partner Extradenticle (Exd) might serve as DII cofactors specifically in the developing antenna (Dong et al., 2000). In vitro and in vivo biochemical analyses have confirmed that Exd and Hth can form complexes with Dll (J. Chu and G. P., unpublished). As these molecules possess multiple vertebrate homologs, Meis1-Meis3 and Prep1 for Hth, and Pbx1-Pbx3 for Exd, it could be that particular complexes of various Dll and Dlx proteins with specific members of these families exhibit unique DNA-binding site preferences and tissue or temporally distinct functions. Consistent with this possibility, specific Dlx genes are coexpressed with specific Pbx and Meis genes in the telencephalon (Toresson et al., 2000).
expressed in the neural ridge (e.g. Dlx3 and Dlx5) could regulate Wnt1.

Dlx3 has been implicated directly in the activation of several genes, including those encoding a human chorionic gonadotropin subunit in the placenta (Roberson et al., 2001) and profilaggrin in differentiating keratinocytes (Morasso et al., 1996). The binding sites through which Dlx3 regulation occurs have been identified (Fig. 7C,D) (Morasso et al., 1996; Roberson et al., 2001). These sites share a TAAT core with the recognition sites for other Dlx (and other homeodomain) proteins.

Dlx4 (previously DLX7) activates both GATA1 and MYC in hematopoietic cells (Shimamoto et al., 1997). Ectopic Dlx4 also can inhibit apoptosis via upregulation of expression of intercellular adhesion molecule 1 and intercellular adhesion molecule 1 is direct. Three isoforms of DLX4 have been identified (Chase et al., 2002; Fu et al., 2001). All encode the homeodomain and bind identical DNA sequences found in β-globin silencer elements, but differ in their ability to repress β-globin transcription (Berg et al., 1989; Chase et al., 2002; Fu et al., 2001). DLX4 downregulation is correlated with hematopoietic stem cell differentiation in culture, and DLX4 is upregulated in some leukemias.

Several targets of Dlx5 have been identified during bone formation. The first of these, osteocalcin, initially was found to be repressed by Dlx5 (Ryoo et al., 1997). However, more recent work suggests that Dlx5 actually is a weak activator of osteocalcin transcription but that it potentiates osteocalcin transcription mainly by interfering with the osteocalcin repressor Msx2 (Newberry et al., 1998). Both Msx2 and Dlx2 can bind to the same recognition sequence in OC-Box 1 (Fig. 7F) (Newberry et al., 1998; Ryoo et al., 1997) Two other genes activated by Dlx5 during bone differentiation are those encoding collagen 1A1 (Dodig et al., 1996) and bone sialoprotein (Benson et al., 2000). The Dlx5-binding sites in the regulatory regions for these genes have been characterized (Fig. 7E,G) (Benson et al., 2000; Dodig et al., 1996). Finally, there is evidence that Dlx2 is involved in BMP-mediated induction of chondroblast differentiation and collagen2a1 expression (Xu et al., 2001).

An important issue when considering vertebrate Dlx functions and targets is the possibility that there are species specificities. For example, in mice, the Dlx5/Dlx6 pair plays a dominant role in ear development, whereas in zebrafish, the dlx3/dlx4 pair seems to carry out an analogous function (Quint et al., 2000; Robledo et al., 2002; Solomon and Fritz, 2002) (M. Westerfield, personal communication). Also, in mice and chick, the anterior neural plate/ectoderm boundary is demarcated early by Dlx5/Dlx6, whereas dlx3 demarcates that boundary in zebrafish (Quint et al., 2000). One explanation for this is that the enhancers responsible for certain aspects Dlx expression already were present in the original pair of Dlx genes, duplicated with the coding regions as the subsequent gene pairs were generated, and then were lost differentially during evolution from particular Dlx gene pairs in distinct vertebrate lineage (Quint et al., 2000). One implication of these differences in the use of Dlx proteins is that key interaction surfaces outside of the homeodomain may be conserved even between paralogous Dlx proteins.

Recent evidence suggests that Dll and the Dlx proteins share targets, i.e. that the genetic hierarchies in which they are involved have been conserved during evolution. For example, in the first branchial arch, Dlx5/6 mutants have abnormal expression of Alx4, Barx1 and Dlx3; this provides evidence for conservation of Drosophila genetic circuitry, as Drosophila Distal-less regulates homologs of these genes (aristaless,
BarH1 and Distal-less itself, respectively) (M. Depew, T. Lufkin and J. L. R. R., unpublished). As mentioned above, targets of Dll in the auditory and olfactory appendage of the fly (the antenna) that represent candidate Dlx targets in the vertebrate ear and/or olfactory system include aonal (mouse homolog Math1), splt (human homolog SALL1) and dachshund (mouse homolog Dach). A target of Dlx proteins in the vertebrate brain that represents a candidate target in the fly brain is GAD. Determining whether any of these targets are shared between flies and vertebrates will be an important area of future research.

The evolution of Dll/Dlx function

The expression of Dll and the Dlx genes in the vertebrate and invertebrate nervous systems led to the proposal that the original function of Dll/Dlx was in the nervous system and that functions such as that of Dll in Drosophila limbs may have arisen much later in animal evolution (Fig. 8) (Panganiban et al., 1997). Several striking commonalities between Dll and Dlx expression have since emerged that ultimately may allow us to pinpoint the ancestral functions of Dll/Dlx more precisely. For example, the requirements for Dll and Dlx in the auditory and olfactory systems and the mouthparts of both invertebrates and vertebrates suggests not only that at least primitive versions of these systems/structures preceded the divergence of these lineages, but also that Dll/Dlx was involved in their formation of the primitive auditory and olfactory systems and mouthparts prior to that divergence. Evidence is emerging that suggests that Dll and Dlx regulate appendage morphogenesis (fly limb and antenna; mouse branchial arch) through both growth and identity specification.

In addition, the positioning of Drosophila thoracic limb primordia expressing Dll at the lateral edge of the neural ectoderm (R. Bolinger and G. P., unpublished) is analogous to the position of the Dlx-expressing neural crest precursors at the edge of the vertebrate neural plate. Intriguingly, common Wnt/Wingless signaling systems are used to induce formation of both the Drosophila limb primordia and the vertebrate neural crest cells, and the Drosophila limb precursors undergo migrations prior to differentiation, as do the neural crest cells. Thus, specialized migratory cell populations derived from the lateral edges of a primitive neural ectoderm/neural plate and expressing Dll/Dlx also are likely to have predated the divergence of invertebrate and vertebrate lineages. Finally, Dll and the Dlx genes are expressed in the brains of invertebrates and vertebrates, respectively. Although several Dlx functions in the vertebrate brain have been described, it remains to be seen whether any are shared by Dll. If so, it would implicate Dll/Dlx in the differentiation of the ancestral central nervous system.

Obviously, care needs to be taken when attempting to draw parallels between vertebrate and invertebrate development, particularly when genes with pleiotropic phenotypes are concerned [see Erwin and Davidson (Erwin and Davidson, 2002) for detailed discussion]. However, if Dll and Dlx targets and co-factors can be identified that are, for example, targets and co-factors only during GABAergic interneuron differentiation in both flies and vertebrates, it would lend strong support to a model in which Dll played a similar role in GABAergic interneuron differentiation in the last common protostome-deuterostome ancestor. Critical avenues of future research therefore will include the identification and comparison of tissue-specific Dll and Dlx targets and co-factors.

The authors thank Marc Ekker for comments on the text, Arturo Alvarez-Buylla, Brian Condie, Michael Depew, Marc Ekker and Monte Westerfield for allowing them to cite unpublished results, and Carol Dizack for assistance with figure making. G. P. is the recipient of a Young Investigator Award in Molecular Studies of Evolution from the Sloan Foundation and the National Science Foundation. This work also was supported in part by NIH grant #GM59871-01A1 to G. P. J. L. R. R. was supported by research grants from: Nina Ireland, NARSAD and NIMH K02 MH01046-01.
REFERENCES


