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REVIEW ARTICLE

Developmental functions of the *Distal-less*/Dlx homeobox genes

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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; Akimenko et al., 1994; Depew et al., 1999; Quint et al., 2000; Robinson et al., 1991; Yang et al., 1998; Zhao et al., 1994). 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 (Qiu et al., 1995). Whether *Dll* 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; Gorfinkiel et al., 1997), *spineless* (*ss*) (Duncan et al., 1998), *aristaless* (*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 (Godt 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 *atonal* (*ato*) (Dong et al., 2002). Intriguingly, all

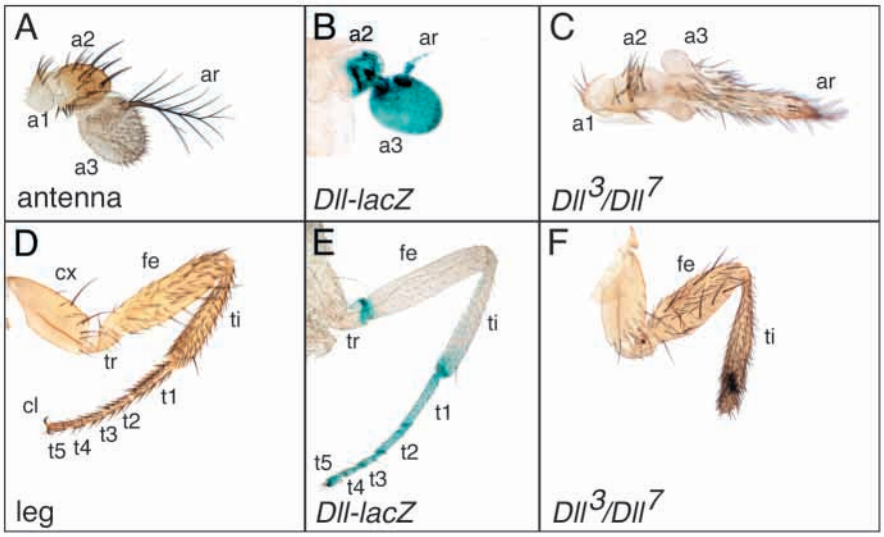

Fig. 1. Expression and phenotypes of *Dll* in the *Drosophila* antenna and leg. (A) Wild-type adult *Drosophila* antenna. The arista (ar) vibrates in response to sound, putting torque on the third antennal segment (a3), which then rotates. A large chordotonal organ, the Johnston's organ, inside the second 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 proximal part of the arista are transformed to medial leg structures. (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.

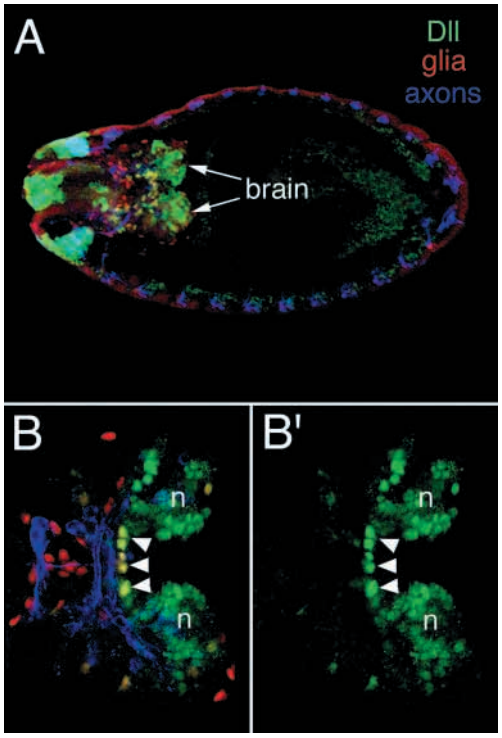

Fig. 2. Expression of *Dll* in the embryonic *Drosophila* brain. Dorsal view of a late stage *Drosophila* embryo stained for Dll (green), the glial marker RK2 (red) and the axonal marker Fasciclin 2 (blue). (A) Low-magnification view of entire embryo with the two lobes of the brain indicated by arrows. (B,B′) High magnification views of the brain where Dll is expressed in neurons (n) in the brain, as well as some of the glia lining a transverse axon commissure. These glia, indicated by arrowheads, also express RK2 and therefore are yellow in B.

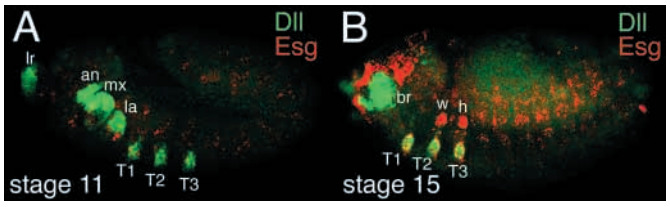

Fig. 3. Expression of *Dll* in embryonic *Drosophila* limb primordia. Lateral views of stage 11 (A) and 15 (B) *Drosophila* embryos stained for Dll (green) and Escargot (Esg; red) protein. Anterior is towards the left. Esg is expressed in imaginal tissues that ultimately give rise to adult structures. Dll is expressed in the first to third thoracic segments (T1-T3) in clusters of cells that give rise to both the leg imaginal discs and the larval Keilin's organs. Dll also is expressed in the precursors of the labral (lr), antennal (an), maxillary (mx) and labial (la) larval sense organs. At stage 15, Dll is expressed in some brain (br) precursors, but appears not to be expressed in head imaginal tissues. The wing (w) and haltere (h) discs express Esg, but not Dll.

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 *Hoxc* could account for the present complement of

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

New name	Original or other name(s)
Human (*Homo sapiens*)	
DLX1	
DLX2	
DLX3	
DLX4	*DLX7*, *DLX8*
DLX5	
DLX6	
Mouse (*Mus musculus*)	
Dlx1	
Dlx2	
Dlx3	
Dlx4	*Dlx7*
Dlx5	
Dlx6	
Zebrafish (*Danio rerio*)	
dlx1a	*dlx1*
dlx2a	*dlx2*
dlx2b	*dlx5*
dlx3b	*dlx3*
dlx4a	*dlx8*
dlx4b	*dlx7*
dlx5a	*dlx4*
dlx6a	*dlx6*

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 *hoxda*.

Mouse gene nomenclature, http://www.informatics.jax.org/mgihome/nomen/
Human gene nomenclature, http://www.gene.ucl.ac.uk/nomenclature
Zebrafish gene nomenclature, http://www.zdb.wehi.edu.au:8282/zf_info/nomen.html

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., 1998; Ellies et al., 1997b; Stock et al., 1996). Table 1 contains a list of the known Dll 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

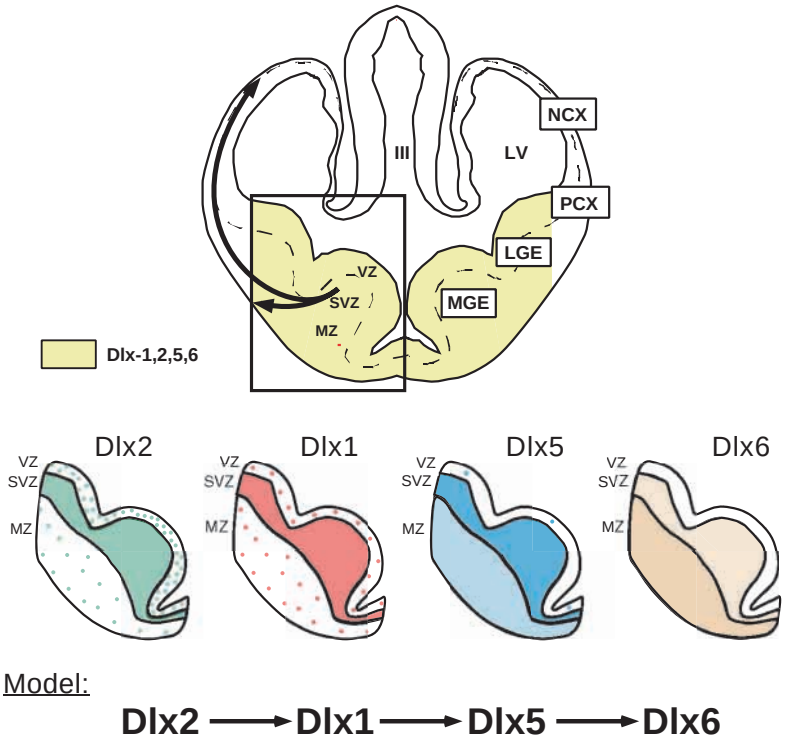

Fig. 4. Expression domains of *Dlx1*, *Dlx2*, *Dlx5* and *Dlx6* during mouse brain development. (Top) Schema of a transverse section through the E12.5 mouse telencephalon showing the combined expression of Dlx transcripts. Most cells in the subpallial telencephalon express *Dlx1*, *Dlx2*, *Dlx5* or *Dlx6* at some stage of their differentiation. The arrows indicate the migration from the subpallium to the pallium (cortex) (Marin and Rubenstein, 2001a). The boxed region on the left is used in the middle section to show the expression of *Dlx2*, *Dlx1*, *Dlx5* and *Dlx6*. *Dlx2* is primarily expressed in undifferentiated cells; it is expressed in scattered cells in the ventricular zone (green dots), in most cells in the subventricular zone (uniform green) and in scattered cells in the mantle zone (green dots). *Dlx6* is primarily expressed in differentiated cells in the mantle zone (uniform peach). *Dlx1* (red) and *Dlx5* (blue) are expressed in intermediate patterns. (Bottom) A hypothesized genetic and biochemical pathway that proposes the sequential role of *Dlx2*, *Dlx1*, *Dlx5* and *Dlx6* at different stages of differentiation. Telencephalic regions are as follows. Pallium: neocortex (NCX) and palliocortex (PCX). Subpallium: lateral ganglionic eminence (LGE). Medial ganglionic eminence (MGE). Stages of differentiation: ventricular zone (VZ); subventricular zone (SVZ); mantle zone (MZ). LV, lateral ventricle (ventricle of telencephalon); III, third ventricle (ventricle of the diencephalon).

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* also are 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; Myojin 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 *Dll*/Dlx 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 (hematopoeitic 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 (Bei 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 nascent chick limb (Ferrari et al., 1999). FGF19, in combination with Wnt8c, can induce *Dlx5* expression in the developing inner ear (Ladher et al., 2000). FGF8 induces *Dlx1* and *Dlx2* expression in murine dental mesenchyme (Bei 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 *dlx6a* (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, *dlx3b* (previously *dlx3*) is required for the activation of *dlx5a* (previously *dlx4*) (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 *dlx3/dlx4* 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) (D. Wu, M. Depew and J. L. R. R., unpublished). In addition, 12-28% of *Dlx5* mutants also lack fusion of the dorsal midline of the rostral neural tube, leading to exencephaly (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* show 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 labryinthine 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. Hrabe 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. Hrabe 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 *Oct6/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; Parnavelas, 2000; Parnavelas 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

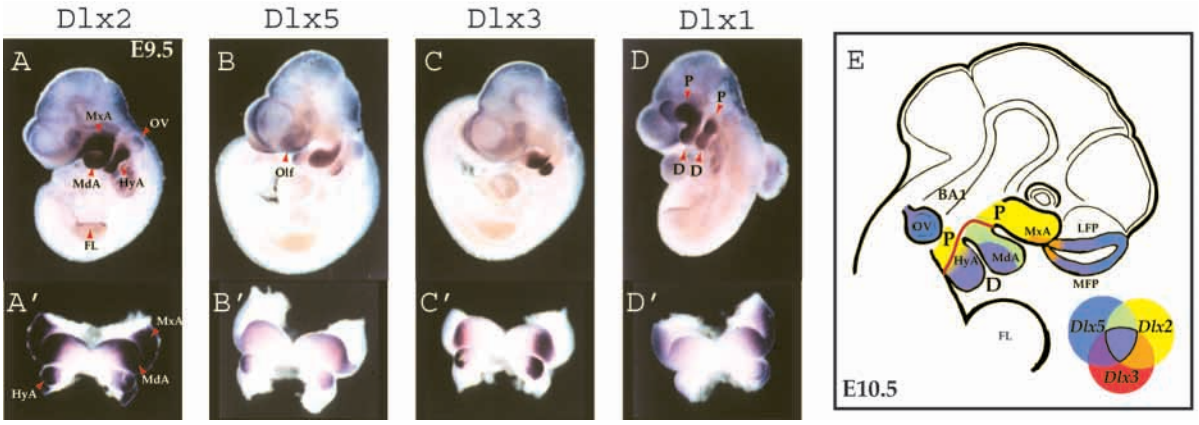

Fig. 5. Expression of three type A Dlx genes (*Dlx2*, *Dlx5* and *Dlx3*; A-C) and one type B Dlx gene (*Dlx1*; D) in E10.5 mouse embryos shown by whole-mount in situ hybridization. The top pictures show a lateral view of the entire embryo (A-D); the bottom pictures show frontal views of dissected branchial 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: *Dlx2*, *Dlx3* and *Dlx5*. These colors are used in the schema to describe the expression of these genes in the ectomesenchyme of the branchial arches and the ectoderm of the olfactory placode/pit and otic vesicle, respectively.

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.ax.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; Bulfone 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 ectomesenchyme 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

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 (Dodig 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

A

```
Drosophila Dll        mrkprtiyss lqlqqlnrrf qrtqylalpe raelaaslgl tqtqvkiwfq nrrskykkmm k
Dlx1                  irkprtiyss lqlqalnrrf qqtqylalpe raelaaslgl tqtqvkiwfq nkrskfkklm k
Dlx2                  vrkprtiyss fqlaalqrrf qktqylalpe raelaaslgl tqtqvkiwfq nrrskfkkmw k
Dlx3                  vrkprtiyss yqlaalqrrf qkaqylalpe raelaaqlgl tqtqvkiwfq nrrskfkkly k
Dlx4                  lrkprtiyss lqlqhldqrf qhtqylalpe raqlaaqlgl tqtqvkiwfq nkrskykkll k
Dlx5                  vrkprtiyss fqlaalqrrf qktqylalpe raelaaslgl tqtqvkiwfq nkrskikkim k
Dlx6                  irkprtiyss lqlqalnhrf qqtqylalpe raelaaslgl tqtqvkiwfq nkrskfkkll k
consensus             .rkprtiyss .ql..l..rf q..qylalpe raelaa.lgl tqtqvkiwfq n.rsk.kk.. k
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B

```
Extended HD          20 aa 5′ to HD                              20 aa 3′ to HD
Drosophila     fsi.sdkc.edsglrvngkgkk              aaggpgtnsgmplggggpnpgq

Dlx1           .sekstvv.eggevrfngkgkk               qggaalegsalangralsag
Dlx4           esek..lalslvpsqqqsltrk               qssgepeedfsgrppslsph
DLX4*          ..ekprlspepserrpqapakk               gnsggqegdfpgrtfsvspc
DLX6*          .qqkttvi.engeirfngkgkk               qgsnphesdplggsaalspr
consensus      .sek.tv..e.ge.rfngkgkk               qgs...egdfl....alsp.

Dlx2             epdkedlepeirivngkpkk               sgeipteqhpgasasppcas
Dlx3             vsvkeepeaevrmvngkpkk               ngevplehspnnsdsmacns
Dlx5             qpekevaepevrmvngkpkk               ngemppehspsssdpmacns
consensus        .p.ke..epe.rmvngkpkk               nge.p.ehsp..sdsmacns
```

C

```
                starting residue          DllA domains          conserved residues
Dlx2                  44          sssnsslhkpgesptlpvstatdssyyt          19/28
Dlx3                  16          ssslschagskdsptlpestvtdlgyys          19/28
Dlx5                  22          ptsaamhhpsqesptlpessatdsdyys          18/28
consensus                         sss.s.hh.sqesptlpest.tds.yys
```

D

```
                aminoterminal                carboxyterminal
Dll             tqapwdqkp                    vpqywylpe
Dlx1            vppqwnpns                    sytswypsa
Dlx2            apaswdfga                    gnypwyhqa
Dlx3            spalwdtss                    ptnswyhtq
Dlx4            lpfiwglpk                    hfgawyqhr
DLX4            lpylwdlpk                    sfgpwyqhh
Dlx5            spavwepqg                    nsaswypsa
DLX6            lppvwdvsa                    gyshwyssp
consensi        lpalwdpsa                    sy.pwyps.
                  pv    s                        h
```

E

```
        start residue
Dll              91          syappcasppkddf
Dlx2            228          sasppcasppvsap
```

F

```
        start residue
Dll             247          lspefpptglspptqapwd
Dlx1            199          langralsagsppvppgwn
Dlx2            228          saspppcasppvsappaswd
Dlx3            199          pnnsdsmacnsppspalwd
Dlx4            187          sgrppslsphspalpfiwg
DLX4            296          pgrtfsvspcspplpylwd
Dlx5            207          pssspdpmacnspqspavwe
DLX6            120          lqgsaalsprspalppvwd
```

Fig. 6. Alignments of various Dll and Dlx subdomains. (A) Dll/Dlx homedomain alignments. The consensus represents amino acids conserved among all family members. Dots in the consensus represent non-conserved residues. (B) Extended homedomain alignments of Dll and Dlx proteins. Underlined residues are conserved among two or more Dlx subgroup members or between Dll and Dlx. The consensi 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 consensi 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 consensi 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.

(Ignatius et al., 1996; Mishra et al., 2000; Tackels-Horne et al., 2001).

Dll/Dlx biochemistry

Dll and the Dlx genes encode homeodomain transcription factors and thus are thought to function via regulation of downstream target genes. Transient transfection experiments demonstrate that Dlx proteins can activate transcription from both artificial (Feledy et al., 1999b; Masuda et al., 2001; Zhang et al., 1997) and authentic enhancers (Benson et al., 2000; Dodig et al., 1996; Iler et al., 1995; Morasso et al., 1996; Roberson et al., 2001; Stühmer et al., 2002a; Yu et al., 2001; Zerucha et al., 2000). There also is evidence that they can function as repressors on artificial reporter genes (Ryoo et al., 1997; Yu et al., 2001).

Outside of the 61 amino acid DNA-binding homeodomain (Fig. 6A), Dll and the Dlx proteins share other, more limited, regions 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 TFIID (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 homedomains, tryptophan residues and proline-rich domains, there are several other conserved features of Dll/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 'DllA' 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 Ca^{2+} (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 homedomain 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 necdin- 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 Dll 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).

Dlx targets

A variety of genes have been identified as targets of Dlx regulation, including the Dlx genes themselves. For example, Dlx1, Dlx2 and Dlx5 all can activate transcription from the mouse *Dlx5/Dlx6* and zebrafish *dlx5a/dlx6a* (previously *dlx4/dlx6*) intergenic enhancers in tissue culture cells (Yu et al., 2001; Zerucha et al., 2000) and in slices of embryonic mouse brain (Stühmer et al., 2002a). Dlx1, Dlx2 and Dlx5 also can activate a glutamic acid decarboxylase enhancer (B. Condie, personal communication). As described above, this is likely to be important in the differentiation of particular GABAergic neurons in the brain. Dlx-binding sites that mediate this regulation have been identified (Fig. 7A) (Zerucha et al., 2000).

Dlx2 also is thought to regulate Wnt1 directly in the developing telencephalon (Iler et al., 1995). A single binding site, termed HBS-1 (Fig. 7B) (Iler et al., 1995), mediates this Dlx2 regulation. In vivo, it is more likely that Dlx genes

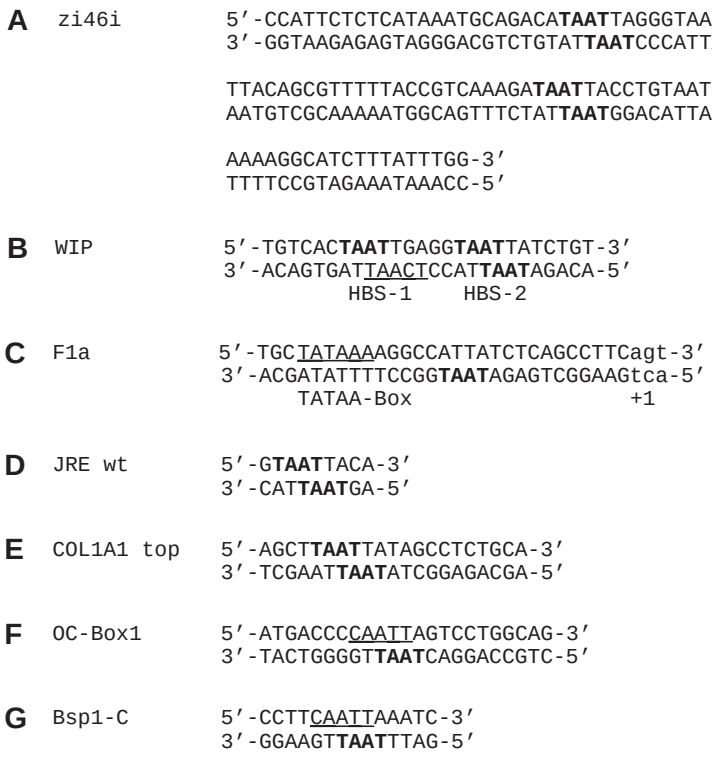

Fig. 7. Native Dlx binding sites. (A) Dlx1, Dlx2 and Dlx5 binding sites in the *Dlx5/Dlx6* intergenic region (Zerucha et al., 2000). (B) Dlx2 binding site in *Wnt1* (Iler et al., 1995). This interaction occurs predominantly via HBS-1 and the result is repression, not activation. (C) *Xenopus* Dlx3-binding site in human profilaggrin promoter (Morasso et al., 1996). (D) Dlx3-binding site in gene encoding the alpha subunit of human chorionic gonadotropin (Roberson et al., 2001). (E) Dlx5-binding site in collagen 1A1 (Dodig et al., 1996). (F) Dlx5 binding site in osteocalcin (Ryoo et al., 1997). (G) Dlx5-binding site in bone sialoprotein (Benson et al., 2000). We note that in several of these native binding sites, there are pairs of 5′-TAATT-3′ sequences on opposite strands, and propose that these might bind Dlx dimers. Intriguingly, in vivo site selection with Dlx3 recovered opposing 5′-TAATT-3′ sequences in 17/30 selected sites (Feledy et al., 1999b). We also note that the WIP sequence, which mediates repression by Dlx2, and OC-Box 1, which initially was reported to mediate repression, have 5′-CAAT-3′ instead of 5′-TAATT-3′ opposing a 5′-TAATT-3′.

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 gonadtropin 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 hematopoeitic cells (Shimamoto et al., 1997). Ectopic DLX4 also can inhibit apoptosis via upregulation of expression intercellular adhesion molecule 1 (Shimamoto et al., 2000). However, it is not known whether the activation of *GATA1*, *MYC* or 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 *collagen2α1* 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 lineages (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*,

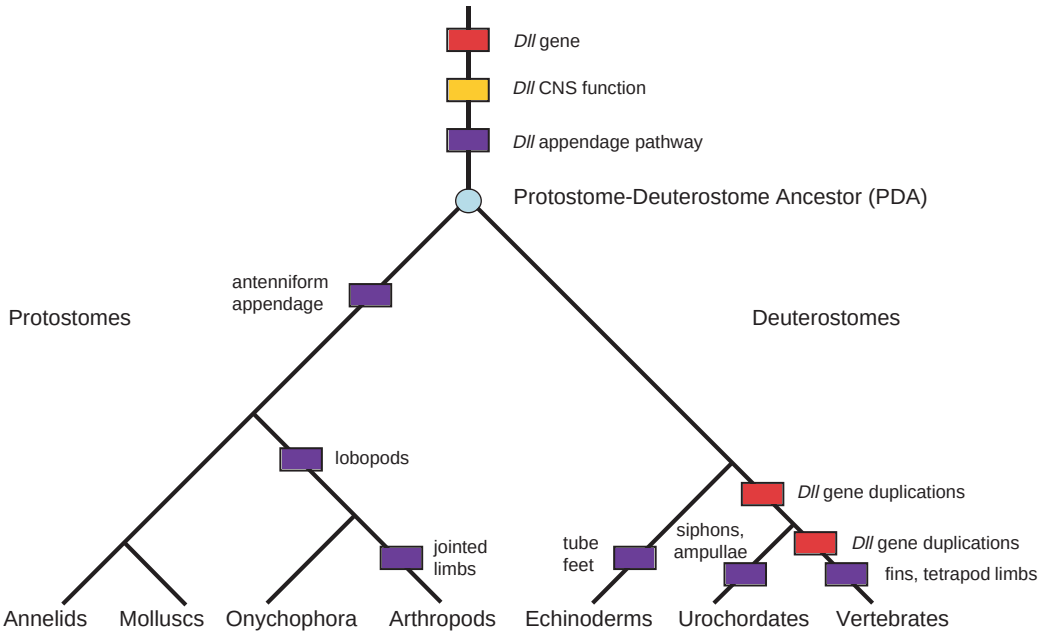

Fig. 8. Possible evolution of the *Dll* and Dlx genes and their functions. Modified from Panganiban et al. (Panganiban et al., 1997). The ancestral *Dll* gene may have functioned first in the developing nervous system, acquiring roles in appendage development later in evolution. *Dll* was duplicated multiple times in the deuterostome lineage to give rise to the present day six Dlx genes in mice and humans and the eight Dlx genes in zebrafish. See text for details. Not shown is the acquisition of *Dll*/Dlx functions in other tissues, including the branchial arches, the otic and olfactory systems, and hematopoietic system. It is not yet known when these roles were acquired or whether they predate the divergence of the protostomes and deuterostomes.

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 *atonal* (mouse homolog *Math1*), *spalt* (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.

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