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**Regulation, function and evolution of the *Distal-less*-
related genes in the pharyngeal arches**

Steven M. Sperber

Thesis is submitted as a partial fulfillment of the Doctor of Philosophy program
in Cellular and Molecular Medicine

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Statement of contribution

The thesis, “Regulation, function and evolution of the *Distal-less* related gene in the pharyngeal arches,” contains three investigations, two of which were collaborative efforts.

In the first investigation, “Intergenic enhancers with distinct activities regulate *Dlx* gene expression in the mesenchyme of the branchial arches,” the following people contributed to the work: former members of Dr. Marc Ekker’s laboratory, Wei Lin and Genny Giroux technically assisted in the cloning of the *I12a* enhancer element of the *Dlx1/2* genes. *I56i* enhancer characterization was previously reported in Zerucha *et al.* 2000. The creation of transgenic mouse lines was performed by Mr. Gary Hatch, and Mrs. Adrianna Gamboratta, with assistance by Mr. Noel Ghanem. Dr. Keon Park with the assistance of Mr. Steven Sperber performed the expression analysis. Both Dr. Park and Mr. Sperber performed a series of arch culture bead implantation experiments. Dr. Bethan Thomas and Ms. Anuradha Choudhury performed the *bosentan* experiments. The article was collaboratively written by Dr. Park, Mr. Sperber, and Dr. Ekker.

Work performed in the second investigation, “Zebrafish *dlx2a* maintains the identity of the hindbrain neural crest, and is necessary for the differentiation of the sensory ganglia and functions with *dlx1a* in the maturation of the arch cartilage elements” was wholly done by Mr. Sperber with editorial assistance by Dr. Ekker.

The last investigation, “*OdiDll*, a unique *distal-less* homeobox gene of the tunicate *Oikopleura dioica*, and the origin of vertebrate *Dlx* bigene clusters,” was a collaborative effort. Cloning of the *OdiDll* genomic sequence and comparative analysis was performed by Mr. Sperber assisted by Dr. Hee-Chan Seo. Analysis of the genomic neighbourhood was performed by Dr. Seo with technical assistance by Mrs. Marit F. Jensen. Expression analysis was performed by Mr. Sperber, as well as Ms. Jana Mikhaleva. The paper was written by Mr. Sperber, Dr. Chourrout and Dr. Ekker.

Abstract

The vertebrate *Dlx* homeodomain-containing genes are generally organized as three convergently transcribed bigene clusters. Paired genes share highly conserved overlapping expression patterns in the forebrain, the pharyngeal arches, sensory placodes and limb/fin buds. Little is known about how the *Dlx* genes are targeted to their sites of expression, or what particular roles individual genes play in development. In the pharyngeal arches, *Dlx* genes are expressed in a nested manner, which defines proximodistal identity. We identified two distinct *cis*-acting enhancers, I12a and I56i situated between *Dlx1/2* and *Dlx5/6* pairs respectively that regulate expression in the mandibular arch. In transgenic animals, the two enhancers targeted reporter gene expression to distinct populations of mesenchymal cells in the mandibular arch. Both enhancers responded to epithelial signaling cues, such as FGF8 and BMP4, similar to endogenous *Dlx* genes. Therefore, the combinatorial arch expression is achieved through interaction between signaling factors and intrinsic cellular factors. To investigate the individual roles of the paired *Dlx1/2* gene in patterning the pharyngeal arches, we silenced zebrafish *dlx1a* and *dlx2a* using morpholino antisense oligonucleotides. Loss of *dlx2a* function in zebrafish embryos resulted in reductions of arch neural crest markers as well as perturbation of neurogenic and chondrogenic derivatives. Loss of *dlx1a* resulted in malformations of cartilage elements indicating a role in arch patterning. To explore further the evolution of the *Dlx* gene family, we characterized the *Distal-less* homologue, *Odidll*, in the protochordate species, *Oikopleura dioica*. *Odidll* is not part of a bigene cluster suggesting either that a paralogue was lost or that the tandem duplication event occurred subsequent to the species' divergence. *Odidll*, was expressed in ectodermally-derived tissues suggesting a role that may have been observed in a common chordate ancestor. These results contribute to our understanding of *Dlx* regulation, function and evolution.

Résumé

Les gènes à homéoboîte *Dlx* des vertébrés sont généralement organisés en trois complexes bigéniques dont la transcription est convergente. Les deux gènes formant un complexe montrent des chevauchements de leurs profils d'expression dans le cerveau antérieur, les arcs pharyngiaux, les placodes sensorielles et les bourgeons de membres/nageoires. On connaît très peu les mécanismes gouvernant l'expression des gènes *Dl*, ni le rôle joué spécifiquement par chacun des membres de la famille au cours du développement. Dans les arcs pharyngiaux, les gènes *Dlx* sont exprimés de manière imbriquée et l'on croit que cette expression définit l'identité des cellules selon l'axe proximo-distal. Nous avons identifié deux séquences agissant en *cis*, de type *enhancer*, I12a et I56i, situées entre les gènes *Dlx1* et 2 et *Dlx5* et 6, respectivement. Chacune de ces séquences contrôle l'expression dans l'arc mandibulaire. En transgénèse, les deux *enhancers* dirigent l'expression de gènes rapporteurs dans des populations distinctes de cellules mésenchymateuses de l'arc mandibulaire. Les deux *enhancers* répondent à des signaux provenant de l'épithélium, tel que FGF8 et BMP4, de manière analogue aux gènes *Dlx* endogènes. Conséquemment, l'expression *Dlx* combinatoire dans les arcs est le fruit de l'interaction entre des facteurs de signalisation et des facteurs intrinsèques. Afin d'étudier les rôles spécifiques des gènes du complexe *Dlx1/2* au cours de la morphogénèse des arcs pharyngiaux, nous avons aboli l'activité des gènes *dlx1a* et *dlx2a* du poisson-zèbre, *Danio rerio*, à l'aide d'oligonucléotides anti-sens de type morpholino. La perte de fonction de *dlx2a* chez les embryons de *Danio* résulte en une diminution d'expression de marqueurs des cellules des crêtes neurales ainsi qu'en la perturbation du développement de leurs dérivés neurogéniques et chondrogéniques. La perte de fonction de *dlx1a* a provoqué des malformations d'éléments cartilagineux, indiquant un

rôle pour ce gène dans la morphogénèse des arcs. Afin d'explorer plus à fond l'évolution de la famille *Dlx*, nous avons caractérisé un homologue de *Distal-less*, *Odidll*, chez le proto-cordé, *Oikopleura dioica*. *Odidll* ne fait pas partie d'un complexe bigénique ce qui suggère, soit la perte d'un paralogue, soit que l'événement de duplication en tandem menant à un complexe bigénique s'est produit après la divergence de cette branche évolutive. *Odidll* est exprimé dans les tissus dérivés de l'ectoderme, ce qui suggère que cette fonction existait chez un ancêtre commun des cordés. Ces résultats contribuent à notre compréhension de la régulation, fonction, et évolution des gènes *Dlx*.

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List of abbreviations

A/P, anterior/posterior

AER, apical ectodermal ridge

AmphiDll, amphioxus *Distal-less*

BCIP, 5-bromo-4-chloro-3-indolyl phosphate

bHLH, basic helix-loop-helix

BMP, bone morphogenic protein

bp, base pair

BP1, beta protein 1; DLX4 isoform that represses the *β -globin* gene

BSA, bovine serum albumin

BSP, bone sialoprotein; extracellular matrix component involved with biomineralization

C-, carboxy

CATCH22, cardiac, abnormal facies, thymic hypoplasia, cleft palate, hypocalcemia

cDlx, chick *Dlx*

cDNA, complementary deoxyribonucleic acid

ChIP, chromatin immunoprecipitation

CNS, central nervous system

CS, calf serum

D/V, dorsoventral

DAB, 3,3'-diaminobenzidine tetrahydrochloride

dHAND, a bHLH transcription factor

Dll, *Distal-less* gene

Dlx, *Distal-less related* vertebrate homologue

DMEM, Dulbecco's minimal essential media

DNA, deoxyribonucleic acid

dpf, days post-fertilization

DV, dorsoventral

EDTA, (disodium) Ethylenediamine tetraacetate (acid)

ENS, enteric nervous system

ENU, N-ethyl-N-nitrosourea

ET-1, endothelin-1

EtOH, ethanol

fez, forebrain embryonic zinc finger

FGF, Fibroblast growth factor

GABA, γ -amino butyric acid

GAD, glutamic acid decarboxylase

GAL-4, yeast transcriptional activator

GFP, green fluorescent protein

HB, homeobox

HBS, homeodomain binding site

HD, homeodomain

hDLX, human *DLX*

Hox, homeobox transcription factor

hpf, hours post fertilization

hy, hyoid

kb, kilobase

lacZ, β -galactosidase

mb, megabase

mDlx, mouse *Dlx*

MeOH, methanol

Mn, mandible

MO, morpholino oligonucleotide

mRNA, messenger ribonucleic acid

Msx, msh homologue

Mx, maxilla

mya, million years ago

N-, amino

NBT, nitroblue tetrazolium

NLS, nuclear localization signal

O/N, overnight

°C, degrees Celsius

OdiDll, *Oikopleura dioica* *Distal-less* related gene

PBS, phosphate buffered solution

PBST, phosphate buffered solution and Tween-20

PCR, polymerase chain reaction

PD, proximal distal

PFA, paraformaldehyde

RA, retinoic acid

RNA, ribonucleic acid

RT, room temperature

SHFM, split hand/split foot mutation

Shh, sonic hedgehog

TGF- β , transforming growth factor β

TUNEL, TdT (terminal deoxynucleotidyl transferase)-mediated dUTP-biotin nick end
labeling

vgo, *van gogh* zebrafish mutant

WT, wild-type

xDlx, *Xenopus Dlx*

zdlx, zebrafish *dlx*

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Thank you.

1. Introduction

The underlying molecular mechanisms of craniofacial formation are gradually being elucidated. Of particular interest is vertebrate oropharyngogenesis, as it details the development of the stomatognathic apparatus used for mastication, respiration, as well as auditory reception and speech creation, all highly derived and evolutionarily novel morphological innovations. The *Dlx* family of gene regulators, which is related to the *Drosophila Distal-less (Dll)* gene (Cohen *et al.*, 1989), is associated with these morphological innovations. The focus of my thesis research was to investigate the function of the *Dlx* genes in pharyngeal arch formation, their regulation, and evolution. I examined each of these aspects of the *Dlx* genes using three different experimental approaches. The first aspect of my research was to investigate how the *Dlx* genes are patterned within the pharyngeal arches, utilizing transgenic mice possessing the *lacZ* reporter gene targeted to the mandibular mesenchyme by intergenic enhancer sequences. The second aspect of my work was to characterize zebrafish *dlx2a* and *dlx1a* loss of function using a morpholino-mediated gene silencing approach. Lastly, my research focused on characterizing the *Dll*-related/*Dlx* gene in the planktonic urochordate species, *Oikopleura dioica*, a proxy-model of a protochordate to investigate evolutionary aspects of the *Dlx* gene family. Here, I briefly summarize pharyngeal arch development and give an overview of the *Dlx* gene function in development.

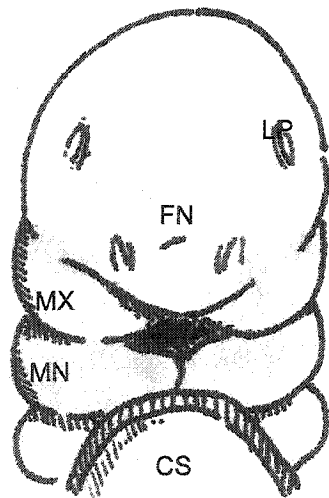
1.1. Craniofacial patterning and Oropharyngogenesis

Craniofacial development is a process of outgrowth and fusion of the facial prominences, organization of the cranial neural crest, and coordination of epitheliomesenchymal interactions necessary for form and function. The face is derived from the facial primordia, five prominent swellings of undifferentiated tissue that encircle the future oral cavity – the frontonasal mass, and the paired maxillary and mandibular prominences (Fig. 1.1A). The buds are comprised of cephalic neural crest that surround a core of mesenchyme derived from paraxial mesoderm, and are encapsulated by an outer ectodermal layer, and an inner endodermal surface (reviewed in Graham, 2001; Sperber, 2001). The median frontonasal mass, containing the medial and lateral nasal processes, covers the forebrain giving rise laterally to the optic diverticula of the eye primordia. The upper jaw is derived from the frontonasal prominence merging with the two lateral maxillary prominences (Fig. 1.1B). The lower jaw forms from the fusion of the paired mandibular primordia (Sperber, 2001). Ectomesenchyme derived from the migrating neural crest cells of the neural tube gives rise to skeletal and cartilaginous elements, nerve ganglia, and the connective tissues of the arches. The mesoderm differentiates into the musculature, endothelial cells and angioblasts (reviewed in Francis-West *et al.*, 1998; Graham, 2001). The cranium is composed of the neurocranium or brain case, and of particular interest, the partially segmented viscerocranium, which includes the pharyngeal arch skeleton.

The pharyngeal arches are repetitive ventrolateral segmented outgrowths of the pharyngeal region that form in a rostrocaudal manner. The arches are organized as pouches, separated by grooves. The jaws and gills of teleosts and the jaws and laryngopharyngeal

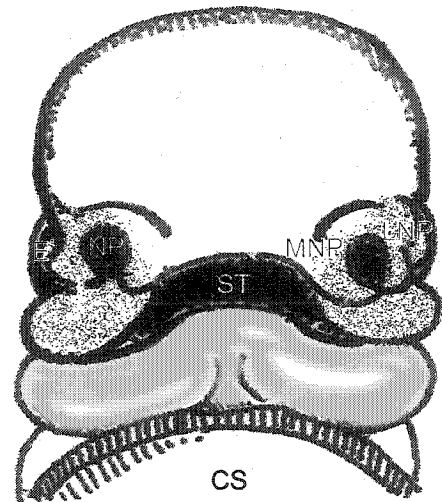
Figure 1.1 Schematic depiction of human embryonic facial development at four and five weeks of gestation. Swellings of the primordial buds surround the stomodeum adumbrating the future facial features. CS, cardiac swelling; FN, frontonasal prominence; LNP, lateral nasal process; LP, lens placode; MNP, medial nasal process; E, eye; NP, nasal pit; MN mandibular prominences; MX, maxillary prominences; ST, stomodeum. Adapted from Sperber (2001).

A



4 weeks

B



5 weeks

apparatus of all other vertebrates are derived from the arch primordia (reviewed in Sperber and Sperber, 1996). Each arch produces a shared pattern of derivatives consisting of an arch artery, nerve, cartilage and muscle. The derivatives have uniquely shaped features particular to that arch. For example, the mammalian first arch contains Meckel's cartilage that presages the jaw bone, the malleus and incus of the middle ear, as well as the external acoustic meatus, eustachian tube, and tympanum necessary for sound transmission and pressure regulation. The second arch features the stapes and hyoid elements as well as the tonsillar fossa (Sperber and Sperber, 1996). Therefore, individual arches have evolved unique structures and functions.

Craniofacial development is an extraordinarily complex process. Many defects and syndromic dysmorphologies arise because of poor developmental organization (Cohen, 2002). Examples of human syndromic conditions include Treacher Collins auriculofacial hypoplasia (1 in 50,000 live births), a presumed failure of cranial neural crest to migrate, leading to small facial bones, cleft palate, deafness and external ear malformation (Wilkie and Morriss-Kay, 2001). DiGeorge (velo-cardio-facial) syndrome (1 in 4000 live births) shows defects predominantly in the third and fourth pharyngeal arch neural crest derivatives. Defects include craniofacial malformations, aortic arch and heart problems, as well as deafness (Wilson *et al.*, 1993; Ryan *et al.*, 1997; Wilkie and Morriss-Kay, 2001). The most prevalent facial defects are cleft lip and cleft palate. Clefting results from the failure of medial nasal and maxillary tissue derived palatal shelves, and or lateral nasal processes to merge. Failure may be a result of defects in cellular induction, transformation, migration, and apoptosis necessary for fusion. Clefting occurrence in the general population is 1:500 in

Japanese, 1:1000 in Caucasians, and 1:2000 in African Americans indicating the high incidence, and complex inheritance of facial malformation (Wilkie and Morriss-Kay, 2001). The high incidence of facial defects attests to the numerous factors involved in craniofacial development and the complexity of their interactions.

Facial outgrowth is mediated by epithelial-ectomesenchymal interactions initiated by signaling cascades (reviewed in Francis-West *et al.*, 1998). Prominent signaling factors involved in these interactions include the fibroblast growth factor family (FGFs), the transforming growth factor β (TGF β) family, which contains the bone morphogenic protein family (BMPs), the hedgehog family (HH), the Wnt family, and the Endothelins (Francis-West *et al.*, 1998). The regionalization of facial morphology is recapitulated in the expression patterns of transcription factors that are targets of the signaling factors. Several homeobox-containing gene families are expressed in the developing facial prominences after neural crest migration. These genes include the *Dlx* family members, the *Msx* genes, *Lhx* genes, the bicoid-related homolog *Gsc*, as well as, *Barx*, *Otx*, *Pax* and *Prx* (*Mhox*) families. Additionally, many non-homeodomain-containing transcription factors are also necessary for defining polarity and facial organization (Francis-West *et al.*, 1998; Cobourne and Sharpe, 2003). Many of these genes have been proven, through targeted disruption in mutant mice, to be necessary for patterning the face. For example, deficiency in *Msx1* results in maxillary and mandibular hypoplasia, cleft palate because of fusion failure of the palatine shelves that form the roof of the mouth, as well as arrested tooth development (Satokata and Maas, 1994). Therefore, mutations in these factors tend to have pleiotropic effects influencing patterning of a wide variety of tissues during embryogenesis.

1.1.1. Neural crest contribution to the pharyngeal arches

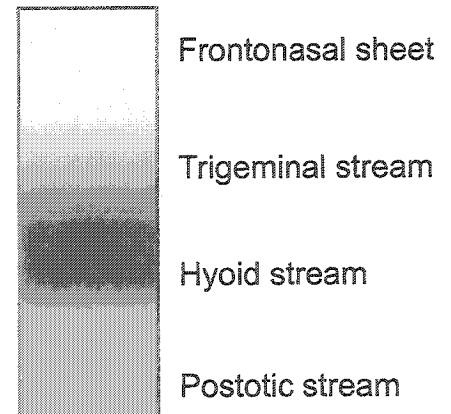
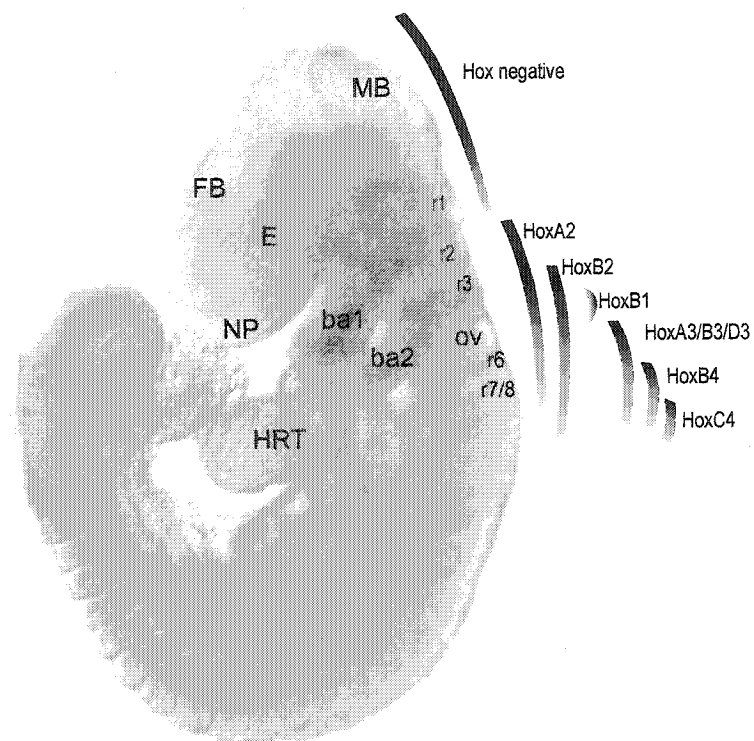
A large component of the pharyngeal arches consists of a unique ectodermally derived cell population. Neural crest cells are a transient pluripotent cell population originating from the apical neural tube folds delineated at the border of the neural plate and presumptive surface ectoderm (Copp *et al.*, 2003). The crest cells are divided into the trunk crest and the cephalic or cranial crest as determined by their anteroposterior (A/P) position along the neural tube, and the subsequent tissues that they will ultimately form. The cells undergo an epithelial to mesenchymal transition, delaminate, and migrate throughout the embryo, differentiating into a wide variety of tissue types that include nerve ganglia, connective tissue, peripheral nerve cells, melanocytes, and the enteric nervous system. Only the cranial portion of neural crest differentiates into mesenchymal tissues including connective tissues, cartilage, bone and crestal components of teeth, therefore contributing to the majority of the head skeleton (Couly *et al.*, 1993).

The frontonasal mass is populated by neural crest cells from the fore- and midbrain that migrates as a contiguous sheet (Osumi-Yamashita *et al.*, 1994; Richman and Lee, 2003). Three distinct streams of cephalic neural crest originating from the mid- and hindbrain regions migrate into the pharyngeal arches in a rostrocaudal manner (reviewed in Cobourne, 2000; Graham, 2001). The most rostral, trigeminal, stream originates from the posterior midbrain and rhombomeres 1 and 2 of the hindbrain and travels to the first or mandibular arch. The hyoid or second arch crest cells originate mostly from rhombomere 4. The postotic stream is derived from rhombomeres 6 and 7 and populates the caudal pharyngeal

arches and some of the enteric nervous system and heart (Graham, 2001). The tripartite segmentation of the migrating streams from the rhombencephalon is crucial for organizing the proper afferent innervations of the hindbrain, and the cranial sensory ganglia (Fig. 1.2) (Graham *et al.*, 2004). In mammalian and avian species but not in zebrafish, rhombomere segments 3 and 5 neural crest generally undergo BMP4 mediated apoptosis, and therefore contribute a smaller number of cranial neural crest cells to the pharyngeal arches (Schilling and Kimmel, 1994; Graham, 2001). Segmentation of the neural crest cells is a result of the lack of migrating neural crest from rhombomeres 3 and 5, in addition to peripheral inhibitory cues which direct and maintain the streams (Graham *et al.*, 2004).

Cranial neural crest cells are not entirely pre-specified but plastic in their fate determination depending on intrinsic and extrinsic factors (Schilling and Kimmel, 1994). Neural crest fate is correlated with the timing and route of migration. Cranial neural crest cells that migrate early, travelling laterally and over tissues, tend to fill the arches and differentiate into cartilage, intramembranous bone and connective tissue (Richman and Lee, 2003). Later migrating cells, generally travelling ventrolaterally through tissues, form the ganglionic neurons, glia, Schwann cells and pigmented melanocytes (Graham *et al.*, 2004). The surrounding cell-community, as well as environmental cues emanating from the epithelium, mesoderm and endoderm influence the ectomesenchyme (reviewed by Trainor and Krumlauf, 2001). Plasticity has been shown in hindbrain transplant studies. In mouse (Trainor and Krumlauf, 2000), and zebrafish studies (Schilling *et al.*, 2001), grafting of single or small groups of rhombomere cells result in the changing of the intrinsic *Hox* gene expression pattern of these cells concordant with their new A/P location (Trainor and Krumlauf, 2001). Timing and size of transplants suggest the existence of cell community

Figure 1.2 Schematic depiction of the cranial neural crest migration pathways. An E9.0 (16 som) mouse embryo is overlaid with the fate maps of the migrating frontonasal sheet (yellow), emanating from the fore- and anterior midbrain, and the three streams originating from the posterior mid- and hindbrain: the trigeminal (red), the hyoid (blue), and the postotic (green). The neural crest migrates in a rostrocaudal manner beginning at ~E6.0 with the medial frontonasal sheet extending over the forebrain. The trigeminal stream begins at approximately E8.5. Dorsal lines indicate the anteroposterior expression domains of the *Hox* genes in the rhombomeres, and in the migrating neural crest. *Hox* genes are not expressed in the first arch. ba, branchial arch; FB, forebrain; HRT, heart; MB, midbrain; r, rhombomere, NP, nasal placodes; ov, otic vesicle. Adapted from Richman and Lee (2003), Graham (2004), and Cobourne (2000).



influence, as well as a temporal window to which the cells will respond before becoming irreversibly committed (Trainor and Krumlauf, 2001). Large transplants of neural crest cells impart species-specific organization of the facial tissues. Quail and duck reciprocal grafts of cranial neural crest reproduce their intrinsic morphology in the chimeric animal, such that duck neural crest in quail forms a bill. Conversely, the quail neural crest cells in duck produce a beak (Schneider and Helms, 2003; Tucker and Lumsden, 2004). Thus, the donor-derived neural crest maintains inherent patterning information in an independent species-specific manner. Therefore, patterning of the arch derivatives is attained by combined intrinsic and extrinsic cues.

In addition to the cranial neural crest cells, other tissues also participate in organizing the pharyngeal arches. Cues emanating from the pharyngeal endoderm determine the unique skeletal elements. Loss or ectopic transplantation of foregut endoderm alters the form and orientation of skeletal elements indicating its role as an inducer of skeletal identity (Couly *et al.*, 2002). In the zebrafish *tbx1/van gogh* (*vgo*) mutant, the endoderm fails to differentiate properly, causing the neural crest streams to be disorganized, fusing and resulting in dysmorphic skeletal elements (Piotrowski and Nusslein-Volhard, 2000; Piotrowski *et al.*, 2003). In chick, removal of neural tube segments prior to neural crest delamination reveals that pharyngeal pouches develop with correct regionalization, and arch identity (Veitch *et al.*, 1999; Graham, 2001). These studies illustrate tissues other than the neural crest are involved in patterning the neural crest and the pharyngeal arches. Thus, epithelial signaling patterns the neural crest cells, while the neural crest cells interpret the patterning according to their intrinsic positional identity (reviewed in Santagati and Rijli, 2003).

1.1.2. *Hox* gene regulation and protein interactions

In addition to the external influence of the surrounding tissues, the migrating neural crest cells express a combinatorial code of homeobox-containing transcription factors that confer A/P identity. Homeobox-containing genes are a large group of regulatory factors categorized based on chromosomal organization, and on sequence conservation of the homeodomain (Duboule, 1994). The homeobox is a 180 bp sequence encoding for a 60-amino acid helix-turn-helix DNA binding homeodomain (HD). *Hox* gene products act as sequence-specific transcription factors illustrated by their ability to regulate promoters in cell culture and *in vivo* (Di Rocco *et al.*, 1997; Maconochie *et al.*, 1997). How the *Hox* gene activity is effectuated remains under wide investigation. Hox proteins determine cell fate decisions by transcriptionally regulating downstream genes by binding to a core TAAT DNA motif assisted by combinations of cofactors. Two homeodomain-containing families of cofactors include the Pbx and Meis families that interact forming heterodimers or higher-order configurations that augmented Hox function by modulating affinity and specificity of DNA interactions for optimal binding with promoter elements (Shanmugam *et al.*, 1999; Sagerstrom, 2004).

Originally discovered in the fruit fly, *Drosophila melanogaster*, homeobox genes, organized as the Homeotic complexes (HOM-C), are master regulators arranged in a co-linear manner conferring segment identity, and determine the initial body plan. The vertebrate homologues, the *Hox* genes, of which there are at least four clusters, are organized in a similar co-linear fashion, such that their axial expression patterns relate to their position

in the gene complex. The result is a combinatorial expression code of distinct overlapping *Hox* domains that specifies anteroposterior segment identity along the axis.

Hox transcription factors are expressed in a spatiotemporal manner according to their chromosomal position. The relationship of gene activation and organization likely uses several different regulatory mechanisms to maintain the “*HOX* code” of combinatorial distribution, along the embryonic anteroposterior axis. Three mechanistic models attempt to resolve the correlation of anteroposterior sequential *Hox* gene expression beginning at late primitive streak stage in the mouse with vertebrate rostrocaudal developmental progression, as well as the posterior prevalence of more 3' genes having dominance over 5' genes within the *Hox* cluster (Kmita and Duboule, 2003).

Hox colinear expression may be a result of progressive transcriptional availability, whereby activation starts at the posterior end of the complex and progresses down the cluster likely depending on changes in the chromatin state incrementally opening more anterior genes to activation through possibly elevating repression from silencing factors and or transcription of earlier genes (Kondo and Duboule, 1999). The molecular aspects of cluster polarity are unclear. However, this mechanism is supported by increases in transcriptional stimulation of early *Hoxb* genes responding to short RA-treatments, suggesting a 3' chromatin decondensation or looping out event susceptible to initial activation (Roelen *et al.*, 2002; Chambeyron and Bickmore, 2004). A second mechanism for *Hox* gene regulation suggests the use of locally shared *cis*-acting regulatory elements dispersed throughout the cluster. Thus, expression of neighbouring genes in a cluster may be coordinated. A

putative signaling factor with varying affinities to the elements could regulate a series of elements having unequal influence on the surrounding genes and therefore differently control activation (Duboule, 1998). Global enhancer sequences located outside the cluster is a possible third mechanism of regulation. For example, *Hoxd* expression in the presomitic mesoderm coordinate with somitogenesis requires an external element activating over several genes at different times (Zakany *et al.*, 2001) therefore possibly keeping segmentation in phase with expression. Alternatively, gene clustering may be necessary to maintain the patterning rather than establish it. Initiation of gene activity must be maintained to effect change in patterning tissues. Premature or delays of activation produce no stable alterations in spatial distribution of transcripts. The mechanisms indicated here may ultimately work in combination, reflected in the complexity of the patterning that has evolved and adapted to produce the necessary distribution of *Hox* gene products in the right place at the right time (Duboule, 1998).

1.1.3. *Hox* gene patterning of the pharyngeal arches

Neural crest cells derived from the hindbrain contribute to the mesenchyme of the pharyngeal arches. In the hindbrain, each rhombomere has a unique combinatorial *Hox* gene expression code, shared by the migrating neural crest cells originating from each segment (Fig. 1.2). The *Hox* code is anteriorly limited to the second rhombomere (r2) expressing *Hoxa2*. Although r2 expresses *Hoxa2*, the derived neural crest cells rapidly down-regulate expression (Prince and Lumsden, 1994). Consequently, the frontonasal sheet, and the trigeminal neural crest stream contributing to the first pharyngeal arch, are *Hox*-negative (reviewed in Kuratani *et al.*, 1997; Graham *et al.*, 2004). Therefore, the code extends

regional identity to the ectomesenchyme and epithelia of the developing head and pharyngeal arches.

The *Hox* genes specify each of the distinct types of skeletal elements formed within the pharyngeal segments. For example, the first arch, in addition to the masticatory apparatus, forms the auditory ossicles, incus and malleus of the middle ear (Sperber, 2001). The second arch forms Reichert's cartilage derivatives that include portions of the hyoid bone and stapes. The more caudal arches provide cartilage elements for the pharynx and larynx in tetrapods and gill arches in teleosts. While the trigeminal stream is *Hox*-negative, the hyoid stream expresses *Hoxa2*, and the post-otic stream expresses *Hoxa3*, *-b3* and *-d3*. Mouse null mutants reveal the role of *Hox* genes in patterning the type of skeletal elements formed from the arches. The genetic loss of both *Hoxa3* and *Hoxd3* causes third and fourth arch cartilage dysmorphology (reviewed in Graham and Smith, 2001). A more dramatic example is the loss of the *Hoxa2* resulting in a homeotic duplication of *Hox*-negative first arch skeletal elements in the second arch (Gendron-Maguire *et al.*, 1993; Rijli *et al.*, 1993). Conversely, overexpression of *Hoxa2* in the first arch leads to hyoid-like elements in *Xenopus* tadpoles (Pasqualetti *et al.*, 2000), and chick (Grammatopoulos *et al.*, 2000). Gain and loss of function studies in zebrafish with *hoxa2* and its redundant paralogue *hoxb2* generate similar homeotic conversions (Hunter and Prince, 2002). Transformations of first arch elements, Meckel's cartilage and the quadrate, into second arch-like elements illustrate the influence of the *Hox* genes in specifying the A/P skeletal morphology in the arches.

1.1.4. Evolution of the jaws

Pharyngeal segmentation is a chordate feature observed in all of the urochordates, such as, the pharyngeal slits of the filter-feeding *Ciona intestinalis* larvae, the gills of the cephalochordate, amphioxus, as well as the highly derived masticatory and laryngopharyngeal apparatus of vertebrates. Jaws are a unique and significant vertebrate evolutionary innovation, not found in other chordates, requiring a jointed skeleton and supporting musculature (reviewed in Kimmel *et al.*, 2001). Comparative studies speculate that jaws developed from the anterior-most preexisting gill-bearing arch through a transformation. However, no fossil record exists to substantiate this theory (Forey and Janvier, 1993). The ancestor of gnathostomes is long extinct (390-420 mya), but modern examples, the jawless (agnathan) lamprey and hagfish, provide clues as to the morphological and molecular changes that occurred. Lampreys and hagfish are eel-like, lacking paired fins, having a circular mouth and possess a single median nostril. Unlike gnathostomes, lampreys develop a velum for the sucking of water, and have cartilage rods supporting the upper and lower lips, with no joint (Horigome *et al.*, 1999).

The fossil record suggests that jaws appeared suddenly, and how jaws evolved remains unclear (Forey and Janvier, 1993). Differences between gnathostome vertebrates and lampreys suggest how jaws may have evolved. Molecular studies postulate that evolution of a masticatory apparatus is the result of the loss of *Hox* gene expression in the first arch of gnathostomes. Lampreys, similar to gnathostomes, have three streams of neural crest migrating into the arches (Horigome *et al.*, 1999). However, unlike gnathostomes, the lamprey species, *Lampetrea fluviatilis* expresses a *Hox* genes in the first arch (Cohn, 2002).

HoxL6 is expressed in all the arches and later in the upper and lower lips, co-localizing with *Dlx* immunostaining, a marker of lamprey cranial neural crest (Cohn, 2002). Shigetani and colleagues (2002) proposed that a heterotopic shift of epitheliomesenchymal tissue interaction was responsible for the transition based on comparison of ortholog gene expression in ectomesenchyme of chick versus lamprey. Gene cognates of FGF, BMP, *Dlx* and *Msx* showed conserved developmental mechanisms in lamprey as in other jawed vertebrates. However, the lamprey *Dlx1/6* cognate expression pattern is broader in the ectomesenchyme than in gnathostomes, possibly due to the expanded distribution of FGF8 in the epidermis (Shigetani *et al.*, 2002). Recently published data suggests however that some lamprey species may lack *Hox* gene expression in the first arch (Takio *et al.*, 2004). Therefore, it is probable that jaws evolved from existing developmental mechanisms that have been adapted into forming novel morphology.

1.1.5. FGF, BMP and endothelin signaling in the pharyngeal arches

Initiation of many of the molecular cascades that shape the pharyngeal arches is the result of the signaling events occurring between the ectomesenchyme and the adjacent epithelium and endothelium. Several signaling families, the FGFs, BMPs and endothelins, have particular importance in influencing arch development and *Dlx* gene expression. To afford greater understanding of their influence on the *Dlx* genes, a brief review is necessary.

Fibroblast growth factors

Fibroblast growth factors, of which there are twenty-three known ligands, play an integral part in facial patterning (Bachler and Neubuser, 2001). Several family members, *Fgf8*, -9

and -4, are expressed in the oral epithelium of the first arch (Kettunen and Thesleff, 1998). *Fgf8* is expressed in several key signaling centers that influence patterning of the face. *Fgf8* and Sonic-hedgehog (*Shh*) are expressed in the frontonasal ectodermal zone (fez), which induces and coordinates D/V polarity of maxillary outgrowth (Hu *et al.*, 2003). The isthmus, midbrain-hindbrain border, secretes FGF8 which inhibits *Hoxa2* expression (Trainor *et al.*, 2002), and is necessary for neural crest cell survival (Trumpp *et al.*, 1999; Abu-Issa *et al.*, 2002). Additionally, FGF8 is involved in determining oral/aboral polarity of the first arch (Tucker *et al.*, 1999a). *Fgf8* is expressed in two domains that presage the mandibular arch (Shigetani *et al.*, 2002), and positively influence *Dlx* and *Barx1* expression in the mandible (Thomas *et al.*, 2000). The significance of FGF signaling in craniofacial development is revealed in the number of human syndromes that are a result of mis-sense mutations in the FGF receptors (FGFR). These syndromes include Apert Syndrome, Beare-Stevenson Syndrome, Crouzon Syndrome, Jackson-Weiss Syndrome and Pfeiffer Syndrome (Passos-Bueno *et al.*, 1999; Wilkie and Morriss-Kay, 2001; Ornitz and Marie, 2002).

Deficiencies in FGF signaling lead to dysmorphogenesis. In mice, a hypermorphic FGFR1 allele causes aberrations in neural crest migration into the second arch (Trokovic *et al.*, 2003). Conditional loss of FGF8 results in the absence of first branchial arch structures (Trumpp *et al.*, 1999). Additionally, *Fgf8* hypermorphs exhibit craniofacial defects akin to DiGeorge syndrome (Abu-Issa *et al.*, 2002; Frank *et al.*, 2002) and CATCH-22-like aberrations (a cluster of disorders originating from the q arm of chromosome 22) (Wilson *et al.*, 1993), suggesting *Fgf8* participates in pharyngeal arch patterning and derivation of the cranial neural crest. In chick, FGF imbued beads implanted in the face shifts patterning,

exemplified by alterations in skeletal elements (Mina *et al.*, 2002). In zebrafish, perturbation of *fgf3* by morpholino (MO) mediated gene silencing results in the absence of ceratobranchial cartilages. A double knockdown with *fgf8* MO leads to the loss of all head cartilage elements (Walshe and Mason, 2003). Concordantly, zebrafish embryos subjected to increasing concentrations of SU5402, a pharmacological inhibitor of FGFR activity, showed increasing loss of head cartilage elements, indicating the necessity of FGF signaling in facial patterning (Walshe and Mason, 2003).

Bone morphogenic proteins

Members of the TGF- β superfamily, the bone morphogenic proteins (BMPs) are signaling molecules that also participate in patterning the facial form. BMP expression specifies the maxillary processes that undergo midline fusion to form the palatal shelves separating the nasal cavity from the mouth. Inhibition of BMP signaling with noggin coated beads with additional retinoic acid (RA) coated beads transforms maxillary processes into supplementary frontonasal processes (Lee *et al.*, 2001b). Similar to FGF bead implantation experiments, misexpression of BMP imbued beads alters patterning of downstream transcription factors in chick facial prominences (Barlow and Francis-West, 1997; Mina *et al.*, 2002). BMP2 and BMP4 signaling has been shown to positively influence *Msx1* mesenchymal expression in the first arch (Tucker *et al.*, 1998b-a), while antagonizing the effects of FGF signaling (Ferguson *et al.*, 2000). BMP4 inhibits *Barx1* and *Dlx*, altering tooth shape from incisiform to molariform (Tucker *et al.*, 1998a). Therefore, the combination of the two opposing signals induces regionalization and creation of distinct domains within the head (Neubuser *et al.*, 1997).

Endothelin-1

In addition to FGF and BMP signaling, the endothelin-1 signaling pathway is also involved in the specification, and patterning of the ventral or most distal portion of the pharyngeal arches (Tucker *et al.*, 1999a). *Endothelin-1* (*Et-1*), encodes a peptide expressed in the vascular endothelial cells that was initially characterized as a powerful vasoconstrictor regulating blood pressure (Yanagisawa *et al.*, 1988). *Et-1* also plays a developmental role in patterning the major arch arteries and heart. *Et-1* is expressed in the arch epithelium, pharyngeal pouch endothelium and arch core mesoderm in mouse and zebrafish (Clouthier *et al.*, 1998; Miller *et al.*, 2000). Loss of function of *Et-1* or the endothelin type A receptor (*EndrA/ETA*), in mice and chick have indistinguishable defects that include small tongues, micrognathia, cleft palate, and cardiovascular malformations (Kurihara *et al.*, 1994; Clouthier *et al.*, 1998; Kempf *et al.*, 1998). Similarly, the zebrafish *et-1* mutant, *sucker* (*suc*), is a ventral arch mutant (Miller *et al.*, 2000). Recently *Et-1* has been shown to regulate *Dlx6* expression along with *dHAND* in the most distal aspect of the mandible (Charite *et al.*, 2001). *dHAND* is a bHLH transcription factor that patterns ventral skeletal elements of the first and second arches (Yanagisawa *et al.*, 2003). Therefore, signaling molecules such as FGFs, BMPs and ET-1 are involved in the induction events necessary for proper facial patterning, by regionalizing and influencing downstream factors involved in tissue identity and cell differentiation.

1.2. The *Distal-less/Dlx* related gene family

1.2.1. Vertebrate *Dlx* genes

The *distal-less-related/Dlx* genes are homeodomain-containing transcription factors found in all metazoans studied to date (reviewed in Merlo *et al.*, 2000; Zerucha and Ekker, 2000; Panganiban and Rubenstein, 2002). Initially, the *distal-less* (*Dll*) gene was characterized, in *Drosophila melanogaster*, as being involved with distal patterning of facial and limb appendages (Cohen *et al.*, 1989; Vachon *et al.*, 1992). Similarly, in vertebrates, *Dlx* genes function in the proximodistal (P/D) patterning of the pharyngeal arches, as well as a wide variety of other morphogenetic programs.

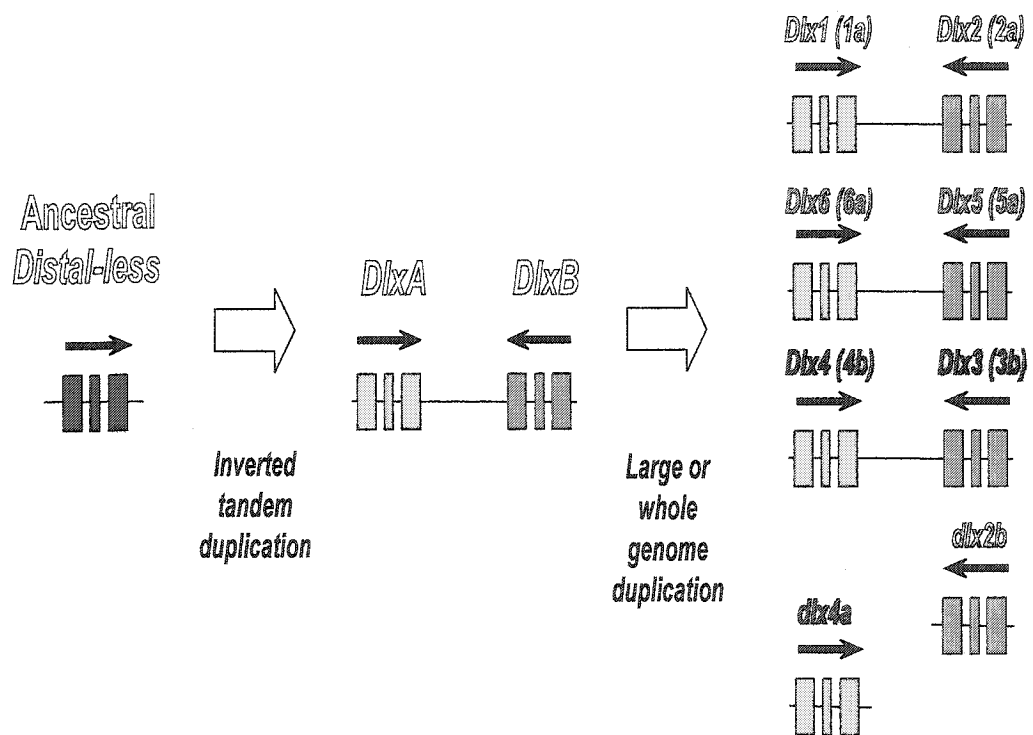
Orthologous *Dlx* genes of divergent vertebrate species generally show similar patterns of overlapping expression. The *Dlx* genes have been investigated in mammals, such as humans (Simeone *et al.*, 1994; Scherer *et al.*, 1995; Nakamura *et al.*, 1996; Davideau *et al.*, 1999b), mouse (Porteus *et al.*, 1991; Price *et al.*, 1991; Robinson *et al.*, 1991; Simeone *et al.*, 1994; Weiss *et al.*, 1995; Nakamura *et al.*, 1996; Liu *et al.*, 1997), and rat (Zhao *et al.*, 1994), as well as other model organisms, such as, chick (Ferrari *et al.*, 1995; Pera and Kessel, 1999), amphibians including newt (Beauchemin and Savard, 1992), frog species *Xenopus laevis* (Asano *et al.*, 1992; Dirksen *et al.*, 1993; Papalopulu and Kintner, 1993), and *Eleutherodactylus coqui* (Fang and Elinson, 1996), turtle (Fernandez *et al.*, 1998), teleosts, such as zebrafish (Ekker *et al.*, 1992; Akimenko *et al.*, 1994), and pufferfish species *Spheroides nephelus* and *Takifugu rubripes* (Ghanem *et al.*, 2003), along with lampreys, which also have been investigated (Neidert *et al.*, 2001). The *Dlx* genes are expressed at sites associated with morphological innovations. The vertebrate *Dlx* genes are expressed

primarily in the forebrain, pharyngeal arches, sensory placodes and limbs/fins. To explore how the *Dlx* genes function in development, reviewed here is a summation of their most salient aspects.

1.2.2. Genomic organization of the *Dlx* genes

Vertebrate *Dlx* genes are typically organized as convergently transcribed bigene clusters with overlapping expression patterns (reviewed in Zerucha and Ekker, 2000; Panganiban and Rubenstein, 2002). In mouse and humans, there are three sets of paired genes (Fig. 1.3). The genes are linked as *Dlx1/2* (McGuinness *et al.*, 1996), *Dlx3/4* (formally *Dlx3/7*) (Scherer *et al.*, 1995; Nakamura *et al.*, 1996), and *Dlx5/6* (Simeone *et al.*, 1994) (revised nomenclature reviewed in Panganiban and Rubenstein, 2002). Interspecies comparison of *Dlx* gene organization reveals that the intergenic region is relatively well conserved, and range in mammals from 8-10 kb for *Dlx1/2* to 4-7 kb in teleost species (Zerucha *et al.*, 2000; Sumiyama *et al.*, 2002; Ghanem *et al.*, 2003). The *Dlx* genes are physically linked approximately 1 to 2 mb from the *Hox* complexes (Stock *et al.*, 1996; Amores *et al.*, 1998; Ruddle *et al.*, 1999). The human *DLX1/2* bigene cluster is linked to the *HOXD* cluster on chromosome 2q32 (Ozcelik *et al.*, 1992; McGuinness *et al.*, 1996), *DLX5/6* are linked to the *HOXA* cluster on chromosome 7q21.3-q22.1 (Simeone *et al.*, 1994) and *DLX3/4* are linked to the *HOXB* cluster at 17q21.3-q22 (Scherer *et al.*, 1995; Nakamura *et al.*, 1996). The *HOXC* complex does not have a *DLX* cluster. Zebrafish, which diverged from a common vertebrate ancestor approximately 450 mya, have eight *dlx* genes organized in a similar

Figure 1.3 Model of *Distal-less*-related gene family evolution. An initial inverted tandem duplication of an ancestral *Dll* gene (red) created an ancient bigene cluster physically linked to the Hox complex. Whole or large genomic duplications created the three pairs, *Dlx1/2*, *Dlx3/4* and *Dlx5/6* found in gnathostomes. Zebrafish *dlx* gene family indicated in parenthesis have an additional two single genes, thought to be the result of extra duplications events and subsequent gene loss. The *Dlx* genes are divided into two major clades. The *Dlx1/4/6* clade (orange), and the *Dlx2/3/5* clade (green). The three *Dlx* exons are indicated by boxes; the direction of transcription is indicated by the black arrows. Adapted from Zerucha and Ekker (2000).



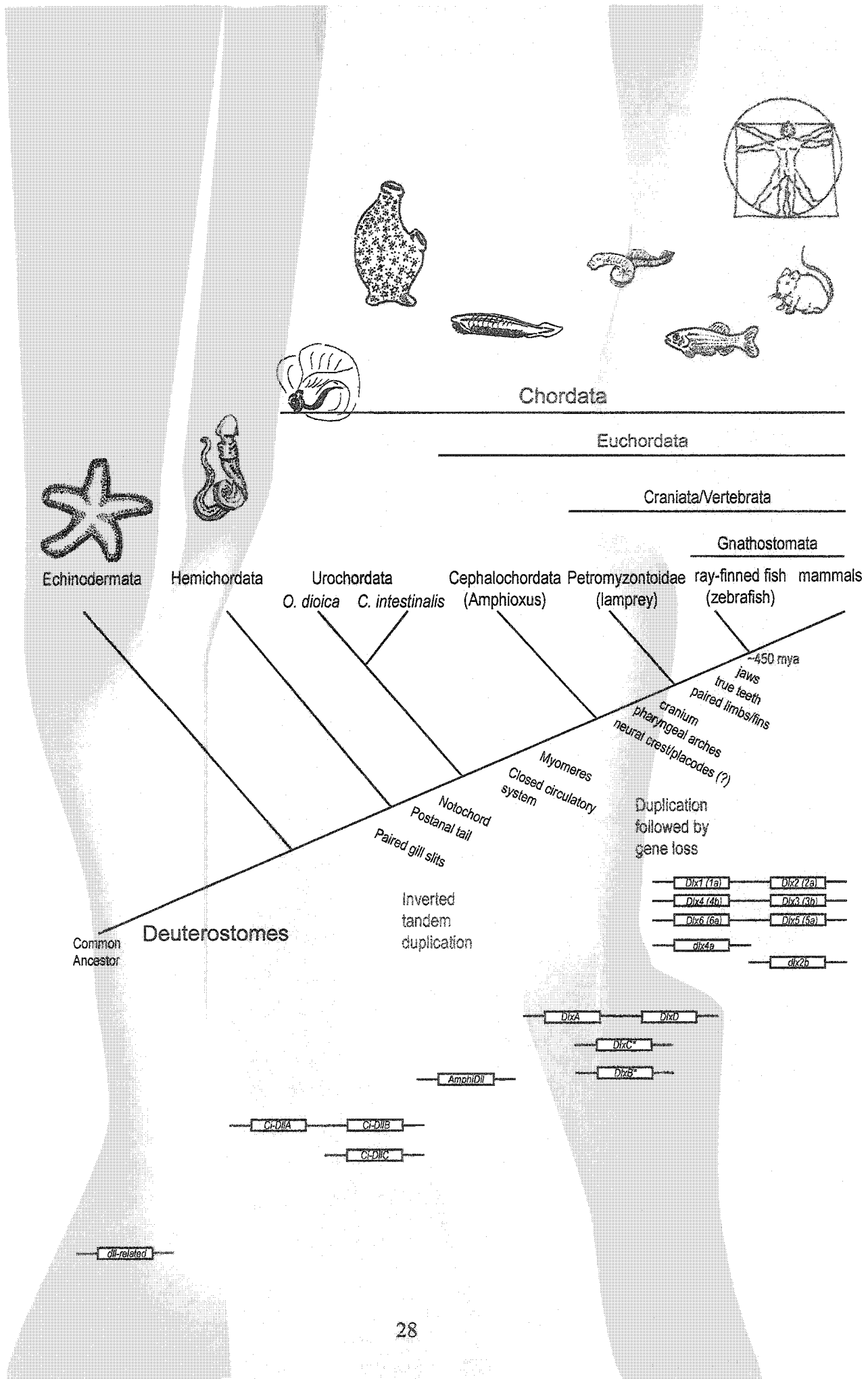
manner to their mammalian orthologs as *dlx1a/2a*, *dlx3b/4b*, and *dlx5a/6a* (formerly *dlx4/6*). Zebrafish also have an additional two unlinked genes, *dlx2b* (formerly *dlx5*), and *dlx4a* (formerly *dlx8*) (Stock *et al.*, 1996; Ellies *et al.*, 1997a; reviewed in Zerucha and Ekker, 2000).

Vertebrate *Dlx* genes are organized as three exons with a common intron-exon structure, and a highly conserved intron splice site dividing the homeodomain (HD) between exon two and three (Price *et al.*, 1991; McGuinness *et al.*, 1996; Ellies *et al.*, 1997b; Liu *et al.*, 1997). The most conserved region is the HD, with short regions of similarity outside the domain (Stock *et al.*, 1996). Many of the *Dlx* genes have multiple transcripts of varying lengths, either due to several start sites, as described for *Dlx1* (McGuinness *et al.*, 1996), or as a result of alternative splicing observed for *Dlx4* and *Dlx5* (Nakamura *et al.*, 1996; Liu *et al.*, 1997; Yang *et al.*, 1998). Antisense isoforms of unknown significance have also been detected for *Dlx1* and *Dlx6* (McGuinness *et al.*, 1996; Liu *et al.*, 1997). Furthermore, *Dlx5* has been shown to produce cytoplasmic truncated proteins (δ Dlx5) lacking the HD and putative NLS (Liu *et al.*, 1997; Yang *et al.*, 1998; Eisenstat *et al.*, 1999). In addition to the conservation of the HD, non-coding intergenic regions separating linked pairs of approximately 400-500 bp show a high sequence similarity. These conserved sites have important *cis*-acting enhancer activity, to be discussed in more detail in section 1.2.6.2. (Zerucha *et al.*, 2000; Sumiyama *et al.*, 2002; Ghanem *et al.*, 2003).

1.2.3. Evolution of the *Dlx* gene family

A paradigm of evolution is revealed in the increase in genes arising from multiple large or whole genome duplications correlated with the increase in body complexity. A well-characterized example is the evolution of the chordates. Increasing complexity is observed in comparing the simplest urochordates such as the ascidians and larvaceans, followed by the cephalochordates exemplified by amphioxus and at the apex of the phylum the vertebrates (Figure 1.4). All members of the chordata phylum share primitive characteristics that were inherited from a common ancestor (plesiomorphic traits). Such traits include a notochord, paired and segmented muscles, post-anal tail, and pharyngeal gill slits. Conversely, differences between species within a class, or comparisons between classes reveal apomorphic or derived characteristics specific to those animals. Examples of derived traits include such morphological innovations as paired limbs, sensory placodes, true teeth and a jaw (Shimeld and Holland, 2000). Many of the morphological evolutionary innovations are thought to be the result of the increase of the master regulatory genes (Chipman, 2001) and evolution of the developmental mechanisms they control (Rudel and Sommer, 2003). Analysis of genomes from different species, from each of the three chordate classes indicate that urochordate species and amphioxus have only one cluster of *Hox* genes (Holland *et al.*, 1994; Schilling and Knight, 2001; Dehal *et al.*, 2002). Vertebrates have multiple clusters, with gnathostomes, jawed vertebrates, such as tetrapods having at least four (Amores *et al.*, 1998). Many teleost species have more than four *Hox* clusters correlating with their large increase in phenotypic diversity and species radiation (Amores *et al.*, 1998; Taylor *et al.*, 2003; Venkatesh, 2003).

Figure 1.4 *Dlx* genes are involved in morphological innovations. A cladogram schematizes the phylogenetic relationships between chordate species, incorporating a partial list of morphological innovations and associated evolution of the *Dlx* gene family. Coloured ribbons link illustrated model organisms with *Dlx* gene number and organization. Parentheses within the gene boxes indicate zebrafish orthologues; asterisks indicate unknown linkage relationships. Adapted from Neidert *et al.*, (2001).



The conserved nature of the vertebrate *Dlx* bigene cluster and their physical linkage to the *Hox* clusters suggests a model for their evolution (reviewed in Zerucha and Ekker, 2000). Only one *Dll* gene has been characterized in arthropods and other invertebrates, such as the nematode, *Caenorhabditis elegans* (Aspöck and Burglin, 2001), or mollusk species (Lee *et al.*, 2001a) suggesting that a single *distal-less* gene existed in a common metazoan ancestor (Fig. 1.4). An inverted tandem duplication occurred, producing an ancestral *DlxA* and *DlxB* cluster after the hemichordate divergence, and most likely during the primitive chordate radiation. Examples include the single *Dll*-related gene in the hemichordate species *Ptychodera flava* (Harada *et al.*, 2001) and three *Dlx* homologs in the urochordate *Ciona intestinalis*, organized as one convergently linked pair, *Ci-Dll-A/B*, and a third unlinked *Ci-Dll-C* (Di Gregorio *et al.*, 1995; Caracciolo *et al.*, 2000). A single *AmphiDll* gene has also been characterized in the cephalochordate, amphioxus, which presumably diverged after the urochordates radiation yet prior to vertebrates (Holland *et al.*, 1996; Sumiyama *et al.*, 2003a). Two subsequent polyploidization events associated with the linked *Hox* gene cluster duplications occurred in the common vertebrate ancestor. Molecular clock analysis suggests a period of 350-650 mya when a large scale DNA-duplication occurred in an ancestral chordate (McLysaght *et al.*, 2002). Four *Hox* clusters were generated during these genomic duplications; only three *Dlx* bigene clusters exist in mammals, suggesting that one pair was lost (Stock *et al.*, 1996). The most basal level vertebrate examined, the agnathan lamprey, *Petromyzon marinus*, has four *Dlx* genes organized as one bigene cluster (*DlxD/A*) and two unlinked genes (*DlxB*, *DlxC*) suggesting a loss of paralogues (Neidert *et al.*, 2001; Sumiyama *et al.*, 2003a). In more advanced ray-finned teleosts, an additional round of polyploidization or partial genomic duplication occurred, and is thought to have led to the

additional *dlx* genes in zebrafish (Amores *et al.*, 1998; Robinson-Rechavi *et al.*, 2001).

Cluster duplication has led to two *Dlx* clades of *trans*-paralogues, whereby, *Dlx2*, -3 and -5 are more closely related than *Dlx1*, -4 and -6. These clades are supported by amino acid sequence similarities within and outside of the HD (Stock *et al.*, 1996). Therefore, sequence similarity exists less between linked paralogues, as they were created by the more ancient inverted tandem duplication producing the initial bigene cluster. *Hox* cluster topology relationships have been suggested as A:D::B:C; whereby A and D are the most primitive and B and C are more divergent (Bailey *et al.*, 1997). This topology is consistent for the *Dlx* cluster evolutionary relationships of *Dlx1/2:Dlx5/6::Dlx3/4*. These relationships are supported by *Dlx1/2* and *Dlx5/6* having similar overlapping expression patterns and *Dlx3/4* having a more divergent pattern of expression (Bailey *et al.*, 1997). Of all the vertebrate *Dlx* genes, *Dlx1* appears to be the closest resembling a founding gene, as it is the most similar to the *Drosophila Dll*, and amphioxus, *AmphiDll* genes (Holland *et al.*, 1996; Stock *et al.*, 1996). Therefore, the *Dlx* gene family provides an interesting example to investigate evolutionary relationships of conservation and divergence between orthologs.

1.2.4. Functional role of *Dlx* genes in development

Dlx genes tend to be expressed in highly derived structures associated with vertebrate evolutionary innovations, including hard tissues of the head, the axial and appendicular skeleton, as well as the forebrain, pharyngeal arches, sensory placodes, and developing limb/fin buds (reviewed in Bendall and Abate-Shen, 2000; Merlo *et al.*, 2000; Panganiban and Rubenstein, 2002). These expression patterns are well conserved between highly

divergent vertebrate species such as mouse and zebrafish. Generally, *Dlx1/2* and *Dlx5/6* are expressed in similar patterns. *Dlx3/4*, however, is expressed in more neomorphic vertebrate organs such as the placenta and erythropoietic lineages (Takeshita *et al.*, 1993; Quinn *et al.*, 1997; Quinn *et al.*, 1998; Morasso *et al.*, 1999).

Overlapping patterns of linked *Dlx* genes in a bigene cluster often makes them difficult to distinguish from each other and suggests functional redundancy (Price *et al.*, 1991; Robinson *et al.*, 1991; Dolle *et al.*, 1992; Papalopulu and Kintner, 1993; Akimenko *et al.*, 1994; Morasso *et al.*, 1994; Robinson and Mahon, 1994; Simeone *et al.*, 1994; Ellies *et al.*, 1997b; Quint *et al.*, 2000; Zerucha and Ekker, 2000). However, subtle differences in expression patterns exist. Differences include apparently higher levels of *Dlx5* transcripts in the subventricular zone of the forebrain compared to the mantle. Conversely, *Dlx6* shows the inverse relative expression pattern (Liu *et al.*, 1997). Additionally, *Dlx2* shows much higher expression in the first arch ectoderm than *Dlx1* (Bulfone *et al.*, 1993a; Qiu *et al.*, 1997). These slight differences may be the reason for the different phenotypes observed in comparing single *Dlx* mutants of a bigene cluster.

Genetic null mutations have been generated for most of the murine *Dlx* genes, (*Dlx6*^{-/-} and *Dlx4*^{-/-} single mutations have yet to be published) and reveal their functional role during development. *Dlx1*^{-/-} mice have defects in the enteric nervous system and proximal skeletal elements of the first and second pharyngeal arches (Qiu *et al.*, 1997). *Dlx2*^{-/-} mutants have malformation in first and second arch derivatives, such as dysmorphic and ectopic skeletal elements, as well as abnormal differentiation of olfactory bulb neurons (Qiu

et al., 1995). Additionally, *Dlx2*^{-/-} mice have reduced *Dlx1* expression in the mandibular and hyoid arches, as well as in the lateral ganglionic eminence of the prosencephalon (Qiu *et al.*, 1997; Eisenstat *et al.*, 1999). *Dlx5*^{-/-} null mice exhibit severe dysmorphology in the auditory vestibular organ, the proximal mandibular arch skeleton, and hypoplastic nasal capsules with some mice showing exencephaly due to poor fusion of the neural tube. Defective mineralization in the bone calvaria of the skull is also prevalent (Acampora *et al.*, 1999; Depew *et al.*, 1999). Moreover, loss of function in *Dlx1*, -2, or -5 reveal defects in the cranial neural crest derivatives, such as, malformation of facial skeletal elements, and distortions of the enteric nervous system (Qiu *et al.*, 1995; Qiu *et al.*, 1997; Acampora *et al.*, 1999). Loss of *Dlx3*^{-/-} in mice leads to midgestation lethality due to lack of vascularization of the placenta (Morasso *et al.*, 1999), while gain-of-function results in skin differentiation defects (Morasso *et al.*, 1996).

Loss of single paralogues results in milder abnormal phenotypes than would be reasonably expected considering the patterns of expression. Loss of function of both genes in a cluster results in a more severe abnormal phenotype with novel malformations, indicating genetic compensation between linked genes. Partial redundancy diminishes the severity of the mutant phenotypes due to overlapping patterns of family members. Loss of bigene function has been characterized for *Dlx1/2*^{-/-} (Qiu *et al.*, 1997), *Dlx5/6*^{-/-} (Beverdam *et al.*, 2002; Depew *et al.*, 2002; Merlo *et al.*, 2002; Robledo *et al.*, 2002), and knockdown of both *dlx3b/4b* in zebrafish, using synthetic antisense morpholino oligonucleotides (Solomon and Fritz, 2002). Deletion of the *Dlx1/2* locus results in far more severe dysmorphic craniofacial skeletal elements including the absence of maxillary molars (Qiu *et al.*, 1997;

Thomas *et al.*, 1997). Forebrain defects include an abnormal striatal subventricular zone having poor differentiation of striatal neurons and migration defects of neocortical interneurons (Anderson *et al.*, 1997a; Anderson *et al.*, 1997b). Moreover, *Dlx1/2*^{-/-} mutants also exhibited a decrease of *Dlx5/6* forebrain expression.

The *Dlx5/6*^{-/-} double null mutant phenotype includes severe exencephaly, skeletal dysmorphology, and a mirror duplication of the maxillary component of the first arch replacing the mandibular, as well as, severe limb deformities (Beverdam *et al.*, 2002; Depew *et al.*, 2002). It is unknown as to whether the defects are the result of quantitative effects perhaps disrupting positional information, or qualitative differences between family members, necessary for patterning tissues. Analysis of the single and double *Dlx* mutants suggests that both genes of a pair have partially redundant functions as well as having unique developmental roles (Qiu *et al.*, 1995; Qiu *et al.*, 1997; Acampora *et al.*, 1999; Depew *et al.*, 1999; Beverdam *et al.*, 2002; Depew *et al.*, 2002).

1.2.4.1. *Dlx* function in appendage development

Dll/Dlx expression in developing appendages is highly conserved. *Drosophila Dll*, participates in the distal patterning of facial and leg appendages. Viable hypomorphic alleles show progressively shorter and fused leg and antennal segments (Panganiban, 2000). In tetrapods, all the *Dlx* genes are expressed in the apical ectodermal ridge (AER) of the developing limbs (Beauchemin and Savard, 1992; Dolle *et al.*, 1992; Zhao *et al.*, 1994; Ferrari *et al.*, 1995; Morasso *et al.*, 1995). In zebrafish, the *dlx* genes are expressed in the developing median fin fold which presages the unpaired fins, as well as the apical

ectodermal cells of the pectoral fin buds (Akimenko *et al.*, 1994). Single targeted *Dlx* losses as well as *Dlx1/2*^{-/-} double loss of function reveal no changes in limb morphology (Qiu *et al.*, 1995; Qiu *et al.*, 1997; Acampora *et al.*, 1999; Depew *et al.*, 1999). However, the *Dlx5/6*^{-/-} mice have a split-hand/split-foot (SHFM) like-phenotype (Merlo *et al.*, 2002; Robledo *et al.*, 2002), similar to the human ectrodactyly, absence of digits condition (Crackower *et al.*, 1996), which is further characterized with mental retardation and middle ear defects, sites where *Dlx5/6* genes are known to contribute to development. The genital bud, the prospective appendage for sexual reproduction, also requires *Dlx5/6* for its formation, as does the external ear lobes of mice (Merlo *et al.*, 2000). Therefore, *Dlx* gene function is apparent in all outgrowths of a body.

1.2.4.2. *Dlx* expression in the forebrain

The *Dlx* genes are employed in regional specification of the vertebrate forebrain. The basal telencephalon and ventral thalamus express four of the six *Dlx* genes, 2/1/5/6, in overlapping patterns (Porteus *et al.*, 1991; Price *et al.*, 1991; Robinson *et al.*, 1991; Dolle *et al.*, 1992; Bulfone *et al.*, 1993a; Bulfone *et al.*, 1993b; Simeone *et al.*, 1994; Liu *et al.*, 1997; Yang *et al.*, 1998; Eisenstat *et al.*, 1999; Zerucha *et al.*, 2000). Expression is well conserved in the forebrain, with comparable patterns observed for other vertebrate *Dlx* homologs (Papalopulu and Kintner, 1993; Akimenko *et al.*, 1994; Fernandez *et al.*, 1998). *Dlx1/2* are expressed in the ventricular and subventricular zones during neuroblast proliferation. *Dlx5* is expressed in the subventricular zone and mantle in the differentiating post-mitotic cells. *Dlx6* is mostly expressed in the mantle, the site of terminally differentiated neurons and glia (Porteus *et al.*, 1994; Liu *et al.*, 1997). The *Dlx* spatiotemporal of expression patterns suggests a regulatory

cascade for organizing differentiation of the basal ganglia (Liu *et al.*, 1997). Genetic evidence is shown by *Dlx1/2^{-/-}* mutant mice having a loss of *Dlx5/6* expression in the forebrain (Eisenstat *et al.*, 1999).

Dlx genes are involved in the development of neurons that utilize the γ -aminobutyric acid (GABA) neurotransmitter that contribute to the cerebral cortex, hippocampus, and olfactory bulb (Anderson *et al.*, 1997a; Anderson *et al.*, 1997b; Bulfone *et al.*, 1998; Pleasure *et al.*, 2000; Anderson *et al.*, 2001; He *et al.*, 2001; Letinic *et al.*, 2002; Stuhmer *et al.*, 2002a; Stuhmer *et al.*, 2002b). *Dlx2/5*, and *Dlx1* in a more restricted manner, have been shown to activate an enhancer of the glutamic acid decarboxylase (GAD) gene, the rate-limiting enzyme in the synthesis of the GABA neurotransmitter (reviewed in Panganiban and Rubenstein, 2002). Moreover, ectopic expression of *Dlx2* and *Dlx5* in cortical neurons induces a GABAergic phenotype of large nuclei and expression of *Gad65* and *Gad67* genes (Anderson *et al.*, 1999; Stuhmer *et al.*, 2002a).

Partial genetic redundancy is observed whereby single *Dlx* mutants show minor defects in forebrain development. For example, *Dlx2^{-/-}* mice have decreased numbers of olfactory dopaminergic neurons while *Dlx1/2^{-/-}* mice have severe developmental defects in the basal telencephalon, including a decrease in late-born striatal neurons, and a lack of migratory GABAergic neurons to the neocortex (Anderson *et al.*, 1997b; Qiu *et al.*, 1997; Marin *et al.*, 2000; Pleasure *et al.*, 2000). Therefore, *Dlx* genes, in addition to determining proximodistal limb patterning are necessary for distinct stages of neuronal development in the forebrain and sensory organs.

1.2.4.3. *Dlx* genes in organ development

Dlx genes are expressed in a variety of other tissues in which their role in development has yet to be fully examined. Some of these tissues include *Dlx* expression in the liver and the spleen (Robinson and Mahon, 1994). *Dlx* genes have been also shown to be transcriptional mediators of epithelial-derived organs such as stratified skin (Robinson and Mahon, 1994), the mammalian placenta (Morasso *et al.*, 1999), hair follicles (Beanan and Sargent, 2000), feather bud development (Rouzankina *et al.*, 2004), as well as ameloblast and odontoblast differentiation during tooth morphogenesis (Robinson and Mahon, 1994; Zhao *et al.*, 2000). *Dlx4* was the first family member characterized in leukemia cells and found to play a role during hematopoiesis (Shimamoto *et al.*, 1997; Shimamoto *et al.*, 2000). In *Xenopus*, *Dlx3* is expressed in the cement gland (Beanan and Sargent, 2000), while the chick *Dlx3* ortholog is observed in the tail tip (Pera and Kessel, 1999). Interestingly, the *Xenopus Dlx3* (*Xdll-2*) promoter sequence is capable of recapitulating endogenous expression in the mouse mammary glands and hair follicles (Morasso *et al.*, 1995). This points to the evolutionary nuances and specific adaptations of the developmental programs in which *Dlx* genes participate. Divergence in the species-specific roles indicates the acquisition of novel expression domains and functions.

1.2.4.4. *Dlx* function during chondro- and skeletogenesis

Increasingly, the role of *Dlx* genes in chondrogenesis is being investigated in precursors of skeletal tissue. *Dlx2* is involved in BMP-mediated expression of collagen type II (*col2a1*), a fibril-forming extracellular matrix protein prevalent in cartilage (Xu *et al.*, 2001). *Dlx5/6*

genes are expressed in the endochondral and membranous derived skeletal tissues of ectomesenchyme, and skeletal tissues of mesodermal origin (Simeone *et al.*, 1994; Zhao *et al.*, 1994; Ferrari *et al.*, 1995; Chen *et al.*, 1996; Ryoo *et al.*, 1997; Yang *et al.*, 1998; Acampora *et al.*, 1999; Depew *et al.*, 1999; Xu *et al.*, 2001). In chick and mouse, *Dlx5* participates in chondrocyte proliferation and maturation (Ferrari and Kosher, 2002; Bendall *et al.*, 2003). Additionally, BMP signals have been shown to regulate *Dlx5* expression in the neural crest derived membranous bone of the chick skull (Holleville *et al.*, 2003), and alveolar bone of the mouse jaw (Zhang *et al.*, 2003). Microarray investigation of BMP2 responsive genes in osteoblasts show that *Dlx2/5* are induced, and maintained until mineralization (Harris *et al.*, 2003). Loss of *Dlx5* function causes reduction in the endosteal component of long bone diaphyses, and these animals have a decrease in the periosteal lamina causing delays in certain dermal bone maturation (Acampora *et al.*, 1999; Depew *et al.*, 1999). Several osteogenic specific genes involved in bone formation are regulated by *Dlx5* function. *Dlx5* regulatory targets include osteocalcin, a bone matrix protein that binds calcium and functions in mineralization in rat calvarial cells (Ryoo *et al.*, 1997; Newberry *et al.*, 1998). Additional genes influenced by *Dlx5* include *collagen 1A1* expression (Dodig *et al.*, 1996), and *bone sialoprotein*, BSP (Benson *et al.*, 2000), as well as other osteogenic promoters in MC3T3-E1 osteoblastic cells (Miyama *et al.*, 1999).

1.2.4.5. *Dlx* patterning of the neural plate border and sensory placodes

Early in gastrulation, *Dlx* genes have been shown to play a role in patterning neural and non-neural surface ectoderm. The neural plate border, dividing the neuroectoderm from the epidermis, is the site of inductive signals that give rise to a variety of different cell-types.

Neural border-derived features include the neural crest, sensory placodes, as well as the dorsal midline of the neural tube (Copp *et al.*, 2003). *Dlx* genes participate in the specification of the border, which is necessary for subsequent fate determination of neural crest, and sensory placodes (Woda *et al.*, 2003). During gastrulation, in mouse and chick, *Dlx5* demarcates the rostral boundary of the ectoderm-neuroectoderm plate border extending caudally to the presumptive neural crest cells and sensory placodes (Yang *et al.*, 1998; Quint *et al.*, 2000; McLarren *et al.*, 2003). *Dlx5* is subsequently expressed in the olfactory epithelium in mouse and chick (Yang *et al.*, 1998; McLarren *et al.*, 2003). In early zebrafish embryos, *dlx3b/4b* are expressed in the bilateral ectodermal stripes adjacent to the midline and are progressively restricted to the olfactory and otic placodes, while *dlx5a/6a* are subsequently expressed in the sensory primordia (Ekker *et al.*, 1992; Akimenko *et al.*, 1994; Ellies *et al.*, 1997b). Similar expression is observed for *Xenopus Dlx3* (formerly *Xdll-2*), and *xDlx5/6* (previously *Xdll-3*, and *Xdll* respectively; revised nomenclature according to Luo *et al.*, 2001) in unique overlapping patterns in the anterior non-neural ectoderm (Asano *et al.*, 1992; Dirksen *et al.*, 1993; Papalopulu and Kintner, 1993). In contrast, mouse *Dlx3* is minimally expressed in the equivalent tissue of the anterior neural ridge ectoderm, but is apparently compensated by *Dlx5/6* expression (Yang *et al.*, 1998; Quint *et al.*, 2000) and by *Dlx5* expression in chick (Pera *et al.*, 1998). The zebrafish *dlx5a* ortholog is not expressed in the gastrulating rostral ectoderm (Akimenko *et al.*, 1994). However, *dlx3b/4b*, as is the *Xenopus* ortholog *Xdll-3*, are expressed similar to *Dlx5* expression during gastrulation, suggesting their functional equivalence (Papalopulu and Kintner, 1993; Quint *et al.*, 2000).

The overlapping expression patterns of divergent species' orthologs suggest that the ancestral *Dll/Dlx* gene may have been expressed at all these sites. The amphioxus *Dlx* homologue, *AmphiDll*, is expressed in the ectodermal cells surrounding the neural plate, suggesting an ancestral role of the chordate *Dlx* gene in specifying the neural crest cells (Holland *et al.*, 1996). Therefore, this is an example of evolutionary divergence, whereby allocation of functional roles may differ between vertebrate species.

1.2.4.6. *Dlx* genes in the sense organs

Dlx genes are associated with advanced morphological features. For example, they are expressed in sensory placodes, bilateral ectodermal thickenings that differentiate into the sensory structures of vision, auditory and olfactory organs. The role of *Dlx* gene expression in sensory organs is conserved throughout evolution. In *Drosophila*, *Dll* is necessary for the determination of the antennae which serve as the fruit fly's auditory and olfactory sense organ (Dong *et al.*, 2000). Analogous structures in vertebrates also require *Dlx* gene expression for proper eye, auditory and olfactory development (Robinson *et al.*, 1991; Ekker *et al.*, 1992; Akimenko *et al.*, 1994; Robinson and Mahon, 1994; Zhao *et al.*, 1994; Liu *et al.*, 1997; Zerucha *et al.*, 1997; Yang *et al.*, 1998; Acampora *et al.*, 1999; Depew *et al.*, 1999; Mendonsa and Riley, 1999; Pera and Kessel, 1999; Quint *et al.*, 2000; Solomon and Fritz, 2002). *Dlx5* plays a prominent role in murine ear (Depew *et al.*, 1999; Merlo *et al.*, 2002), as well as olfactory bulb development (Levi *et al.*, 2003; Long *et al.*, 2003). In zebrafish, *dlx3b/dlx4b* perform an equivalent task with expression in the olfactory and otic placodes (Robledo *et al.*, 2002; Solomon and Fritz, 2002). *mDlx1/2* have been found in sensory neuronal precursors (Dolle *et al.*, 1992; Eisenstat *et al.*, 1999), and *cDlx3* is in the

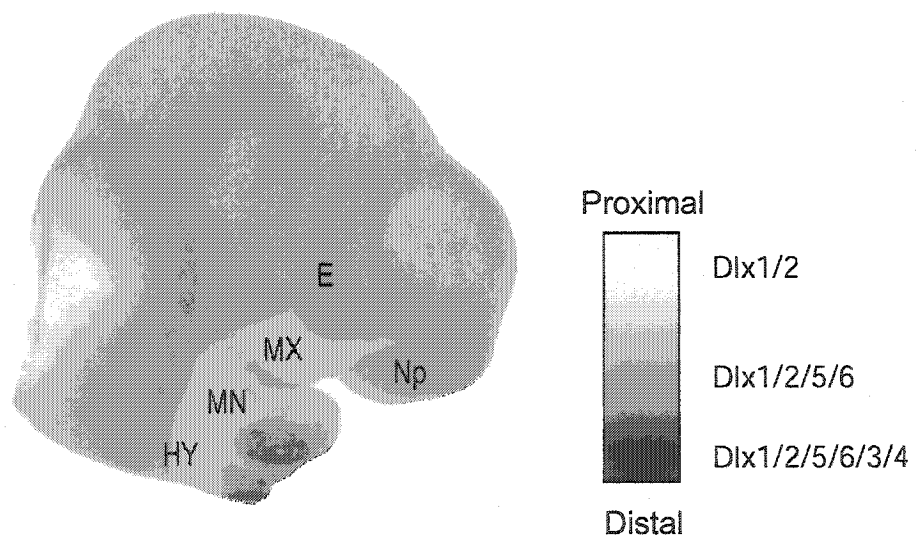
optic cup and neural retina (Dhawan *et al.*, 1997). These diverse sites indicate the multifunctional roles of *Dlx* genes in sensory organ development.

1.2.4.7. Proximodistal patterning of the pharyngeal arches

Dlx genes participate in the P/D specification of the pharyngeal arches and their derived tissues, including the masticatory apparatus and teeth (Dolle *et al.*, 1992; Robinson and Mahon, 1994; Weiss *et al.*, 1994; Zhao *et al.*, 1994; Weiss *et al.*, 1995; Zhao *et al.*, 2000). The *Dlx* role in facial appendage patterning is conserved, as the *Drosophila Dll* gene is necessary for distal patterning of its analogous maxillary palps (O'Hara *et al.*, 1993; Gorfinkiel *et al.*, 1997; reviewed in Panganiban, 2000).

In gnathostomes, *Dlx2* is the first family member expressed in the cranial neural crest migrating from the rhombencephalon to the arches (Bulfone *et al.*, 1993a; Akimenko *et al.*, 1994; Robinson and Mahon, 1994). Subsequently, other *Dlx* genes are expressed in a dynamic manner in the arch primordia during pharyngogenesis (Akimenko *et al.*, 1994). *Dlx* genes are expressed in a P/D nested pattern in the cranial neural crest derived ectomesenchymal cells (Graham, 2001; Graham and Smith, 2001; Graham, 2002; Yelick and Schilling, 2002; Schilling, 2003). In the arch primordia, *Dlx1/2* expression is in a broad continuous rostral to caudal domain from the most proximodorsal aspect of the maxilla portion of the first arch to the distoventral tip of the mandible (Fig. 1.5). *Dlx5/6* expression overlaps with *Dlx1/2* in the mandible and *Dlx3/4* in the most distal aspect of the first arch, creating a nested pattern (Qiu *et al.*, 1997).

Figure 1.5 *Dlx* nested patterns of expression in the pharyngeal arches. An E10.5 mouse head is overlaid with the schematized expression patterns of *Dlx1/2* (yellow), *Dlx1/2/5/6* (green) and *Dlx1/2/5/6/3/4* (blue). E, eye; Np, nasal pit; HY, hyoid; MN, mandible; MX, maxilla. Adapted from Depew *et al.*, (2002).



The functional significance of the nested pattern is evident in mouse double mutants. Targeted loss of *Dlx1/2* results in dysmorphology of proximal soft and hard tissue features including an absence of maxillary molars, yet mutant mice retain all other teeth (Qiu *et al.*, 1997; Thomas *et al.*, 1997). *Dlx5/6*^{-/-} mutants exhibit craniofacial defects that included enantiomorphism of both hard and soft tissues of the proximal maxillary portion of the first arch, replacing the distal mandibular features (Beverdam *et al.*, 2002; Depew *et al.*, 2002). Additionally, *Dlx5/6*^{-/-} mutants exhibit defects such as cleft palate, abnormal middle ear and jaw bone dysmorphology (Depew *et al.*, 2002). Therefore, the resulting mutant phenotypes reveal a role in P/D specification of the pharyngeal arches.

An evolutionary example is seen in the more primitive agnathan lamprey species, *Lampetra japonica*, and *Petromyzon marinus*, with uniformly shaped arch morphology. *Dlx* gene cognates in the lamprey species are expressed in non-nested overlapping patterns within the lips of the animals, suggesting that nested expression is necessary for differentiating proximal versus distal identity (Myojin *et al.*, 2001; Neidert *et al.*, 2001). Additionally, in lamprey, three out of the four *Dlx* genes are expressed in the migrating cranial neural crest cells (Neidert *et al.*, 2001). This suggests that *Dlx* expression in neural crest may be an ancestral function. Therefore, *Dlx* genes function in a nested pattern of increasing distalization of tissue, correlated with the increasing number of family members expressed. Determination of whether this is a quantitative or qualitative patterning mechanism has yet to be investigated.

1.2.4.8. *Dlx* expression during odontogenesis

Dlx genes are expressed in the ectoderm, and mesenchyme of the maxillomandibular process of the first arch (Bulfone *et al.*, 1993a). As jaw development progresses, *Dlx* genes participate in odontogenesis (Weiss *et al.*, 1994; Thomas *et al.*, 1995; Weiss *et al.*, 1995; Zhao *et al.*, 2000), and alveolar bone formation which supports the dentition (Zhang *et al.*, 2003). Mammalian teeth are derived from the oral ectoderm, and underlying ectomesenchyme exclusively in the maxillomandibular processes. Epithelial instructive cues, including BMP4 and FGFs, regionalize the underlying ectomesenchyme to become odontogenic in a spatiotemporal manner (Tucker *et al.*, 1998b-b; Tucker and Sharpe, 1999b; Ferguson *et al.*, 2000). Regionally restricted homeobox-containing genes include the *Dlx* genes, *Msx1/2*, *Lhx6/7*, *Alx3*, *Barx1*, *Pitx* genes and *Gsc* (Sharpe, 1995; reviewed in Cobourne and Sharpe, 2003). Theorized by Paul Sharpe, the homeodomain-containing genes participate in an odontogenic homeobox code, specifying tooth-type in the prospective dental mesenchyme. Discrete and overlapping expression patterns regionalize the mesenchyme and are thought to contribute to zones of incisivization and molarization in determining tooth-type (reviewed in Thomas and Sharpe, 1998; Sperber, 2001).

The dynamic expression of the *Dlx* genes in the oral epithelium and ectomesenchyme reveals the complex reciprocal epitheliomesenchymal interactions necessary for odontogenesis (Bulfone *et al.*, 1993a; Weiss *et al.*, 1994; Thomas *et al.*, 1995; Weiss *et al.*, 1995). Additionally, *Dlx* paralogues have differing dynamic expression patterns during odontogenesis, suggesting differential regulation between paired genes (Zhao *et al.*, 2000). Briefly, teeth are ectodermal appendages that undergo several stages of development

beginning with oral epithelial thickening at the site of tooth formation. *Dlx1/2* genes are initially expressed in the mesenchyme and are influenced by FGF and BMP cues in the thickening epithelium (Bei and Maas, 1998). Epitheliomesenchymal interactions induce invagination of the epithelium into the underlying mesenchyme, producing a tooth germ, and a signaling center, the enamel knot. Corresponding with the organization of the enamel knot, *hDLX5* expression is restricted during bud, and cap stages to the vestibular aspect of the tooth bud's epithelium (Davideau *et al.*, 1999a). Subsequently, the bell stage tissues are organized and cells differentiate into the layers undergoing mineralization necessary for tooth formation.

Dlx function in tooth production is not well understood. However, a human autosomal dominant disorder, tricho-dento-osseous syndrome (TDO), is attributed to a 4 bp truncating deletion in *DLX3* affecting epidermal and ectodermal derived tissues resulting in defects that include taurodontism (bull-like teeth with large pulp chambers and short roots, kinked hair, and a thickening of the cranium (Price *et al.*, 1998). The *Dlx5*^{-/-} mouse mutant has poor mineralization of the molar crowns suggesting a role in odontoblast differentiation (Depew *et al.*, 1999). *Dlx1/2*^{-/-} mice replace the loss of maxillary molars with cartilaginous nodules, suggesting that the mesenchyme cannot respond to epithelial cues necessary for odontogenesis (Thomas *et al.*, 1997). Therefore, the role of the *Dlx* genes extends to patterning all types of hard tissues in the body.

1.2.5. Molecular characteristics of *Dlx* genes

Dlx genes are regulators of transcription. The homeodomain confers binding to DNA, as well as mediating protein:protein interactions (Zhang *et al.*, 1997). Overall, *Dlx* proteins are thought to have similar DNA-binding properties (Liu *et al.*, 1997; Zerucha *et al.*, 1997; Zhang *et al.*, 1997; Feledy *et al.*, 1999b; Zerucha *et al.*, 2000). *In vitro* binding studies indicate that the *xDlx3* DNA-binding consensus sequence (Feledy *et al.*, 1999b) works equally well for *zdlx2a* (Zerucha *et al.*, 2000). *Dlx* proteins interact with both artificial DNA regulatory elements, as well as endogenous enhancers (Zhang *et al.*, 1997; Feledy *et al.*, 1999b; Masuda *et al.*, 2001). Examination of preferred binding sites for *Dlx* proteins indicates an optimal consensus sequence (A/C/G)TAATT(A/G)(C/G) (Feledy *et al.*, 1999b).

At present, few *Dlx* downstream transcriptional targets have been characterized. Chromatin immunoprecipitation (ChIP) experiments show *Dlx1/2* proteins bind directly to a conserved *Dlx5/6* (I56i) intergenic enhancer in forebrain, and retinal tissue (Zhou *et al.*, 2004). *Dlx3* regulates several genes through a TAAT core HD DNA-binding motif in trophoblasts, choriocarcinoma cells (Roberson *et al.*, 2001), and differentiating keratinocytes (Morasso *et al.*, 1996). Examples of genes activated by *Dlx* expression include the *bone sialoprotein* (BSP) gene (Benson *et al.*, 2000), the α subunit of chorionic gonadotropin (Roberson *et al.*, 2001), and glutamic acid decarboxylase (GAD) genes (Stuhmer *et al.*, 2002a). *Dlx5* is suggested to both positively and negatively regulate the osteocalcin gene during different stages of osteoblast differentiation (Ryoo *et al.*, 1997; Miyama *et al.*, 1999). BP1, an isoform of hDLX4, functions as a transcriptional repressor of the β -globin gene by interacting with silencer elements (Fu *et al.*, 2001; Chase *et al.*, 2002).

How *Dlx* regulation is modulated remains unclear. Outside the HD, several short amino acid sequences are conserved among *Dlx* proteins that may have some significance. Within the *Dlx* clades, conservation of amino acids surrounding the HD is apparent. The biological significance of these regions remains unclear. However, the amino- and carboxy-terminal domains of *Dlx3* potentiate transcriptional activation, as well as function as a bipartite nuclear localization signal (NLS) necessary for DNA binding (Bryan and Morasso, 2000). The *Dlx2/3/5* clade shares a well conserved 'DIIA' domain of unknown significance (Akimenko *et al.*, 1994; Robinson and Mahon, 1994). Functional analysis of the *Dlx5* N-terminal sequence, in a GAL-4 DNA binding fusion protein, acts as an activation domain. Similarly, both *Dlx3* N- and C-terminal domains assist in transcriptional activation with the HD (Feledy *et al.*, 1999b).

Dlx function is likely context-dependent, requiring additional factors to modify activator or repressor functions. *Dlx* genes have been shown to act in concert with other factors, such as *Dlxin1* to regulate transcriptional activity (Masuda *et al.*, 2001). In tissue culture, *Dlx2* and -5 are capable of complexing as heterodimers with *Msx1* and -2 HDs in antagonistic interactions (Zhang *et al.*, 1997). Yeast two-hybrid screens indicate the GRIP1b PDZ-domain protein, previously designated *DLX interacting protein-2* (DIP2), interacts with the N-termini of *Dlx2* and *Dlx5* (Eisenstat *et al.*, 1999; Yu *et al.*, 2001). Chiba and colleagues have shown that *DLX1* interacts with *Smad4* to inhibit activin A signaling in hematopoietic cells (Chiba *et al.*, 2003). Therefore, the observed positive and negative influences of the *Dlx* genes in different cell-types and cellular processes may be the result of cell-specific cofactors.

1.2.6. Regulation of the *Dlx* gene expression

1.2.6.1. Context dependent regulation of the *Dlx* genes

Various signaling factors, such as BMPs and FGFs, have been implicated in influencing *Dlx* gene expression. BMPs both positively and negatively regulate *Dlx* expression depending on tissue type and developmental stage. BMP signaling induces *xDlx3/5/6*, and *cDlx5* in the prospective non-neural ectoderm (Pera *et al.*, 1998; Feledy *et al.*, 1999a; Luo *et al.*, 2001b). During chondrogenesis, BMP2 induces *Dlx2/5* in TMC23 chondroblasts (Xu *et al.*, 2001), and transactivates *Dlx3* expression in keratinocytes (Park and Morasso, 2002). BMP4 induces *Dlx5* in osteoblasts (Miyama *et al.*, 1999), *cDlx3* in ectodermal tissue (Feledy *et al.*, 1999a), and *Dlx1/2* in late dental mesenchyme (Bei and Maas, 1998). However, BMP4 signaling induces *Dlx2* expression in oral epithelium and inhibits mesenchymal expression prior to odontogenesis, suggesting a stage-dependent regulatory mechanism (Thomas *et al.*, 2000). FGF signals have been shown to stimulate *Dlx* expression in such tissues as denervated axolotl limb ectoderm (Mullen *et al.*, 1996), and in chick limb primordia (Ferrari *et al.*, 1999). In an inverse manner to BMP4, FGF8 inhibits *Dlx2* expression in the oral epithelium (Thomas *et al.*, 2000). However, FGF8 induces *Dlx1/2* in the dental mesenchyme (Bei and Maas, 1998; Thomas *et al.*, 2000; Shigetani *et al.*, 2002). FGF9 and FGF4 signaling in the oral epithelium also contributes to *Dlx1/2* and *Dlx5* maintenance (Trumpp *et al.*, 1999). ET-1 signaling induces *Dlx6* expression, along with the dHAND transcription factor, in the most distal aspect of the mandibular arch (Charite *et al.*, 2001). *Dlx6* expression, however, is retained in *Endr^{-/-}* mice in a more proximal position of the mandible adjacent to the presumptive jaw hinge. Another secreted factor shown to influence

Dlx gene expression is retinoic acid. Retinoic acid (RA) induces *hDLX5* and *hDLX6* in transient transfections of NT2/D1 embryonal carcinoma cells (Simeone *et al.*, 1994). Conversely, RA represses *Dlx* gene expression in zebrafish embryos during pharyngogenesis (Ellies *et al.*, 1997a).

Various transcriptional regulators have been shown to influence *Dlx* expression. Pitx2, a bicoid-like transcription factor, expressed during tooth development, induces a 3.2 kb 5'-flanking sequence of the *Dlx2* promoter in Chinese hamster ovary cells (Espinoza *et al.*, 2002). Misexpression of Fez, an early forebrain marker, ectopically induces *zdlx2a* and *zdlx6a* expression (Yang *et al.*, 2001). Msx1 has been shown to maintain *Dlx2* expression in the arch mesenchyme (Bei and Maas, 1998). Studies in mouse, *Xenopus*, and zebrafish indicate familial cross-regulatory interactions between *Dlx* genes. Examples of cross-regulation include *dlx3b* expression in the zebrafish otic placode that is necessary for *dlx5a* subsequent expression (Zerucha *et al.*, 1997; Mendonsa and Riley, 1999; Solomon and Fritz, 2002). Supporting evidence is seen in co-transfection experiments of *dlx3b* that reveal *dlx5a* activation through a 1.7 kb 5'-flanking region (Zerucha *et al.*, 1997). Correspondingly, loss of *dlx3b* function by MO knockdown causes loss of *dlx5a* expression (Solomon and Fritz, 2002).

1.2.6.2. *Dlx* cis-acting regulatory elements

The highly conserved genomic organization of the *Dlx* genes, and the coincident expression patterns of linked paralogues, suggest sharing of regulatory elements. Phylogenetic footprinting of the non-coding regions surrounding the *Dlx* genes has revealed many sites of

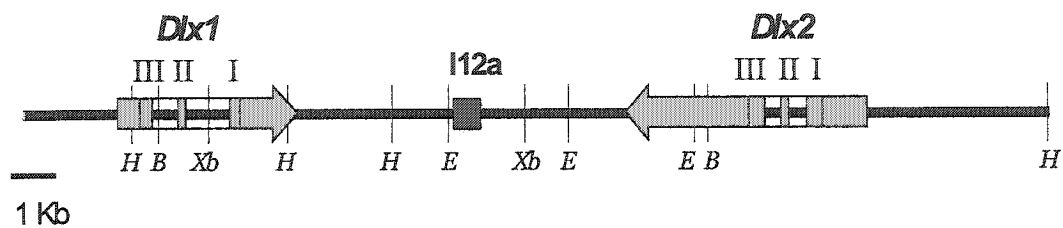
conservation between highly divergent species, such as teleosts and mammals (Fig. 1.6). These short domains, in transgenic animals, target reporter gene expression to regions of the endogenous expression pattern, indicating regulatory function (Zerucha *et al.*, 2000; Ghanem *et al.*, 2003; Sumiyama and Ruddle, 2003b). The conserved elements drive reporter gene expression in the forebrain, the limb buds, and/or the ectomesenchyme of the first and second pharyngeal arches, resembling the endogenous *Dlx* expression patterns, and provide a mechanism for shared expression (Zerucha *et al.*, 2000; Sumiyama *et al.*, 2002; Ghanem *et al.*, 2003).

The zebrafish I56i enhancer, ~440 bp with 81% sequence identity to mouse and human orthologous loci, is situated between *dlx5a* and *dlx6a*, and is capable of recapitulating forebrain expression in zebrafish as well as in mouse (Zerucha *et al.*, 2000; Ghanem *et al.*, 2003). Co-transfection experiments and DNA-protein binding assays indicate that I56i is responsive to *Dlx1/2/5* expression *in vivo* and *in vitro*, indicating cross-regulatory interactions between bigene clusters (Zerucha *et al.*, 2000; Stuhmer *et al.*, 2002a). I56i-*LacZ* forebrain expression is lost in *Dlx1/2*^{-/-} transgenic mice, comparable to the *Dlx5/6* loss, suggesting that *Dlx1/2* may function as positive regulators of *Dlx5/6* expression through the I56i enhancer (Anderson *et al.*, 1997b; Zerucha *et al.*, 2000). Subtle differences exist between endogenous *Dlx5* and *Dlx6* forebrain expression patterns with the I56i enhancer recapitulating *Dlx5* expression more closely, suggesting that the enhancer works differentially between the two linked genes (Zerucha *et al.*, 2000).

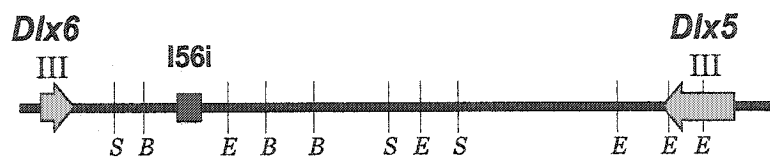
Recent phylogenetic footprinting comparing human and mouse *Dlx3/4* clusters revealed conserved sequences proficient in driving reporter expression in the limbs and

Figure 1.6. Schematization of the *Dlx1/2* and *Dlx5/6* genomic organization. Exons are depicted as orange boxes numbered in Roman numerals. Green arrows indicate direction of transcription and untranslated regions (UTR) of the transcript. Conserved enhancers I12a and I56i are depicted as red boxes. Restriction sites are labeled as the following: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; Xb, *Xba*I; S, *Sac*I/*Sst*I. Adapted from Park *et al.*, 2004, and Zerucha *et al.*, 2000a.

Mouse Dlx1/2



Mouse Dlx5/6



arches of transgenic mice (Sumiyama *et al.*, 2002). The I37-2, 245 bp element, adjacent to *Dlx4*, has high sequence similarity between human and mouse (88%). I37-2 contains a putative homeodomain binding site (HBS), and two conserved DLX binding sites similar in arrangement to I56i, and is necessary for *Dlx3* arch expression (Sumiyama and Ruddle, 2003b). The element targets transgene expression to the mesenchyme of the first and second pharyngeal arches, recapitulating *Dlx3* expression (Sumiyama and Ruddle, 2003b).

The conserved organization of the paralogues separated by a short intergenic sequence indicates the arrangement has an evolutionary advantage. *Dlx* overlapping expression patterns are most likely the result of duplication or sharing of *cis*-acting regulatory elements (reviewed in Zerucha and Ekker, 2000). Enhancer sharing likely maintained the bigene cluster organization. Redundant genes are generally lost if they have not adapted new functional responsibilities. Modification of shared elements may impair both linked genes. Therefore, deleterious changes in the *cis*-acting enhancers may not be viable, which ensures their retention and organization.

Upstream flanking sequences have also been investigated for regulatory activity. Promoter analysis of *xDlx3* (*Xdll-2*) reveals a 1 kb 5'-flanking sequence that confers *lacZ* expression in mouse keratinocytes and *Xenopus* epidermis, as well as distal portions of the limbs (Park and Morasso, 1999). Moreover, transgenic mice harbouring *xDlx3* promoter sequence reproduced endogenous-like expression in hair follicles and mammary epithelium, indicating not only conservation of the regulatory mechanism between divergent vertebrate species, but also its evolutionary adaptation into novel mammalian structures (Morasso *et*

al., 1994; Morasso *et al.*, 1995). The *mDlx2* promoter has also been examined. A 3.8 kb 5'-flanking fragment targets *lacZ* transgene expression to a subset of the maxillomandibular oral epithelium identical to endogenous gene expression, but not to the underlying ectomesenchyme (Thomas *et al.*, 2000). The regulatory flanking region was further shown to be responsive to epithelial BMP4 and FGF8 signals similar to endogenous *Dlx2* expression (Thomas *et al.*, 2000).

Orthologous gene expression patterns seem to be conserved after the genomic duplication events. Selective pressures or specialized functions of paralogues may be maintaining the conserved regulatory mechanisms through evolution. Patterns of *Dlx* expression are well conserved with few exceptions. Expression of the single *Distal-less* gene, *AmphiDll*, in amphioxus is similar to regions of *Dlx* gene expression in vertebrates (Holland *et al.*, 1996). For example, *AmphiDll* expression in the presumptive ectoderm during gastrulation and the cerebral vesicles is considered to be homologous to the vertebrate forebrain (Holland *et al.*, 1996). Therefore, aspects of *Dlx* regulation appear to be inherited from an ancestor that predates vertebrate radiation. Exceptions include early *zdlx3b/4b* and *xDlx3* expression which appears to be performed by *Dlx5/6* in mammals and chicks (Quint *et al.*, 2000) suggesting that the genes are functionally equivalent (Zerucha and Ekker, 2000). In mice and chick *Dlx5/6*, acts in an equivalent manner to *dlx3b/4b* in zebrafish, specifying the neural plate border, suggesting that the enhancers responsible were originally in the initial bigene complex and were subsequently lost or degenerated during vertebrate species radiation (Quint *et al.*, 2000). The functional differences between *Dlx* paralogues have yet to be thoroughly investigated. Although *Dlx trans*-paralogues have overlapping expression

patterns, for example in the limb buds and pharyngeal arches, identified control elements in *Dlx1/2* are not found in *Dlx3/4* or *Dlx5/6* suggesting that the non-coding elements are necessary for evolutionary diversification (reviewed in Sumiyama *et al.*, 2003a).

1.3.Statement of inquiry

The purpose of the research described in the three following chapters was to investigate different aspects of *Dlx* regulation and function in the pharyngeal arches. Furthermore, the genomic organization of the *Dll*-related gene was examined in a basal-level larvacean species, as a proxy animal model of an ancestral chordate. Little is known about how the *Dlx* genes attain their complex spatiotemporal expression pattern, and how they are regulated. In the following chapter, I demonstrate, in collaboration with Drs. Keon Park, and Bethan Thomas, that specific *Dlx* *cis*-acting elements target expression to the mandibular arch mesenchyme, and are influenced by oral epithelial signals. In chapter three, I show the role of *dlx2a* in maintaining cranial neural crest specification in zebrafish, as well as the roles of *dlx2a* and linked *dlx1a* in patterning neural crest arch derivatives. Lastly, in collaboration with Drs. Hee-Chan Seo and Daniel Chourrout, I characterize the *dll*-related gene, *OdiDll*, in the planktonic organism, *Oikopleura dioica*. Therefore, I have extended our understanding of *Dlx* participation in the neural crest and arch development, and contributed to our knowledge of the evolutionary divergence of the *distal-less-related* genes in chordates.

2. Intergenic enhancers with distinct activities regulate *Dlx* gene expression in the mesenchyme of the branchial arches

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Abstract

The vertebrate *Dlx* genes, generally organized as tail-to-tail bigene clusters, are expressed in the branchial arch epithelium and mesenchyme with nested proximodistal expression implying a code that underlies the fates of jaws. Little is known of the regulatory architecture that is responsible for *Dlx* gene expression in developing arches. We have identified two distinct *cis*-acting regulatory sequences, I12a and I56i, in the intergenic regions of the *Dlx1/2* and *Dlx5/6* clusters that act as enhancers in the arch mesenchyme. *LacZ* transgene expression containing I12a is restricted to a subset of *Dlx*-expressing ectomesenchyme in the first arch. The I56i enhancer is active in a broader domain in the first arch mesenchyme. Expression of transgenes containing either the I12a or the I56i enhancers is dependent upon the presence of epithelium between the onset of their expression at E9-10 until independence at E11. Both enhancers respond positively to FGF8 and FGF9, however, the responses of the reporter transgenes were limited to their normal domain of expression. BMP4 had a negative effect on expression of both transgenes and counteracted the effects of FGF8. Furthermore, bosentan, a pharmacological inhibitor of endothelin-1 signaling caused a loss of *I56i-lacZ* expression in the most distal aspects of the expression domain, corresponding to the area of *Dlx6* expression previously shown to be under the control of endothelin-1. Thus, the combinatorial branchial arch expression of *Dlx* genes is achieved through interactions between signaling pathways and intrinsic cellular factors. I56i drives the entire expression of *Dlx5/6* in the first arch and contains necessary sequences for regulation by at least 3 separate pathways, whereas I12a only replicates a small domain of endogenous expression, regulated in part by BMP-4 and FGF-8.

2.1 Introduction

The first branchial arch, that gives rise to the mandible and maxilla, is made of proliferating neural crest-derived ectomesenchyme covered by an epithelial layer that differentiates into a variety of different tissue types. The arch is considered to be regionalized into a large maxillomandibular proximal domain with a smaller distal mandibular domain. The molecular basis for how the tissues are organized relies on epitheliomesenchymal interactions which involve many different factors in a dynamic spatiotemporal cascade (Trumpp *et al.*, 1999). Initial patterning of the mandibular arch involves epithelial signaling factors that include FGF8 (Tucker *et al.*, 1999a), FGF9 (Kettunen and Thesleff, 1998; Colvin *et al.*, 1999), BMP4 (Tucker *et al.*, 1998a; Tucker *et al.*, 1998b-a), and endothelin-1 (Thomas *et al.*, 1998), as well as regionalized expression of homeobox genes, such as *Dlx*, *Msx*, *Barx1*, *Lhx*, *Pax9*, and *Gsc* (Francis-West *et al.*, 1998). Rostrocaudal polarity of the mandibular arch is induced by FGF8 signaling in the oral epithelium (Tucker *et al.*, 1999a). Recent work by Depew and colleagues (2002) and Beverdam *et al.*, (2002) suggest that *Dlx* family members are necessary for specification of the proximodistal axis. In the mouse, the *Dlx1* and *Dlx2* genes are similarly expressed in the epithelial and mesenchymal cells of both the mandibular and maxillary divisions of the first branchial arch (Qiu *et al.*, 1997). The *Dlx5* and *Dlx6* genes are expressed in a nested manner in the mandibular domain, but not the maxillary domain of the first arch prior to initiation of tooth development. Mouse embryos that are homozygous for a mutation that inactivates both *Dlx1* and *Dlx2* exhibit a loss or malformation of proximal arch features typified by the loss of maxillary molars yet retain all other teeth (Qiu *et al.*, 1997). The loss of function of both *Dlx5* and *Dlx6* results in a mirror duplication of maxillary features in the mandible indicating that maxillary expressed *Dlx1/2*

genes pattern proximal tissues (Qiu *et al.*, 1997) while *Dlx5/6* are required for distal tissues (Beverdam *et al.*, 2002; Depew *et al.*, 2002).

To further understand *Dlx* gene function within arch patterning, it is necessary to examine the mechanisms responsible for their spatial expression and for their response to epidermal regulatory cues. The concerted regulation of *Dlx* genes may be related to their genomic organization. The *Dlx1* and *Dlx2* genes are linked on mouse chromosome 2, while the *Dlx5* and *Dlx6* genes are linked on chromosome 7 (Simeone *et al.*, 1992). This genomic organization is also found for a third *Dlx* bigene cluster, *Dlx3/4* (formerly *Dlx3/7*) (Stock *et al.*, 1996; Ellies *et al.*, 1997b; Sumiyama *et al.*, 2002) and orthologous bigene clusters have been found in human and in teleost fish (Simeone *et al.*, 1994; Stock *et al.*, 1996; Ellies *et al.*, 1997b; Ghanem *et al.*, 2003). The genomic organization of vertebrate *Dlx* genes may be related to their overlapping expression in several tissues through enhancer-sharing (Ellies *et al.*, 1997b; Zerucha *et al.*, 2000). The short (about 3-15 kb) intergenic region of *Dlx* bigene clusters contains some of the *cis*-acting sequences that are involved in *Dlx* gene regulation (Zerucha *et al.*, 2000; Sumiyama *et al.*, 2002). Thus, our previous work has shown that evolutionary conserved enhancer elements within the intergenic region of *Dlx5/6* are capable of inducing reporter gene expression in the forebrain that replicates endogenous patterns (Zerucha *et al.*, 2000). A distinct enhancer with forebrain activity was recently found in the *Dlx1/2* intergenic region (Ghanem *et al.*, 2003), suggesting that enhancers unrelated in sequence can target *Dlx* expression with overlapping patterns.

One of the forebrain enhancers in the *Dlx5/6* intergenic region, I56i, is also able to target expression of a reporter transgene to the first branchial arch (Zerucha *et al.*, 2000). Interspecies sequence comparisons of the *Dlx1/2* loci have further revealed a highly conserved sequence of about 550 bp, I12a, which shows enhancer activity in the first branchial arch of transgenic mice, but not in the forebrain (Ghanem *et al.*, 2003). Here we examine the spatial and temporal activity of the I56i and I12a enhancers and determine the effects of epithelial factors on the expression of *lacZ* reporter constructs containing either I56i or I12a in the mandibular mesenchyme of transgenic mice. The two sequences are unrelated, showing no evidence of conservation, but are both transiently dependent on signals from the epithelium. Both enhancers are similarly regulated by FGF-8 and BMP-4, but only I56i is regulated by ET-1.

2.2 Materials and methods

2.2.1 Construction of *lacZ* reporter constructs and transgenic mouse production

Constructs #2, 3, and 5 (Fig. 2.1) were made in the p1229 or p1230 vectors (Yee and Rigby, 1993) where the *lacZ* gene is placed under the control of a minimal human β -globin promoter. Construct #2 (Fig. 2.1) was made by successive sub-cloning resulting in the introduction of a 1.9 kb fragment of the mouse *Dlx1/Dlx2* locus delineated by *Xba*I and *Eco*R1 restriction sites and containing the conserved I12a sequence. The conserved I12a sequence (Ghanem *et al.*, 2003) and surrounding nucleotides (total size: 713 bp) was introduced into the reporter vector (construct #3) after PCR amplification with the primers: 5'CCAAGCTTCCTATGCTGAGAACAGAG3' and 5'CCAAGCTTCGTCAATTCCTCATTGTC3', followed by direct sub-cloning into the *Hind*III site of the p1230 reporter vector.

A 1.6 kb *XhoI-XhoI* zebrafish fragment (zI12a 1.6kb; construct #5), containing the conserved I12a sequence was directly sub-cloned into the reporter vector. A reporter transgene construct containing the entire mouse *Dlx1/Dlx2* intergenic region (mI12a 13.5; Fig. 2.1, construct #1), was made by first inserting a 13.5 kb *BamHI-BamHI* fragment that includes the *Dlx1/2* intergenic region into a pBluescript plasmid. The β -globin promoter-lacZ cassette from the p1229 vector was then amplified by PCR and inserted downstream of the mouse intergenic fragment. Construct #4 was made by introducing the 1.9 kb *XbaI-EcoRI* fragment identical to that used in construct #2 into the reporter transgene plasmid described by Thomas and collaborators (2000) which also contains a 3.8 kb fragment of the 5'-flanking region of *Dlx2*.

For the production of transgenic mice, the transgene was excised from the plasmid construct and injected at a concentration of 5 ng/ μ l in eggs from FVB/n crosses using standard procedures (Hogan *et al.*, 1986). Transgenes were analyzed in either founder embryos or from established transgenic lines. Presence of the transgene was assayed by PCR on DNA prepared from embryonic tissues as described previously (Zerucha *et al.*, 2000). Production of lines of transgenic mice containing the mouse I56i enhancer has been previously described (Zerucha *et al.*, 2000).

2.2.2 *β -galactosidase staining*

Embryos from hemizygous mice (transgenic FBV males x CD1 females) were harvested at various embryonic stages and assayed for β -gal activity by staining with X-gal as described previously (Zerucha *et al.*, 2000). Noon on the day a vaginal plug was detected is

considered as E0.5. Embryos were staged precisely based on morphological criteria (Kaufman, 1995). X-Gal stained embryos were cryo-sectioned (thickness: 16 μ m) and counter-stained with eosin.

2.2.3 *In situ* hybridization

Whole-mount digoxigenin (DIG) *in situ* hybridization was performed as previously described except that mouse embryos were incubated in 6% hydrogen peroxide for 45 minutes to enhance signal visibility (Wilkinson, 1992). Antisense probes were generated from mouse cDNA clones. *Dlx2* (NM010054) was linearized with *HindIII*, and *Dlx5* (NM010056) with *SmaI*. Antisense riboprobes were transcribed with T3 Pol and DIG-UTP (Roche) producing approximately 0.8 kb and 0.6 kb transcripts respectively. *In situ* hybridization subsequent to X-Gal staining was performed as described by Tajbakhsh and Houzelstein (1995).

2.2.4 *Culture and bead Implantation*

Cultures were carried out using tissues from hemizygous mouse embryos (transgenic FBV x CD1) aged between E9.5 and E11.5. Mandibular arches were dissected out in D-MEM culture media (Invitrogen). When separation of epithelium and mesenchyme was required, the explants were incubated in Dispase (Invitrogen) in calcium and magnesium-free phosphate-buffered saline (PBS) at 2 units per ml for 7-10 minutes at 37°C, depending on age and size. After incubation, the tissues were washed in D-MEM with 10% fetal calf serum (FCS), and the epithelium was dissected using micropincettes. The mesenchyme was

placed on membrane filters (0.1 μm pore size, Millipore) supported by stainless steel grids and beads were placed as required.

For FGF8 and FGF9, heparin acrylic beads (Sigma) were used. These were washed several times in PBS, then incubated overnight at 4°C in FGF8b or FGF9 (R&D Systems) at a concentration of 1 $\mu\text{g/ml}$. For BMP4 and noggin, Affi-Gel-blue beads (Bio Rad) were used. The beads were washed and dried, then soaked in a 100 ng/ml BMP4 (R&D Systems) or 1 $\mu\text{g/ml}$ Noggin (R&D Systems) solution for 1 hour at 37°C. Bovine serum albumin (BSA) control beads (both Affi-Gel-blue and heparin acrylic) were made in a similar manner. The protein-soaked beads were stored for up to 2 weeks at 4°C. Endothelin-1 inhibition was accomplished by culturing dissected arches in medium containing 1 mg/ml of bosentan (Actelion Pharmaceuticals Ltd).

The explants were cultured for 18-24 hours in D-MEM with 10% FCS at 37°C in a humidified atmosphere of 95% air and 5% CO₂. All solutions used contained penicillin and streptomycin at 20 IU/ml. After the period of culture, the explants were fixed and processed for *in situ* hybridization or X-Gal staining.

2.3 Results

2.3.1 *The Dlx1/2 intergenic region targets reporter transgene expression to the branchial arches, forebrain, and limbs*

To determine the presence of *cis*-acting regulatory sequences in the *Dlx1/2* intergenic region, a 13.5 kb fragment that includes the mouse intergenic region separating the 3' exons of the linked genes was cloned into a *lacZ* reporter gene construct containing a human β -globin

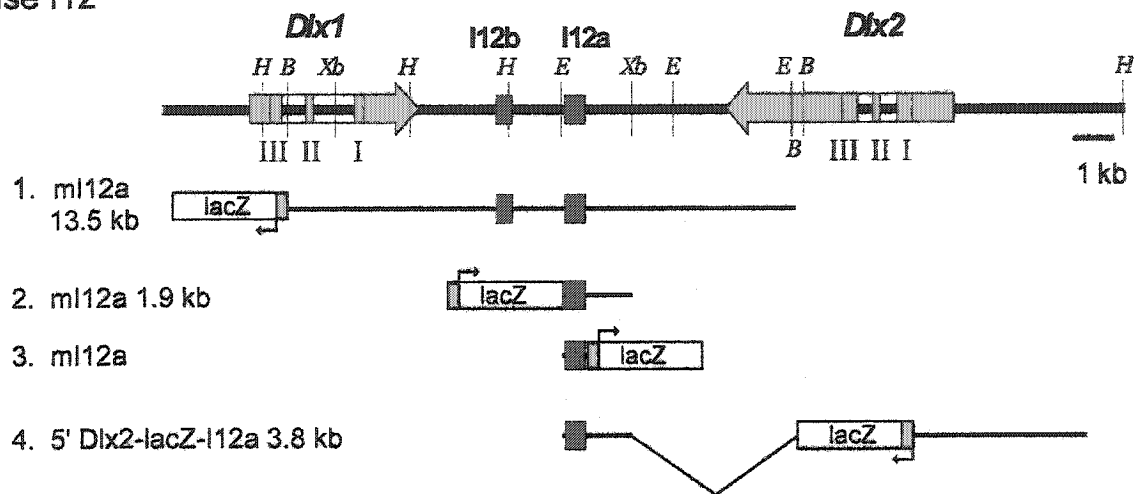
minimal promoter (mI12a13.5; Fig. 2.1, construct #1). Transgene expression was examined in two primary embryos and five lines of transgenic animals at E11.5 (Fig. 2.2A), a time point chosen because it gives a general overview of *Dlx* expression, although other time points were also examined (data not shown). Transgenic animals carrying the above construct showed strong and reproducible expression in several domains of endogenous *Dlx1/2* expression. These included the telencephalic and diencephalic domains of expression in the forebrain, the frontonasal prominence, the apical ectodermal ridge (AER) of developing limb buds, and the genital tubercle (Fig. 2.2A and data not shown). At this time, β -galactosidase activity was also observed in a subset of mesenchymal cells in the mandibular portion of the first arch and in the hyoid arch (Fig. 2.2A and inset). Expression intensity varied between lines, likely due to the copy number and insertional site of the transgene into the genome. None of the primary transgenics or transgenic lines reproduced the entire pattern of *Dlx* expression in the mesenchyme of the first branchial arch. None expressed *lacZ* in the epithelium of the arches, consistent with prior identification of an arch epithelium enhancer upstream of *Dlx2* (Thomas *et al.*, 2000).

2.3.2 Activity of the I12a enhancer in mesenchymal cells of the mandibular component

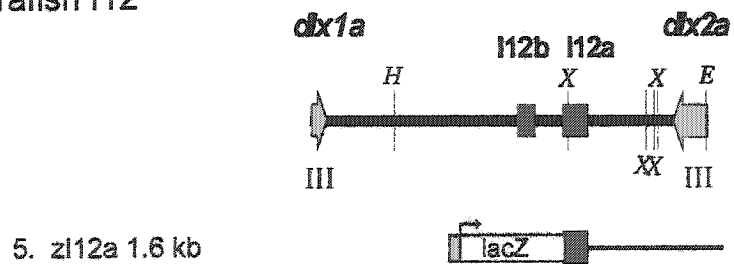
To investigate the genetic elements within the intergenic region responsible for targetting gene expression, we previously carried out interspecies comparisons of the *Dlx1/Dlx2* intergenic sequence from human, mouse, zebrafish, and two species of pufferfish (Ghanem *et al.*, 2003). The similarity between the five species was limited to domains of high sequence conservation, I12a and I12b. In transgenic animals, I12a targeted expression of reporter constructs to the branchial arches with patterns that were identical to those obtained

Fig. 2.1. The mouse and zebrafish *Dlx1/2* loci and *lacZ* transgene constructs. Schematic representation of the mouse and zebrafish *Dlx1/Dlx2* loci with *Dlx1* to the left. Exons are portrayed as orange blocks and numbered in Roman numerals, gene transcripts including untranslated regions (UTR) of *Dlx1* and *Dlx2* are depicted as convergent green arrows. The position of the I12a and I12b *cis*-acting regulatory sequences are indicated by red and blue blocks respectively. Restriction sites are labeled as following: B, *BamHI*; E, *Eco RI*; H, *HindIII*; Xb, *XbaI*; X, *XhoI*. The *lacZ* constructs with the β -globin minimal promoter (grey box) containing I12a used for the production of transgenic mouse lines are numbered and schematized underneath.

Mouse I12



Zebrafish I12

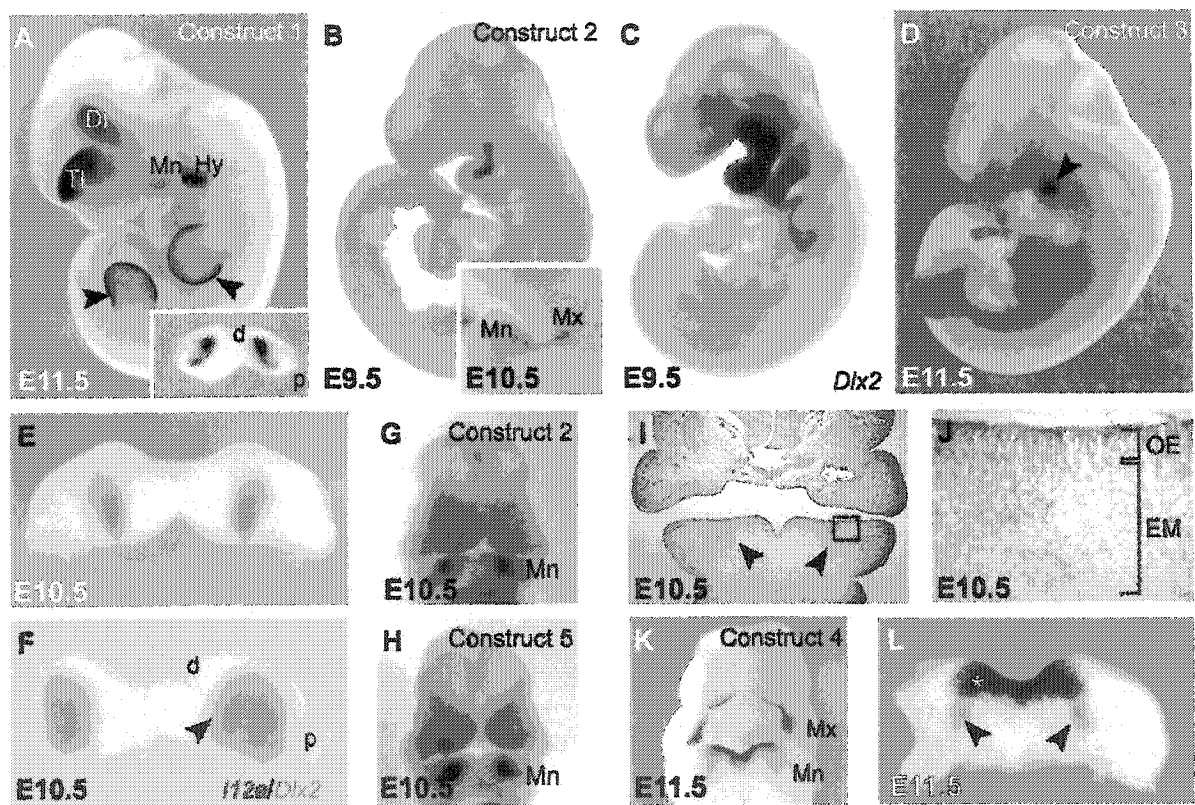


containing the entire intergenic region while I12b showed enhancer activity only in the forebrain (Ghanem *et al.*, 2003). Transgenic mice were made with a *lacZ* reporter construct containing a 1.9 kb *XbaI-EcoRI* fragment (Fig. 2.1) from the mouse locus encompassing I12a (Fig. 2.1, construct #2).

We obtained five lines of transgenic mice with the I12a-containing construct #2 and used three of them to examine in detail the spatial and temporal activity of the I12a enhancer. One line showed *lacZ* expression in a group of ectomesenchymal cells in the first arch at E9.5, corresponding to a subpopulation of *Dlx2*-expressing cells as compared to *Dlx2* whole-mount *in situ* hybridization (Fig. 2.2B, C). As development progressed expression appeared to bifurcate into maxillary and mandibular expression domains (Fig. 2.2B inset). The other two lines did not show similar expression at this early stage. Primary transgenic embryos (4/6) carrying only the mI12a element within the reporter construct (construct #3) were assessed and showed mandibular staining similar to that obtained with the mI12a 1.9 kb construct (Fig. 2.2D).

By E10.5-11, all lines of transgenic mice showed *lacZ* expression in bilateral regions between the lateral and medial portions in the first arch (Fig. 2.2E, F). Whole-mount *in situ* hybridization, for *Dlx2* transcripts, performed after staining for β -galactosidase activity with X-Gal (Tajbakhsh and Houzelstein, 1995), indicates that cells expressing the transgene (Fig. 2.2F, arrowhead) correspond to a sub-population of the *Dlx2*-expressing cells in the most distal aspect of the endogenous expression domain.

Fig. 2.2. Enhancer activity of the *I12a cis*-acting regulatory sequence. (A) Whole-mount β -galactosidase staining of an E11.5 mouse transgenic embryo with a 13.5 Kb *lacZ* reporter transgene containing the entire *I12* intergenic region (construct 1) shows expression in diencephalon (Di) and telencephalon (Tl) of the forebrain, mandibular (Mn) and hyoid (Hy) branchial arches and the limb bud apical ectodermal ridge (AER; arrowheads). (A inset) E11.5 dissected mandibular arch, viewed from the oral surface, from an embryo similar to that shown in (A) exhibits mesenchymal *lacZ* expression in two bilateral domains. Orientation is indicated by p (proximal, dorsal) and d (distal, ventral). (B) E9.5 embryo (line 1486; construct 2) shows *lacZ* expression in the prospective maxilla (Mx) and mandible. (B inset) E10.5, bifurcation of maxilla and mandibular *I12a-lacZ* expression shown in the first branchial arch (line 1486). (C) Whole-mount in situ of E9.5 *Dlx2* expression in the maxilla and mandible and in the hyoid arch. (D) E11.5 primary embryo (construct 3) with *lacZ* expression in the mandible (arrowhead) and hyoid. (E) E10.5 dissected mandible showing bilateral transgene expression in comparison to a mandible (F) stained for β -galactosidase activity (light blue) and *Dlx2* mRNA expression (purple). Transgene activity (arrowhead) is restricted to the most distal (d), aspect of the endogenous mesenchymal expression domain. P: proximal. (G and H) E10.5, frontal views reveal conservation of arch mesenchymal expression pattern in transgenic mice containing the zebrafish *I12a1.6-LacZ* reporter construct (construct 5) in comparison to mouse *I12a1.9-LacZ* (construct 2) enhancer activity. (I; 50x) frontal section at E10.5 shows β -galactosidase activity targeted to the rostral half of the arch (arrowheads). (J) Transgene expression is restricted to the ectomesenchyme (EM) and absent in the oral epithelium (OE) magnification: 400X. Embryos are from transgenic line 1483 (construct 2). Similar results were obtained with two additional lines produced with the same transgene. (K, L) E11.5 primary embryo exhibiting *I12a-lacZ* expression in combination with a 3.8 kb 5' flanking *Dlx2-LacZ* reporter construct (construct 4) (Thomas *et al.*, 2000). Transgene expression is observed in the maxilla and mandibular oral epithelium (star) and in the restricted bilateral mesenchymal domains shown in the dissected mandible (L, arrowheads). (K) Frontal view. All dissected mandibles are orientated distal to the top and oral side facing.



Transgenic mice were also produced with a similar reporter construct containing a 1.6 kb *XhoI-XhoI* fragment of the zebrafish *dlx1/dlx2* intergenic region (Fig. 2.1, construct #5). The sequence similarity between the zebrafish and the mouse genomic fragments used to make the transgene described above is limited to the I12a enhancer. One stable line of transgenic mice was obtained and analysis of the embryos from this line resulted in patterns of transgene expression identical to those obtained with the mouse version of the *I12a-lacZ* transgene (Fig. 2.2G and H). We conclude that the zebrafish intergenic sequence, zI12a, is able to target expression in mouse embryos in a way that is identical to its mouse counterpart.

Sections indicate that the observed β -galactosidase-positive cells are mesenchymal and that there is no epithelial expression (Fig. 2.2I arrowheads, J). The existence of specific interactions between intergenic elements and regulatory sequences located upstream of the *Dlx* promoters might explain the incomplete expression of reporter transgenes containing I12a. Previous work has shown a *lacZ* construct containing 3.8 kb 5'-flanking fragment of *Dlx2* replicates endogenous expression in the epithelium but not in the mesenchyme (Thomas *et al.*, 2000). To determine whether combining the I12a intergenic enhancer to the *Dlx2* 5'-flanking sequences would synergistically reproduce the complete expression of *Dlx2* in branchial arch mesenchymal cells, we added the 1.9 kb *XbaI-EcoRI* mouse fragment containing I12a to the *Dlx2-lacZ* construct previously used by Thomas and collaborators (Thomas *et al.*, 2000). The resulting transgene, *Dlx2-lacZ-I12a* (Fig. 2.1, construct #4) was expressed in transgenic mice with patterns that represent the sum of the expression of *Dlx2-lacZ* and *I12a-lacZ*, that is, the epithelial expression of *Dlx2-lacZ* and the spatially-restricted

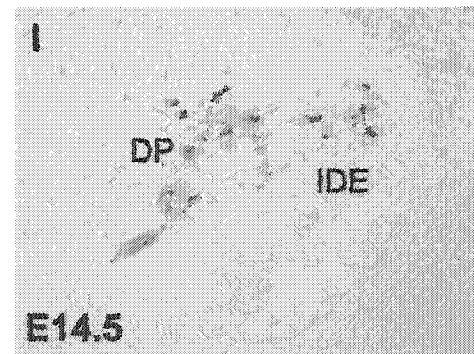
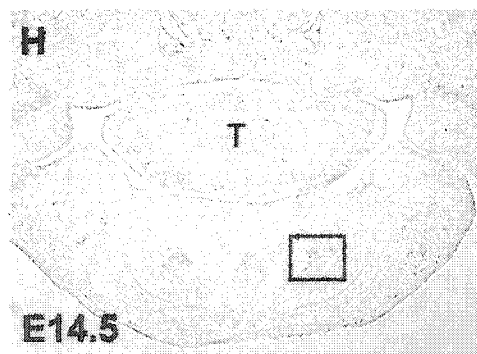
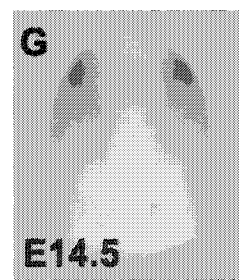
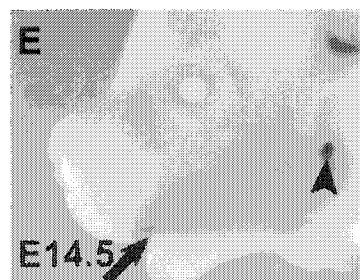
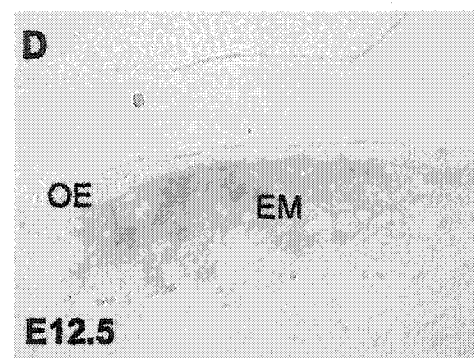
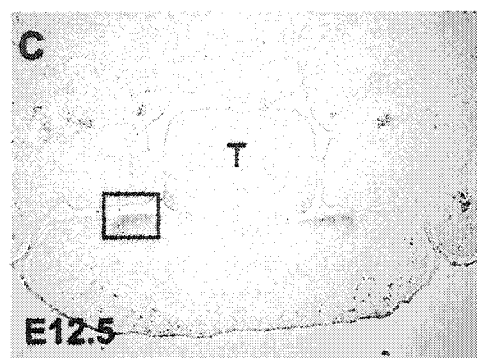
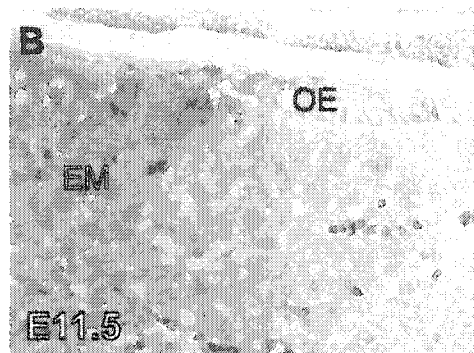
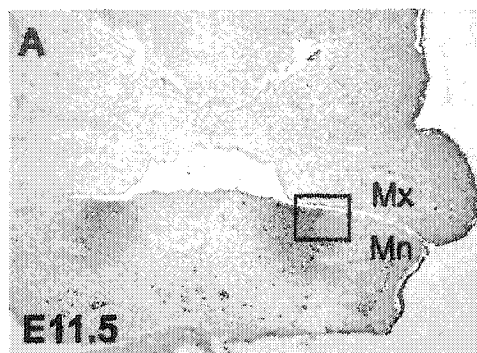
mesenchymal expression of *I12-lacZ* (Fig 2.2K and L). Thus, the inability of *I12a-lacZ* to reconstitute the complete patterns of *Dlx2* expression in the first arch mesenchyme is not due to the lack of specific interactions or synergy between the *cis*-acting regulatory sequences located in the intergenic region (*I12a*) and in the flanking (3.8 kb) region of *Dlx2*, but suggests the existence of additional regulatory elements.

As development progresses, *lacZ* expression in *I12a* transgenic mice is maintained in the mandible (Fig. 2.3A, B; arrowhead) and becomes apparent in bilateral domains of the hyoid arch (Fig. 2.2A and data not shown). Transgene expression remained exclusively mesenchymal and, by E12.5, was restricted to the mesenchyme adjacent to the epithelium in the tongue and mouth floor (Fig. 2.3C, D). At E14.5, expression of the transgene was mainly observed in the intraoral portion (Fig. 2.3E, arrow) and some part of the external acoustic meatus (arrowhead). In the intraoral portion, transgene expression became gradually restricted to cells on the floor of the mouth with distinct distal limits that extended to the areas of developing incisor teeth (Fig. 2.3F) and to bilateral groups of cells on the lateral edges of the tongue (Fig. 2.3G). At E14.5 transgene expression was also observed in the dental papilla mesenchyme of the developing incisor teeth (Fig. 2.3H, I). Expression persisted in all three lines of mice until at least E16.5 (data not shown).

2.3.3 I12a-lacZ expression is regulated by signals produced in the epithelium and is antagonistically regulated by FGF and BMP4 signals

The extensive conservation of the *I12a* enhancer sequence of diverse vertebrates over more than 500 bp suggests that it could be the site of interactions with multiple trans-acting

Fig. 2.3. Expression of *Il2a-lacZ* reporter transgene at various developmental stages. (A-D) Frontal sections at E11.5, and E12.5 reveals an overall loss of X-gal staining in the mandible with a restriction of β -galactosidase activity to the mesenchyme abutting the oral epithelium as development progressed. The arrow and arrowhead in (E) indicate transgene expression in the tongue and in the prospective middle ear, respectively. (F) View of the floor of the mouth. (G) Dissected tongue. (H, I) frontal sections at E14.5 show a targeting of transgene activity to the dental papilla. Embryonic stages are indicated. Original magnification: A, C and H (x 50), B, D, and I (x 400). DP, dental papilla; EM; ectomesenchyme; IDE, inner dental epithelium; OE, oral epithelium; T, tongue. Embryos shown are from transgenic line 1483 (construct 2). Similar results were obtained with two additional lines produced with the same transgene (data not shown).



factors. To identify some of these factors, we performed experiments on mandibles dissected from the lines of transgenic mice carrying the *I12a-lacZ* construct #2. It was previously shown that expression of *Dlx2* in the first branchial arch is dependent on signals from the epithelium (Thomas *et al.*, 2000). Similarly, removal of epithelium at E10.5 resulted in a loss of *I12a-lacZ* expression in the mesenchyme (Fig. 2.4A, B). However, when the epithelium was removed from the explants at E11.5, the expression of *I12a-lacZ* was not affected (data not shown). Therefore, the activity of the *I12a* enhancer in the mesenchyme is transiently dependent upon factors that originate from the epithelium.

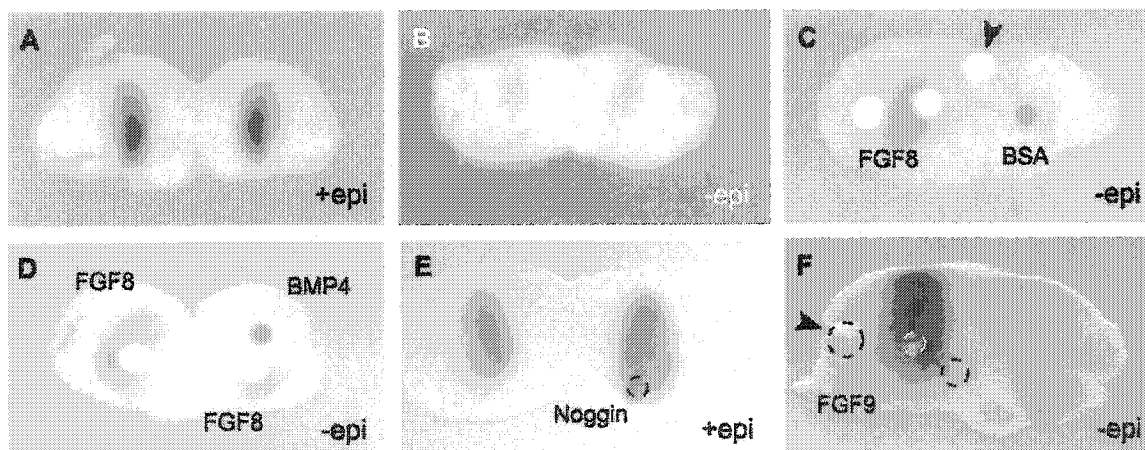
Fgf8 is expressed in patches of proximal oral epithelium of the first arch overlying the *Dlx* mesenchymal expression domain (Grigoriou *et al.*, 1998; Tucker *et al.*, 1999a). FGF8 was shown to induce *Dlx2* expression in the ectomesenchyme but to repress its expression in the epithelium (Thomas *et al.*, 2000). We therefore tested the ability of FGF8 to induce *I12a-lacZ* expression in the mandibular mesenchyme. Beads soaked in FGF8 were placed at various positions in mandibles dissected at E9.5, and transgene expression was determined by X-Gal staining 24 hours later (Fig. 2.4C). FGF8 induced transgene expression in a restricted area around the bead that corresponds to the normal domain of transgene expression (Fig. 2.4A) whereas BSA-soaked control beads placed on the contralateral side had no effect (Fig. 2.4C). The competence to express *I12a-lacZ* transgene in response to an FGF signal is clearly restricted to the normal pattern of expression, as beads implanted in a more distal position (Fig. 2.4C, arrowhead) were not able to induce transgene expression.

In contrast to the effects of FGF8, BMP4 did not induce *I12a-lacZ* transgene expression

but rather antagonized the effects of FGF8 (Fig. 2.4D). Furthermore, BMP4 soaked beads implanted in a mandible with the epithelium left intact also caused decreases in *I12a-lacZ* transgene expression (data not shown). Thus, BMP4 has an overall negative influence on the expression of the transgene. It was previously shown that BMP4 was unable to induce *Dlx2* expression in the mesenchyme when beads were implanted at E10.5 (Thomas *et al.*, 2000) contrary to a previous report that BMP4 can induce *Dlx2* expression in the first arch mesenchyme at later stages of development (Bei and Maas, 1998). The influence of endogenous BMP4 signaling on the *I12a-lacZ* transgene expression was further examined with the use of Noggin beads cultured with dissected arches (Fig. 2.4E). Noggin's ability to bind and inhibit BMP4 signaling revealed an enrichment of the *I12a-lacZ* transgene expression compared to the contralateral control side, however, the expanded expression beyond the initial subset of cells was variable.

Other FGF family members are also expressed in the oral epithelium of the mandibular arch and considered to have redundant functions (Kettunen and Thesleff, 1998). To determine if the *I12a-lacZ* transgene is similarly regulated by other FGFs, FGF9 soaked beads were implanted into dissected arches and cultured with or without the epithelium (Fig. 2.4F and data not shown). Similar to FGF8, FGF9 strongly induced our transgene in the subset of cells. However, as with FGF8, FGF9 was incapable of expanding the expression domain (arrowhead, beads encircled).

Fig. 2.4. Regulation of *I12-lacZ* mandible expression by epithelial factors. *LacZ* transgene expression was analyzed using cultured mandibular arches with and without the epithelium and manipulated with beads impregnated with the indicated proteins. (A, B) *I12a-lacZ* transgene expression is dependent upon presence of the oral epithelium at E10.5. (C) Transgene expression is activated only in its normal domain by FGF8 (white beads) in mandibles from which the epithelium had been removed (-epi). A FGF8 bead (arrowhead) placed outside the normal site of transgene expression does not induce *lacZ* expression in neighboring cells. BSA control (blue bead; right) has no effect on transgene expression. (D) Transgene activation by FGF8 (white beads) is antagonized by BMP4 signaling (blue bead; right). (E) Transgene expression is increased in mandible mesenchyme with epithelium intact following implantation of a bead soaked in Noggin (blue bead encircled), a BMP4 antagonist, compared to the contralateral side. (F) Transgene expression is activated by FGF9 soak bead within its normal domain (white beads circled) but is not inducible outside the typical region (arrowhead). Dissected mandibles are orientated distal to the top and oral side facing.



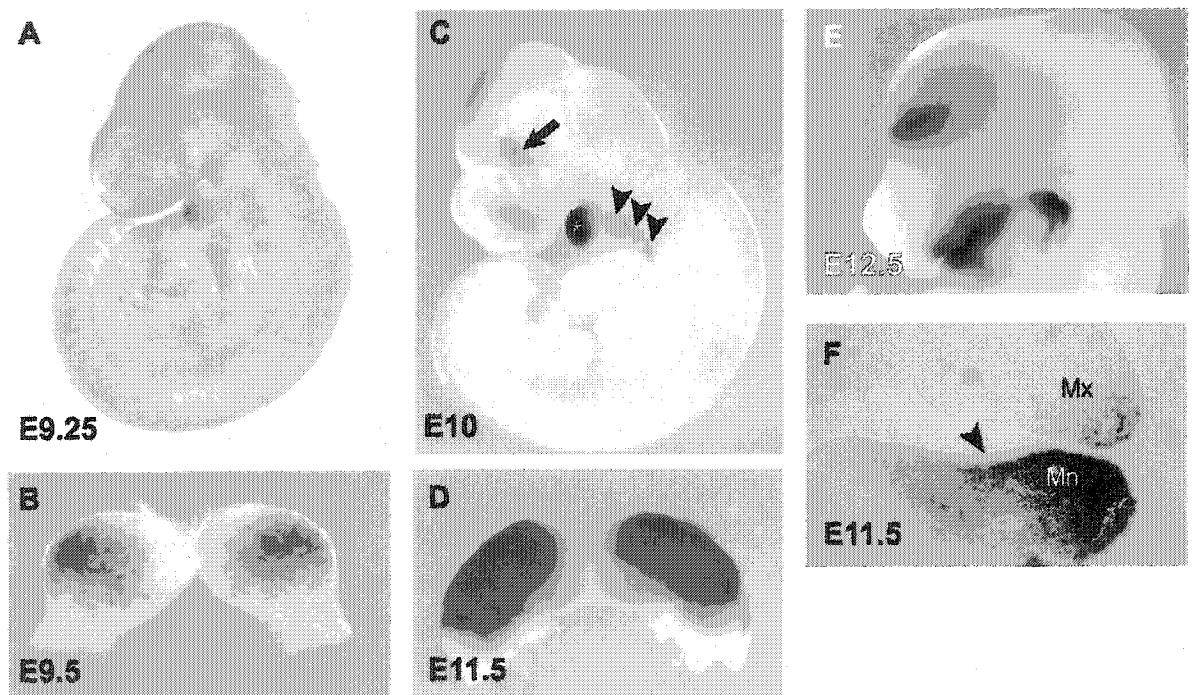
2.3.4 *Activity of the mouse I56i enhancer in the branchial arches is distinct from that of I12a*

We had previously observed that the I56i intergenic enhancer is proficient in targetting transgene expression to the forebrain domain of *Dlx* expression in both zebrafish and mice (Zerucha *et al.*, 2000a). However, the I56i enhancer from mouse, but not its zebrafish counterpart, is also capable of targetting transgene expression to the branchial arches (Zerucha *et al.*, 2000). We examined this activity in greater detail in two of the three stable lines that carry the mouse *I56i-lacZ* transgene (Fig. 2.5). Cells in the first branchial arch first express the *I56i-lacZ* transgene at around E9.25 (Fig. 2.5A, B), persisting at least until late in embryonic life (data not shown). By E10, cells from all branchial arches express *I56i-lacZ* (Fig. 2.5C). Expression of the *I56i-lacZ* transgene recapitulated the entire patterns of *Dlx5* expression in the mandibular component of the first branchial arch (Fig. 2.5D). At later stages, expression of the reporter transgene was also observed in the maxillary component of the first arch as previously observed in mice with targeted integration of the *lacZ* within the *Dlx5/Dlx6* locus (Acampora *et al.*, 1999; Merlo *et al.*, 2000; Robledo *et al.*, 2002). Similar to *I12a*, the *I56i* enhancer targeted expression exclusively to the mesenchyme (Fig. 2.5F).

2.3.5 *The I56i-lacZ transgene is regulated by FGFs and BMPs*

To determine whether *I56i-lacZ* transgene expression is modulated by signals from the epithelium comparable to those that modulate *I12a-lacZ*, we performed mandible explants and bead implantation experiments as those described in Figure 2.4. Mandibles that were dissected at E9.5 and from which the epithelium was removed did not show transgene expression following 18 hours in culture (Fig. 2.6A, B). When the epithelium was removed

Fig. 2.5. Expression of the *I56i-lacZ* transgene during embryogenesis. (A,B) β -galactosidase activity is first observed at approximately E9.25 in the distal portion of the mandible. E10 embryos (C) show expression in the mandibular portion of the 1st branchial arch (star) and more caudal arches (arrowheads) and in the forebrain (arrow). (D,F) By E11.5 arch expression includes distinct regions of the maxilla. (E) E12.5, transgene expression surrounds the oral cavity. (B, D) Dissected mandibles (E9.5 and E11.5, respectively) show progressive expansion of mandibular mesenchymal *lacZ* transgene expression. (F) Frontal section at the level of the first arch with the maxillary division (Mx) on the top and the mandibular (Mn) division at the bottom reveals the absence of transgene expression in the epithelium (arrowhead). Embryonic stage is indicated. Embryos are from transgenic line 7098. Similar results were obtained with line 7089. (A, C, E) lateral views, (F) frontal view. Dissected mandibles (B,D) are orientated with distal to the top.



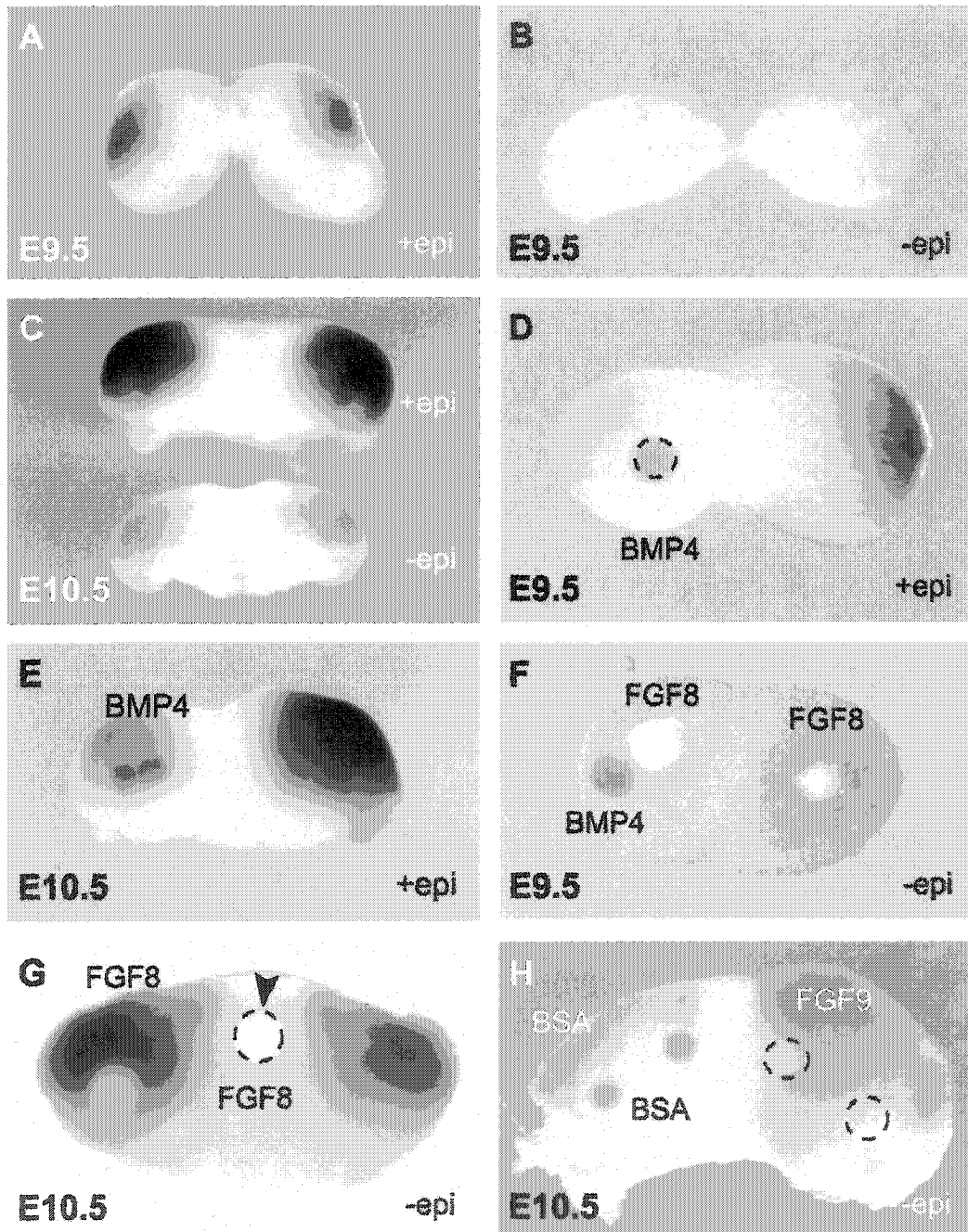
at E10.5, transgene expression was considerably reduced but not abolished (Fig. 2.6C). In mandibles with the epithelium left intact, implantation of a bead soaked in BMP4 resulted in decreases in transgene expression around the bead compared to the contralateral side. Similar effects were observed when the experiment was performed at E9.5 (Fig. 2.6D, blue bead encircled) and at E10.5 (Fig. 2.6E). In contrast, a bead soaked in FGF8 produced increases in *I56i-lacZ* transgene expression (Fig. 2.6F, right) but not as markedly as for the *I12a-lacZ* transgene (Fig. 2.4C). Implantation of a BMP4 (blue) bead close to the FGF8 (white) bead abolished the effects of FGF8 (Fig. 2.6F, left). As well, similar to the subset of cells expressing *I12a-lacZ*, FGF8 beads cultured medially, outside the normal expression domain, were not capable of extending the patterns of reporter gene expression (Fig 2.6G, arrowhead). Experiments carried out with beads soaked in FGF9 (encircled) were able to induce *I56i-lacZ* expression in the absence of epithelium in a similar manner to FGF8 compared to control BSA (blue) beads on the contralateral side (Fig. 2.6H).

2.3.6 Regulation of the *I56i-LacZ* expression domain by endothelin-1 signaling

The endothelin signaling pathway has been implicated in regulating *Dlx6* expression in the most distal aspect of the mandible (Charite *et al.*, 2001). Mice deficient in endothelin-1 (*ET-1*) (Kurihara *et al.*, 1994) or in the endothelin receptor A (*EdnrA*) (Clouthier *et al.*, 2000) result in a similar phenotype which includes retarded and smaller mandibles. Further characterization of the *EdnrA* mutant revealed an absence of *Dlx6* expression in the most ventral portion of the domain yet still retains more distal expression. We investigated the influence of ET-1 signaling on the *I56i-lacZ* transgene. Dissected mandibular arches were cultured with an ET-1 antagonist, bosentan, (Clozel *et al.*, 1994). Previous work has shown

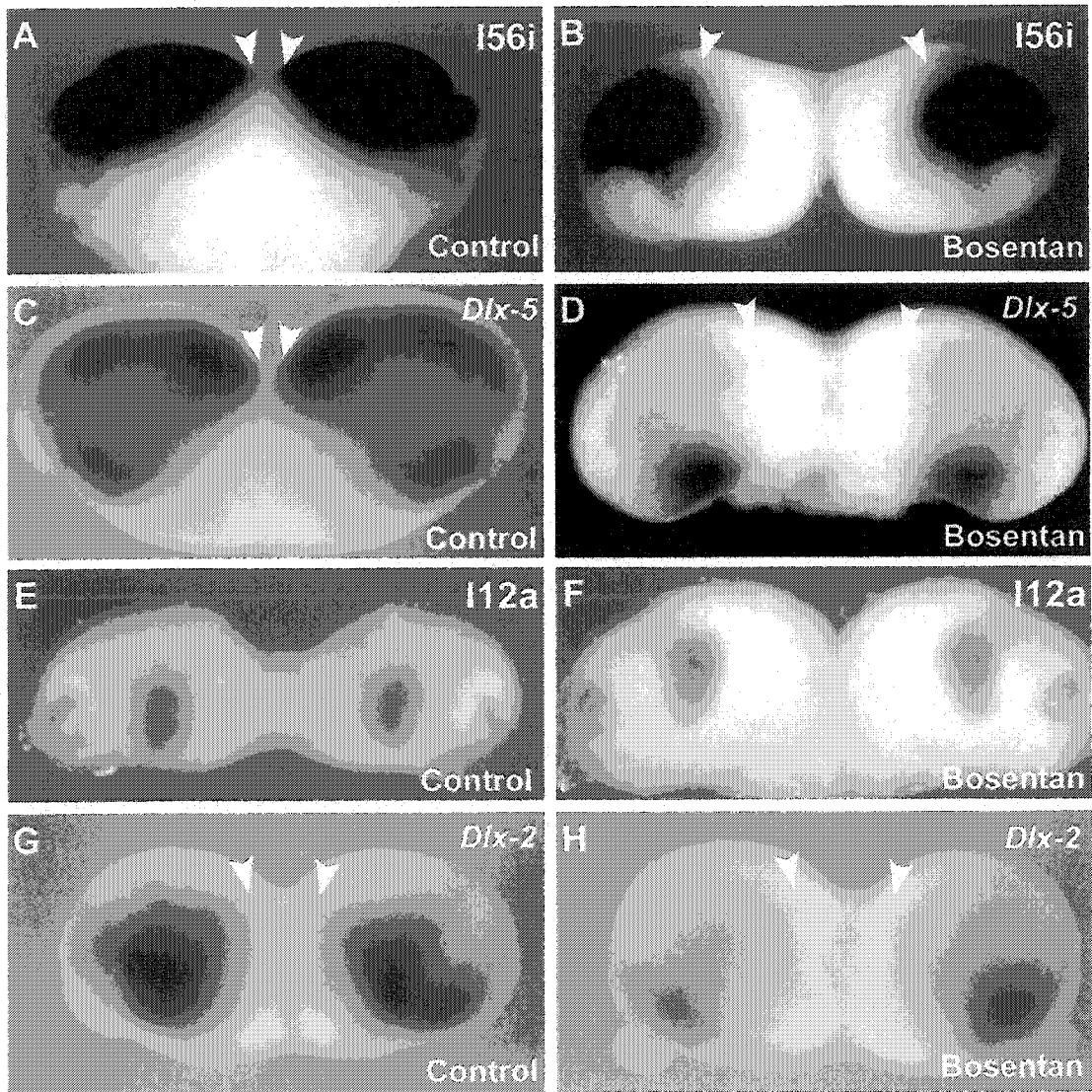
Fig. 2.6. Regulation of *I56i-lacZ* expression by epithelial factors and signaling molecules.

(A, B) Mandibles were dissected from transgenic embryos at E9.5. The epithelium was dissected (B) or left intact (A) and transgene expression was determined 18 hours later. (C) Removal of the epithelium causes decreases in *I56i-lacZ* expression at E10.5 but not as striking as at E9.5 (B). (D) Implantation of a BMP4 bead (encircled) impairs *I56i-lacZ* expression in dissected mandibles with the epithelium left intact. (E) BMP4 is also able to impair transgene expression to a lesser degree in older E10.5 mandibles. (F) Presence of a FGF8 white bead induces or maintains *I56i-lacZ* expression in dissected mandibles from which the epithelium was removed (right), however, this effect is antagonized by BMP4 (blue bead, left) on the contralateral side. (G) Expression of the *I56i-lacZ* transgene is increased by FGF8 activity, but beads placed outside the normal domain of transgene expression have no effect on the surrounding cells (arrowhead). (H) In a similar manner to FGF8, transgene activity in the mesenchyme is also receptive to FGF9 soaked beads (white) as compared to BSA (blue) beads on the opposite side in arches with the epithelium removed. The mandibles shown in panels G, and H were stained for different times. Therefore, effects of FGF beads should be individually compared with the contralateral control side. Dissected arches are orientated distal to the top and oral side facing.



that inhibition of ET-1 function in chick embryos with bosentan (Actelion Pharmaceuticals Ltd.) results in a phenotype similar to that of the *ET-1* and *EdnrA* null mutants in the mouse (Kempf *et al.*, 1998). When *I56i-lacZ* dissected arches were treated with bosentan, a loss of *lacZ* expression was observed in the most distal aspect of the expression domain (Fig. 2.7B), compared to controls (Fig. 2.7A), yet still maintained proximal *lacZ* expression. The loss of distal *lacZ* expression by ET-1 inhibition is similar to the loss of endogenous *Dlx6* expression previously reported by Clouthier and colleagues (Clouthier *et al.*, 1998) in mice deficient for *EdrA* and its effects on spatial distribution of *Dlx5* transcripts (Fig. 2.7C-D). When bosentan was tested on *I12a-LacZ* arches, no effect was observed (Fig. 2.7E, F). The spatial patterns of *Dlx2* expression were not affected (Fig. 2.7G, H). The overall levels of *Dlx2* and *Dlx5* transcripts as estimated by the intensity of the hybridization signals, appeared to be reduced by bosentan (Fig. 2.7, C, D, G, H).

Fig. 2.7. Differential impact of inhibition of ET-1 signaling on I56i and I12a enhancer activity. Dissected mandibular arches (E10.5) cultured in serum (A, C, E and G) are compared with arches cultured with medium containing the ET-1 inhibitor bosentan (B, D, F and H). Arches are shown from the oral aspect with ventral to the top. I56i arches cultured with bosentan and stained with X-gal show loss of distal expression of the transgene (A, B). Whole-mount in situ hybridization with a *Dlx5* riboprobe shows similar loss of distal expression (C,D). Bosentan has no effect on I12a activity in dissected mandibles (E, F), nor does it affect spatial *Dlx2* expression (G,H). Arrowheads indicate distal limit of the expression domain.



2.4 Discussion

2.4.1 *Distinct enhancers target expression of the Dlx1/Dlx2 and of the Dlx5/Dlx6 genes to the mesenchyme of the branchial arches.*

The overlapping expression of *Dlx* homeobox genes in the branchial arches of vertebrates and the phenotype of mutations affecting these genes suggest common regulatory mechanisms and partial functional redundancy. Functional analysis of the *Dlx1/Dlx2* and *Dlx5/Dlx6* bigene clusters suggest that their functional specificity in craniofacial development might be related to the spatial and temporal control of their expression.

The six *Dlx* genes found in most vertebrates are organized as three bigene clusters, *Dlx1/2*, *Dlx3/4* (formerly *Dlx3/7*) and *Dlx5/6*, in which the two genes are organized in an inverted convergent configuration (Panganiban and Rubenstein, 2002) and are separated by a short intergenic region (Simeone *et al.*, 1994; McGuinness *et al.*, 1996; Stock *et al.*, 1996; Ellies *et al.*, 1997a). Highly conserved sequences were found in the intergenic regions of all three bigene clusters (Zerucha *et al.*, 2000; Sumiyama *et al.*, 2002; Ghanem *et al.*, 2003; Sumiyama and Ruddle, 2003b). Enhancer activity has been demonstrated for some of these sequences. Thus, the I56i and I56ii from the *Dlx5/6* locus and I12b from the *Dlx1/Dlx2* locus act as a forebrain enhancer that largely or completely recapitulates expression of the flanking genes in this tissue (Zerucha *et al.*, 2000; Ghanem *et al.*, 2003).

We also found that two conserved intergenic sequences, I56i and I12a, can target expression of reporter transgenes to the branchial arch mesenchyme of transgenic mice, albeit with distinct patterns. *I56i-lacZ* was initially expressed in the distal aspect of the first

arch and extended into all the pharyngeal arches and, later in development, into the maxilla. Thus, the mouse *I56i* enhancer targeted transgene expression to the branchial arches with patterns similar to the endogenous *Dlx5/6* expression (Fig. 2.4) from E9.25 until at least late embryonic life.

In contrast to *I56i-lacZ* which was expressed in the entire mesenchymal domain of *Dlx5* expression, only cells in the most distal part of the mouse *Dlx2* expression domain in the mandibular component of the first branchial arch expressed *I12a-lacZ* reporter transgenes (Fig. 2.1, 2.2 and data not shown). Similar transgene expression was observed in six independent lines containing the entire mouse *Dlx1/Dlx2* intergenic sequence, in three lines containing a 1.9 kb fragment encompassing the *I12a* conserved element, and in 4/6 primary transgenic embryos containing only the *I12a* sequence (Figs. 2.1 and 2.2). Transgene expression in the tongue and in the floor of the mouth at later stages of development (Fig. 2.3) was reminiscent of endogenous *Dlx2* expression. However, only weak expression of the transgene was observed in the developing incisors and molars through these developmental stages and we cannot exclude that additional *cis*-acting regulatory elements involved in odontogenic regulation of *Dlx1/Dlx2* are located elsewhere.

The inability of *I12a* to reconstitute the entire *Dlx* expression pattern in the mesenchyme of the first arch is, at present, unclear. It is surprising that the mesenchyme expression of *Dlx2* is not regulated as a whole (as seen for *Dlx5* via *I56i*), but through the action of enhancers such as *I12a* that regulate *Dlx2* expression only in a subset of cells. The most likely explanation at this time is that additional regulatory sequences, necessary to

recapitulate the complete *Dlx1/Dlx2* expression in the arch mesenchyme are located elsewhere in the *Dlx1/Dlx2* locus, outside the intergenic region and the first 3.8 kb of *Dlx2* 5'-flanking sequence (Thomas *et al.*, 2000). Such additional *cis*-acting sequences might act in coordination with *I12a* to induce mesenchymal expression of *Dlx1/Dlx2*. Alternatively, the two *cis*-acting regulatory elements might target independently two distinct groups of mesenchymal cells.

Mesenchymal expression in the maxilla was only observed in one of the lines of transgenic animals (line 1486). Temporal analysis of *lacZ* expression in animals of this line indicates that the mandibular and maxillary components of the transgene expression domain may result from the separation of a group of expressing cells that is initially contiguous (Fig. 2.1D, and data not shown). The presence of such transgene expression patterns in only one line of transgenic animals is at present unclear. It is unlikely to be due to the coincidental insertion of the transgene near a tissue-specific enhancer with similar activity as transgene expression persists until it results in the same mesenchymal expression as for the other lines (Fig. 2.1). The intensity of β -galactosidase staining in the mandibular mesenchyme of line 1486 was less than in the other transgenic lines, indicating that increased transgene copy number or integration in a chromosomal region more favorable for transcriptional activity are unlikely explanations for our observation. The lower ability of *I12a* to target expression of the reporter transgene to the maxillary component of the first arch is suggestive of distinct regulatory mechanisms for the mandibular and maxillary components, something that was already suggested by the differential expression of *Dlx5* and *Dlx6* in the mandibular and maxillary components of the first arch.

The *I12a* enhancer sequence is 99% identical between mouse and human and more than 90% identical between mammals and teleost fish (Ghanem *et al.*, 2003), a degree of conservation that surpasses that found in the coding region of the *Dlx* genes. We found comparable percentages of conservation between *I56i* sequences of the same vertebrate species. However, *I12a* and *I56i* do not show any striking sequence similarity despite the partial overlap in their branchial arch activity. We had also found that the various forebrain enhancers, *I56i*, *I56ii* and *I12b*, did not show much similarity in their sequence despite a highly overlapping activity (Ghanem *et al.*, 2003). In fact, the similarity in sequence between *I56i* and *I12b* was limited to a short region that contains two potential binding sites for DLX homeodomain proteins, consistent for a role in a *Dlx* cross-regulatory or autoregulatory mechanisms involving at least the *I56i* enhancer (Zerucha *et al.*, 2000).

The mouse *I56i* sequence was able to target expression to the arches, but the orthologous zebrafish *I56i* (*I46i*) could not (Zerucha *et al.*, 2000), suggesting regulatory evolutionary divergence among conserved sequences. This is surprising considering the two sequences are 81% identical. We have ruled out that this difference could be due to orientation of the sequence of the enhancer within the transgene (Zerucha *et al.*, 2000). Therefore, it appears that even small differences in sequence could influence branchial arch expression. Such small differences could be used to identify essential protein binding sites within *I56i*.

Recently, a 245 bp enhancer, I37-2 located in the *Dlx3/Dlx4* (formerly *Dlx3/Dlx7*) intergenic region was shown to target mesenchymal arch expression in a pattern that overlaps with that of I56i but is, overall, distinct (Sumiyama and Ruddle, 2003b). Interestingly, I37-2 and I56i both contain a pair of putative DLX binding sites showing similarity in their arrangements. This arrangement may be essential for auto or cross-regulatory mechanisms that involve DLX proteins (Zerucha *et al.*, 2000).

2.3.1 *Role of epithelial and mesenchymal factors in enhancer activity*

Expression of *Dlx2* in the first branchial arch is dependent on signals, such as BMP4 and FGF8. FGF8 was shown to induce *Dlx2* expression in the ectomesenchyme but to repress its expression in the epithelium where *Dlx2* was induced by BMP4 (Thesleff and Sharpe, 1997; Thomas *et al.*, 2000). Similarly, the early *I12a-lacZ* transgene expression was reliant on signals from the epithelium because removal of the epithelium at E10.5 resulted in a loss of transgene expression in the mesenchyme, but when the epithelium was removed at E11.5, transgene expression was not affected. FGF8 induced the *I12a-lacZ* transgene expression, although the competence to express transgene in response to FGF8 was clearly restricted to the same subpopulation of *Dlx2*-expressing ectomesenchyme observed in intact *I12a-lacZ* transgenic animals. Thus, the restricted *I12a* enhancer activity is unlikely to be the result of its isolation from additional regulatory sequences, as postulated above, resulting in an overall lower responsiveness to FGF. We cannot rule out at this time that the effects of FGFs on transgene expression are indirect and are not the reflection of changes in either the integrity or specification status of the tissue. Other FGF family members are known to be expressed in the oral epithelium. One member, expressed slightly after FGF8 yet prior to tooth development in the epithelium is FGF9 (Tucker *et al.*, 1999a). Repetition of our

experiments implanting beads imbued with FGF9 resulted in similar induction of the transgene as observed for FGF8.

FGFs and BMP4 are known to regulate endogenous *Dlx2* expression and act to restrict expression to the proximal mesenchyme. The effects of FGFs and BMP4 on *I12a-lacZ* expression suggest that this *I12a* is at least partly involved in this regulation. In contrast to the positive effects of FGF8 and FGF9 signaling, BMP4 exhibited overall negative influences on the expression of the *I12a-lacZ* transgene within the mandibular arch. Increased transgene expression resulting from implantation of Noggin coated beads, an inhibitor of BMP4 function, further supported a negative influence for BMP4 (Tucker *et al.*, 1998a).

2.3.2 I56i-lacZ expression was also modulated by FGF8, FGF9 and BMP4 cues emanating from the epithelium.

The results presented here suggest that the effects of FGF and BMP signaling on the expression of at least two *Dlx* bigene clusters, *Dlx1/2* and *Dlx5/6*, in the mesenchyme of the first branchial arch may be mediated through the action of these signaling pathways on intergenic enhancers.

The endothelin-1 (ET-1) signaling pathway has previously been shown to affect *Dlx* gene expression in the arches of both zebrafish and mice. The zebrafish ET-1 mutant, *sucker*, disrupts ventral arch development and exhibits a loss of *dlx3a* expression (Miller *et al.*, 2000). Loss of function of the endothelin-A receptor (*EdnrA*) in mice reveals its

necessity for *Dlx3* expression in the arches and for maintaining *Dlx2* expression in the hyoid arch (Clouthier *et al.*, 2000). Distal expression of *Dlx6* has been shown to be dependent on ET-1 signaling and is lost in *EdnrA* deficient mice (Charite *et al.*, 2001). Here, the expression of the *I56i-lacZ* transgene was shown to be ET-1-dependent, especially in its distal domain (Fig. 2.7). Thus, the I56i enhancer may be involved in mediating the effects of ET-1. Recently, Yanagisawa and colleagues showed that a genetic loss of an arch specific enhancer of *dHAND*, a transcription factor also downstream of ET-1 signaling and influenced by DLX6, results in a loss of ventrolateral expression yet retains its distal (ventral) tip expression (Yanagisawa *et al.*, 2003). The arch enhancer mutant mice exhibit severely truncated Meckel's cartilage but nonetheless forms the distal primordia. In contrast to its effects on I56i and *Dlx6*, Bosentan did not impair *I12a* enhancer activity or spatial expression of *Dlx2* in the mandibular arch at E10.5. This result is consistent with the changes in *Dlx2* expression in the *EdnrA*^(-/-) mice which are observed specifically in the hyoid at E10.5 with no visible changes in mandibular expression of *Dlx2* (Clouthier *et al.*, 2000). The overall levels of *Dlx2* and *Dlx5* transcripts were reduced after bosentan treatments which is consistent with a prior suggestion that ET-1 may be involved in maintenance of *Dlx* expression (Fig. 2.7 G,H, and Tucker *et al.*, 1999a). Thus, activity of the I56i and I12a enhancers reflects the specific effects of ET-1 signaling on *Dlx* paralogs in the mandibular arch.

Advanced morphological innovations, such as the modern vertebrate jaw, have been correlated with changes in gene activity within multigene families (Neidert *et al.*, 2001). It has been suggested that the nested *Dlx* expression pattern assisted in the evolution of the jaw

(Beverdam *et al.*, 2002; Depew *et al.*, 2002), a gnathostome innovation, since jawless vertebrates, such as lampreys, do not exhibit nested *Dlx* expression in the pharyngeal arches (Neidert *et al.*, 2001). Alterations of the *Dlx* gene expression patterns likely arose due to evolutionary changes in the regulatory elements (Quint *et al.*, 2000) in addition to a proposed heterotypic shift of epitheliomesenchymal interactions (Shigetani *et al.*, 2002) that likely facilitated the restricted expression patterns responsible for the proximodistal patterning of the mandibular arch.

Heterodont mammals have distinct types of dentitions arising from different positions around the presumptive jaw. It was previously suggested that the determination of where and what kind of teeth develop arises as the result of the overlapping expression patterns of antagonistic FGF and BMP signaling factors and regionalization of different transcription factor expression patterns in the mandibular and maxillary arches; supported by the *Dlx* bigene loss-of-function phenotypes outlined in a odontogenic homeodomain code that specifies tooth patterning (Sharpe, 1995). FGF8 and BMP4 have been shown to be involved in the spatial patterning of *Dlx2* expression (Thomas *et al.*, 2000) as well as *Barx1* (Tucker *et al.*, 1998a), *Pax9* (Neubuser *et al.*, 1997), and *Lhx* (Grigoriou *et al.*, 1998) and *Msx1* (Ferguson *et al.*, 2000) homeodomain genes. Understanding how the odontogenic process is established requires understanding of the various factors that establish the expression patterns of regulatory genes such as the *Dlx*. We identified regulatory elements that contribute to *Dlx* expression in the mandibular arch mesenchyme and factors that contribute to their activity. The possibility of a specific subset of cells being subjected to differential regulation within the arch mesenchyme suggests an additional level of complexity in the

patterning of the permissive tissue in interpreting the multiple signaling cues from the surrounding epithelium.

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**3. Zebrafish *dlx2a* maintains the identity of the hindbrain neural crest,
and is necessary for the differentiation of the sensory ganglia and functions
with *dlx1a* in the maturation of the arch cartilage elements**

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Abstract

The *Dlx* genes are a family of transcription factors generally organized as bigene clusters with overlapping expression patterns. In zebrafish, *dlx2a* is expressed in the migrating cranial neural crest that contribute to the pharyngeal arches. *dlx2a* arch expression is subsequently followed by the linked *dlx1a* gene, and other *dlx* paralogues that include *dlx5a/dlx6a* and *dlx3b/dlx4b*. To investigate the coordinate patterning and establishment of arch proximodistal polarity in zebrafish we characterized the loss of function of *dlx2a*, using antisense morpholino oligonucleotides, in the migrating neural crest and *dlx1a* in the arches. Loss of *dlx2a* results in hindbrain reductions of *tfap2a*, *crestin* and *sox9a* neural crest expression, in addition to increased levels of apoptosis. Defects in the differentiation of ectomesenchymal derivatives, including sensory ganglia and cartilage elements, indicate a necessity for *dlx2a* in specification and maintenance of cranial neural crest. *Dlx2MO* abrogates subsequently expressed *dlx3a*, *dlx5b* and *dhand* in the gill arches of injected embryos, and causes a loss of expression in the dorsal aspects of the mandibular and hyoid arches. *dlx1a* and *dlx2a* morphants have smaller and dysmorphic arch cartilage elements indicating roles in patterning of hard tissues. Cartilage defects in *dlx1a* morphants were milder than *dlx2a* morphants. The combined loss of *dlx1/2a*, causes severe arch cartilage dysmorphology revealing a role in maturation, and patterning of arch chondrogenesis. Functional analysis of individual *dlx* genes involved in pharyngogenesis contributes to our understanding of coordinate proximodistal patterning and development of the jaws.

3.1 Introduction

The *Dlx* genes, of which gnathostomes have at least six, are homeodomain-containing transcription factors generally arranged as convergently transcribed bigene clusters. Linked paralogs, separated by a short intergenic region, have overlapping expression patterns due to purportedly shared *cis*-acting regulatory elements (Zerucha and Ekker, 2000; Ghanem *et al.*, 2003; Sumiyama and Ruddle, 2003b; Park *et al.*, 2004). *Dlx* genes are affiliated with morphological innovations and are most prominently expressed in the forebrain, pharyngeal arches, sensory placodes, and limbs/fins (reviewed in Panganiban and Rubenstein, 2002). Within the pharyngeal arch primordia, the *Dlx* genes are expressed in a nested pattern. This characteristic is a gnathostome synapomorphy thought to be necessary for the evolution of jaws. *Dlx1* and *Dlx2* are expressed throughout the maxillary and mandibular components of the first arch. *Dlx5/6* and *Dlx3/4* (formerly *Dlx3/7*; reviewed in Panganiban and Rubenstein 2002) are expressed in increasing more ventrodiscal domains of the mandible correlating with their role in proximodistal patterning. Mouse *Dlx1/2*^{-/-} craniofacial defects reveal a loss of proximal features, that include the absence of maxillary molars (Qiu *et al.*, 1997). *Dlx5/6*^{-/-} mutants exhibit enantiomorphism of proximal maxillary hard and soft tissues exemplified by rugae and vibrissae reproduced in the distal mandibular component of the first arch (Beverdam *et al.*, 2002; Depew *et al.*, 2002). An exception to overlapping patterns of *cis*-paralogues, *Dlx2* expression is unlinked from *Dlx1* in the migrating cranial neural crest of mouse and zebrafish (Bulfone *et al.*, 1993a; Akimenko *et al.*, 1994; Robinson and Mahon, 1994). Zebrafish have three streams of cranial neural crest originating from the posterior midbrain and hindbrain regions that migrate into the mandibular, hyoid and gill arches 3-7 in a rostrocaudal manner (reviewed in Eisen and

Weston, 1993; Schilling and Kimmel, 1994; Yelick and Schilling, 2002). During migration, cells divide and express markers that presage their fate. Influenced by intrinsic and extrinsic factors, the cranial neural crest differentiates into bones of the skull, the dentin and pulp of teeth, connective tissues, nerve ganglia and melanocytes (Graham *et al.*, 2004). *Dlx2* may function in either the maintenance of the migrating neural crest cells or differentiation of their derivatives.

To investigate the zebrafish homologue *dlx2a* in the cranial neural crest and its coordinate role with *dlx1a* in the arches, we performed a functional analysis with morpholino antisense oligonucleotides (MO). Our results indicate *dlx2a* participates in the specification, and maintenance of the cranial neural crest necessary for the differentiation of its ectomesenchymal derivatives. Both *dlx1a* and *dlx2a* function in patterning the proximal (dorsal) aspects of the anterior mandibular and hyoid arches, maturation of the arch cartilage elements, and induction of forebrain expression of subsequently expressed *dlx* family members. These results are consistent with the role of the mouse *Dlx1/2* genes, and suggest conservation of the developmental mechanisms.

3.2 Materials and Methods

3.2.1 Animal maintenance and transgenic zebrafish

Zebrafish wild-type embryos were cultured and maintained by the methods indicated by Westerfield (Westerfield, 1995). Embryos were raised at 28.5°C in E3 media with 0.003% phenylthiocarbamide to prevent pigmentation. Embryonic chorions were removed prior to

fixation with watchmaker forceps. Embryos and larvae were fixed in 4% paraformaldehyde (PFA)/phosphate buffered saline (PBS) and dehydrated in methanol for storage at -20°C.

3.2.2 *Whole-mount cartilage staining*

Cartilage staining was performed essentially as in Piotrowski *et al.*, (Piotrowski *et al.*, 1996). Briefly, larvae were fixed in 4% PFA/PBS pH 7.0 overnight (O/N). They were then washed and dehydrated in 70% ethanol (EtOH). Larvae were stained for 6-8 hrs in a solution of 0.1% Alcian blue, 70% EtOH, and 30% acetic acid. Larvae were washed in 70% EtOH, stepwise dehydrated to 100% EtOH, and then rehydrated in PBS. Tissues were then macerated in 1% sodium tetraborate (Borax) and 0.05% trypsin for 1-3 hrs. The larvae were then washed in PBS and bleached in 3% hydrogen peroxide/1% potassium hydroxide for 1-3 hrs.

3.2.3 *In vitro transcription of synthetic RNA*

Antisense riboprobes were labeled with either digoxigenin or fluorescein-12-UTP (Roche). For capped sense mRNA the following templates were used: *dlx1a* (NM131311) in pTL-2 was linearized with *Sall*, and transcribed with T7 resulting in a 1 kb transcript. *dlx2a* (NM131311) in pCS2+ plasmid was linearized with *NotI*, and transcribed with SP6 resulting in a 1.3 kb transcript. Capping was performed with 7' methylguanosine GTP analogue cap (NEB). Transcript length and quality were assessed by agarose gel electrophoresis and quantified by spectrophotometry.

3.2.4 Microinjection

One to two cell stage embryos were microinjected using a Narashigi IM300 microinjector. The microinjection needles were pulled using a P90 micropipette puller (Browning and Flame). Antisense morpholino oligonucleotides (GeneTools; start sites bolded) *dlx1*MO, [5'TAGACTCTCTGGTATTGTAGACATG3'], *dlx2*MO [5'CTCCAGTCATGTTTTTCA TACCGCA3'], and a standard control [5'CCTCTTACCTCAGTTACAATTTATA3'] were diluted in Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO₄, 0.6 mM Ca(NO₃)₂, 5 mM Hepes pH 7.6) and 0.05% Phenol Red. Embryos were tested with approximately 5 nl at the following concentrations: 0.125 mM, 0.25 mM, 0.375 mM, 0.5 mM, and 1 mM. Rescue of morphants was performed by co-injection of equimolar amounts of synthetic capped mRNA.

3.2.5 Whole-mount *in situ* hybridization, and immunohistochemistry

The following labeled antisense riboprobes were used (Accession number, restriction enzyme, polymerase and approximate transcript size are indicated in brackets): *dlx1a* (NM131305; *Bam*HI, T3, 0.77 kb), *dlx2a* (NM131311; *Bam*HI, T7, 1.7 kb), *dlx3b* (NM131322; *Sall*I, T7, 1.9 kb), *dlx5a* (NM131306; *Bam*HI, T7, 1.3 kb) (Akimenko *et al.*, 1994; Ellies *et al.*, 1997b), *dhand* (*hand2*; NM131626; *Bam*HI, T7, 1.8 kb) (Angelo *et al.*, 2000); *crestin* (AF195881; *Eco*RI, T7, 4 kb) (Rubinstein *et al.*, 2000; Luo *et al.*, 2001a); *krox20* (*egr2b*; NM130997; *Pst*I, T3, 2 kb) (Oxtoby and Jowett, 1993); *sox9a* (NM161343; *Eco*RV, T7, 0.7 kb) (Chiang *et al.*, 2001), *tfap2a* (AF457192; *Asp*718, SP6, 1.7 kb) (Knight *et al.*, 2003). Whole-mount *in situ* hybridizations were performed as described by Akimenko *et al.* (Akimenko *et al.*, 1994). Briefly, embryos were rehydrated and washed

twice in PBST. Embryos one day or older were permeabilized with Proteinase K (10 µg/ml) for several minutes depending on stage of development. Embryos were washed and fixed again for 20 min in 4% PFA/PBS at RT. Embryos were washed and prehybridized for 1-3 hrs in PreHyb solution (50% formamide, 5× SSC, 0.1% Tween 20, 5 mg/ml torula RNA, 50 µg/ml heparin) at 65°C. Approximately 5 ng/ml digoxigenin (DIG)-labeled riboprobe was added and hybridized O/N. Embryos were subjected to 10 min washes in the following ratios of hybridization solution and 2× SSC at 65°C: 100% Hyb solution; 3:1; 1:1; 3:1; 100% 2× SSC. Embryos were wash twice for 30 min in 0.2× SSC at 60°C. At RT embryos were washed in following ratios of 0.2× SSC:PBST for 4 min each: 3:1;1:1;1:3; twice in PBST. Prior to detection samples were blocked in for at least 45 min in 2 mg/ml BSA, 2% CS, 1% DMSO. Embryos were incubated with 1/5000 dilution of anti-DIG or anti-Florescein antibody for 3-4 hrs. Antibody was washed out on in four 30 min washes at RT or O/N at 4°C in PBST with mild shaking. Colourimetric reaction was performed with staining buffer (100 mM Tris, pH 9.5, 50 mM MgCl₂, 100 mM NaCl, 0.1% Tween 20). Embryos were washed twice in staining buffer for 10 min each and stained with NBT/BCIP. The reaction was stopped with PBST and embryos were fixed for 20 min at RT or O/N at 4°C. Embryos were washed in PBS and stored in PBS and 10 mM EDTA to inhibit contamination. Two-colour hybridizations included a one hour glycine (100 mM, pH 2.2) incubation to eliminate any carryover alkaline phosphatase activity for the second antibody incubation and staining. Red staining was performed with Fast Red tablets (Roche) in staining buffer (100 mM Tris-Cl, pH 8.2).

For antibody staining, embryos were rehydrated in PBS and blocked in maleic acid blocking buffer (150 mM Maleic acid, 100 mM NaCl, 0.5% Nonidet, 0.1% Tween-20, 2 mg/ml BSA, 2% CS) for 1-3 hrs. All steps were performed on a shaking platform. Primary antibodies, anti-Dll 1/80 dilution (Gifts from G. Boekhoff-Falk, formerly Panganiban, and J. Liu) and zn-5 (Eugene, Oregon), 1/150 dilution (Trevarrow *et al.*, 1990), were incubated with embryos for 4 hrs at room temperature (RT). Antibody was washed out in blocking buffer O/N at 4°C. Anti-Rabbit and anti-mouse biotinylated secondary antibodies (Vectastain) were incubated for 4 hrs at RT, and washed out O/N with several changes of blocking buffer. Embryos were washed in PBS and incubated for 15 min in 1 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB). Colourimetric staining was performed with hydrogen peroxide and DAB as the chromogen. Staining was stopped by washing in PBS and embryos were fixed in 4% PFA/PBS.

3.2.6 Cell death assays

Apoptosis was determined by acridine orange staining and TUNEL labeling. Live dechorionated embryos were bathed in acridine orange (16.66 µg/ml) for 20 minutes in the dark. The embryos were washed and immediately examined under a fluorescent microscope. TUNEL labeling was performed with Terminal transferase (Roche), and dUTP-DIG (Roche) as described in Ellies *et al.*, (1997a)

3.2.7 Photography

Samples were digitally photographed with a Leica MZ FLIII fluorescent dissecting microscope connected to a computer running Eclipse 6.0 image capture software (Empix

Imaging Inc., Mississauga). Specimens were mounted in either PBS or 3% methylcellulose. For higher magnification, embryos were cleared in methanol; benzylbenzoate:benzylalcohol (2:1, v/v); benzylalcohol:glycerol (6:1, v/v), mounted on bridged slides, coverslipped, and digitally photographed using a Zeiss Axioscope.

3.3 Results

3.3.1 Inactivation of *dlx2a* and *dlx1a* mRNA

To examine the loss of function of the zebrafish *Dlx1* and *Dlx2* orthologues, we used an antisense morpholino oligonucleotide (MO) approach (reviewed in Nasevicius and Ekker, 2000; Heasman, 2002). MOs complementary to the translational start site of *dlx2a* and *dlx1a*, were microinjected into one and two cell-stage wild-type embryos. The effective concentration of injected *dlx* MOs was determined empirically (Table 3.1). A dose-dependent phenotype was observed for both the *Dlx1*MO and *Dlx2*MO injections. After injection, embryos were examined for developmental perturbations. Embryos defective for gastrulation were removed. Embryos injected with greater than 20 ng resulted in non-specific effects that included early lethality, tail defects and tectal necrosis characterized as opacity in the mid- and hindbrains. Control MO was injected in concurrent trials at similar concentrations. To ensure specificity of the morpholinos, and to resolve any non-specific deleterious effects, rescue of the resulting mutant phenotypes was performed by co-injecting equimolar amounts of corresponding synthetic capped mRNA (Table 3.2).

Table 3.1 The effects of morpholino “knock-down” during embryogenesis

Injected Concentration	n (6 hpf)*	n (1 dpf/5 dpf)	% dead	% WT	% abnormal
Control Uninjected	935	894	4	99 (883)	1 (11)
Control MO 2 ug/ul	39	32	18	100 (39)	0
Control MO 4 ug/ul	714	592	17	91 (536)	9 (56)
Control MO 8 ug/ul	190	160	16	99 (158)	1 (2)
Dlx1MO 2 ug/ul	43	43/29	33	72 a	28 (8) a
Dlx1MO 4 ug/ul	157	155	1	99 (153) b	1 (2) b
Dlx1MO 4 ug/ul	342	338/264	22	48 a	52 (136) a
Dlx1MO 6 ug/ul	46	46/40	24	28 (11) a	73 (29) a
Dlx1MO 8 ug/ul	101	97	4	91 (88) b	9 (9) b
Dlx2MO 1.5 ug/ul	64	58	10	66 (38)	34 (20)
Dlx2 MO 2 ug/ul	139	122	12	52 (63)	48 (59)
Dlx2MO 3 ug/ul	51	47	8	10 (5)	89 (42)
Dlx2MO 4 ug/ul	710	423	40	47 (198)	53 (225)
Dlx2MO 8 ug/ul	221	75	66	41 (31)	58 (44)
Dlx1/2MO 2 ug/ul	578	487	16	56 (274)	44 (212)
Dlx1/2MO 4 ug/ul	52	37	29	11 (4)	89 (33)

Data pooled from several injection trials

(n), number of embryos is indicated in brackets

* Unfertilized embryos or embryos failing to gastrulate were removed

a. phenotype assessed at 5 dpf

b. phenotype assessed at 1 dpf

Table 3.2 Rescue of Dlx1, Dlx2, Dlx1/2 morphants

Injected Concentration	n (6 hpf)	n (1 dpf/5 dpf)	% dead	% WT	% abnormal
Control Uninjected	479	447	3	99	1
Control MO 4 ug/ul	189	183/155	15	97 (150) a	3(5) a
Control MO 4 ug/ul, <i>dlx2a</i> mRNA 2.0 ug/ul	121	110	9	85 (93)	15 (17)
Control MO 4 ug/ul, <i>dlx2a</i> mRNA 4 ug/ul	47	36	23	100 (36)	0
<i>dlx2a</i> mRNA 4 ug/ul	142	141	1	94 (131)	6 (8)
Dlx2MO 4 ug/ul	75	72	4	43 (31)	57 (41)
Dlx2MO 4 ug/ul, <i>dlx2a</i> mRNA 1.25 ug/ul	31	29	6	45 (13)	55 (16)
Dlx2MO 4 ug/ul, <i>dlx2a</i> mRNA 1.5 ug/ul	81	39	52	49 (19)	51 (20)
Dlx2MO 4 ug/ul, <i>dlx2a</i> mRNA 2.0 ug/ul	69	64	7	62 (40)	38 (24)
Dlx2MO 4 ug/ul, <i>dlx2a</i> mRNA 3 ug/ul	27	15	44	87 (13)	13 (2)
Dlx2MO 2 ug/ul, <i>dlx2a</i> mRNA 2 ug/ul	236	223	6	97 (217)	3 (6)
Dlx2MO 4 ug/ul, <i>dlx2</i> mRNA 4 ug/ul	50	42	25	75 (31)	25 (11)
<i>dlx1a</i> mRNA 4 ug/ul	112	84/84	25	79 (66) a	20 (18) a
Dlx1MO 4 ug/ul	160	160/147	8	42 (61) a	58 (86) a
Dlx1MO 4 ug/ul, <i>dlx1a</i> mRNA 4 ug/ul	346	332/299	10	78 (234) a	22 (65) a
Dlx1/2MO 4 ug/ul, <i>dlx1/2a</i> mRNA 4 ug/ul	146	142	3	96 (137)	4 (5)

Data pooled from several injection trials

(n), number of embryos is indicated in brackets

* Unfertilized embryos or embryos failing to gastrulate were removed

a. phenotype assessed at 5 dpf

3.3.2 Proper head outgrowth and arch cartilage maturation require *dlx2a* and *dlx1a*

Dlx genes have been implicated in zebrafish arch cartilage formation (Ellies *et al.*, 1997a). Chondrogenesis begins around 52 hours post fertilization (hpf), and develops rapidly by 4 days post fertilization (dpf) in the larvae (Schilling and Kimmel, 1997). Injected embryos were grown until 5 dpf to examine head and cartilage formation; a time when head cartilages are mostly differentiated and easily observable (Fig. 3.1A-D). *Dlx* morphants revealed a high level of mortality between 2.5 and 3 dpf. High mortality rates have been previously observed in many of the 109 arch mutations classified in the chemical mutagenesis screen (Schilling *et al.*, 1996). Embryos injected with control MO showed no abnormal phenotype (Fig. 3.1A). During pharyngogenesis (24-48 hpf, Kimmel *et al.*, 1995), *dlx1*MO injected embryos were indistinguishable from control MO injected embryos (not shown). The normal phenotype during early pharyngogenesis suggests that other *dlx* family members may be compensating for the loss of *dlx1a* function. *Dlx1*MO injected embryos did show signs of dysmorphology, beginning at approximately 2.5-3 dpf. Head outgrowth of *dlx1a* morphants appeared stunted compared to the control injected embryos (Fig. 3.1B). In comparison to the *dlx1a* morphants, functional reduction of *dlx2a* revealed a more severely afflicted phenotype. *dlx2a* morphants showed growth retardation during early pharyngogenesis. The experimental embryos were smaller than normal with stunted head growth. Development at 5 dpf revealed poor embryonic head outgrowth similar to *dlx1a* morphants (Fig. 3.1C). Co-injection of *dlx1*MO and *dlx2*MO (*dlx1/2*MO) produced a malformed phenotype more severe than *dlx2a* morphants. Loss of *dlx1/2a* function resulted

in developmental retardation during pharyngogenesis with smaller heads and a lack of facial outgrowth (Fig. 3.1D).

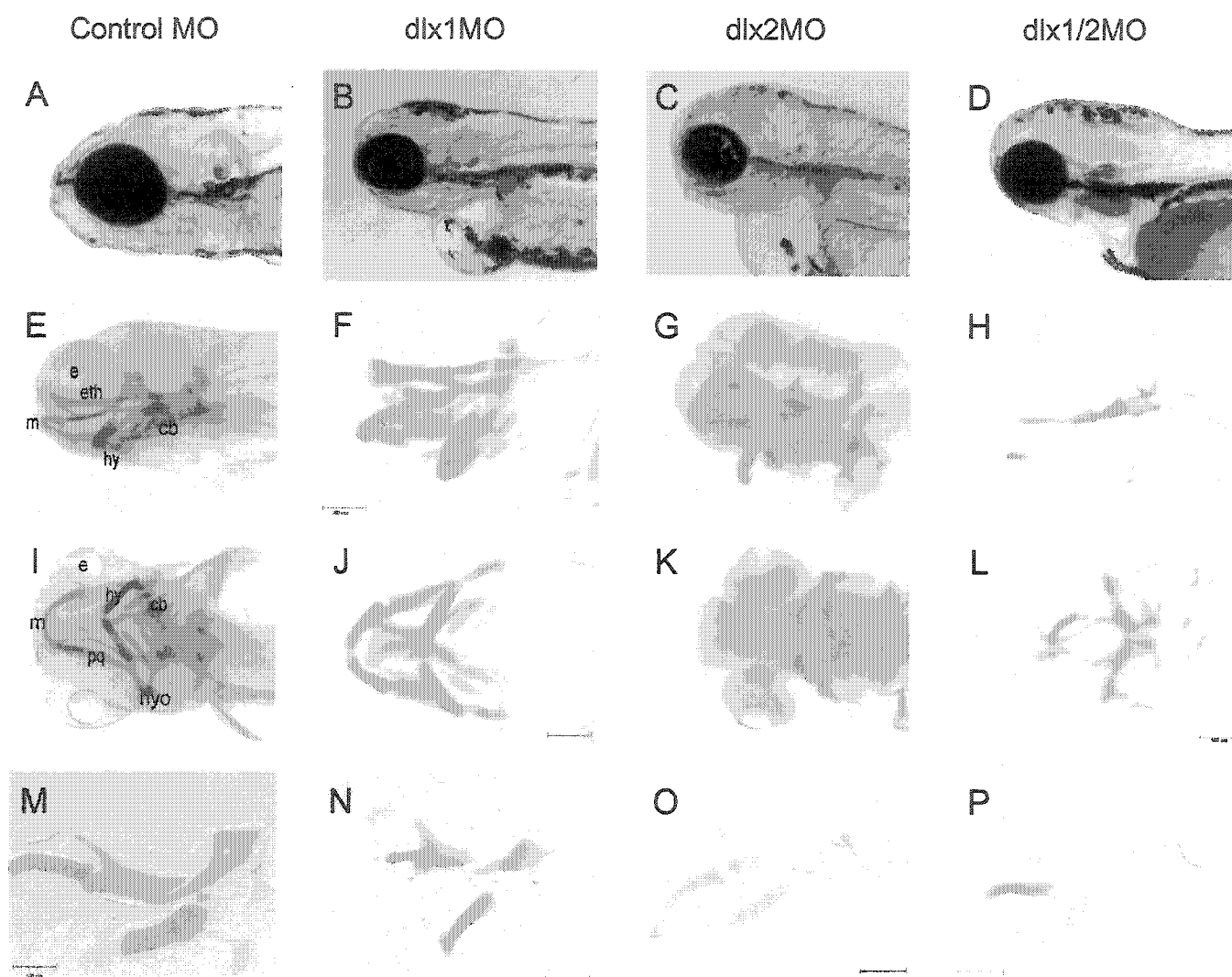
Heart edema was observed for the most severely affected *dlx*MO injected embryos. Heart edema is suggested to be a nonspecific effect, however, it has been previously characterized in zebrafish craniofacial mutants and may be a result of poor circulation in the arch arteries (Neuhauss *et al.*, 1996). Posterior rhombencephalic neural crest contributions to cardiac development, and their deficiencies may contribute to a defective heart complicating the diagnosis of the edema (Li *et al.*, 2003). Laser ablation of cardiac-fated neural crest has been shown to cause heart looping failures and pericardial effusion (Li *et al.*, 2003) similar to our *dlx1/2* morphant phenotypes.

By 5 dpf, *dlx* morphants showed similar flat facies and smaller heads when compared to controls. A lack of anterior facial protrusion correlates with jaw failure to move forward. The eye and otic vesicle, were smaller than in the control larvae. In chick posterior mesencephalic cranial neural crest contribute to the endothelium and stoma of the cornea (Noden, 1978; Couly *et al.*, 1996). Presently, it is not clear whether anterior hindbrain *dlx2a* expressing neural crest cells contribute to the zebrafish eye. *dlx2a* and *dlx1a* expression in the medial fin appeared normal (data not shown). However, in some cases their tails tended to curve (not shown), an observation previously made for several N-ethyl-N-nitrosourea (ENU)-induced arch mutants (Schilling *et al.*, 1996).

Dlx genes are expressed in the zebrafish arch chondrogenic condensations (Ellies *et al.*, 1997a). *dlx1/2a* are expressed in both the dorsal and ventral arch cartilage elements. The ventral cartilage elements are comprised of the mandibular or Meckel's cartilage in the first arch, the basihyal, interhyal and ceratohyal of the second arch, and the basibranchials of the remaining posterior arches. The more dorsal elements include the palatoquadrate in the first arch, and the hyosymplectic in the hyoid arch. To determine the effect of *dlx1a* and *dlx2a* loss of function on cartilage formation, 5 dpf larvae were stained with alcian blue. Alcian blue binds to proteoglycan components of the chondrogenic extracellular matrix. Compared to control larvae (Fig. 3.1E, I and M), *dlx1*MO (Fig. 3.1F, J and N), *dlx2*MO (Fig. 3.1G, K and O), and *dlx1/2*MO injected larvae (Fig. 3.1H, L and P), showed an increasing severity of cartilage malformation. Variability in cartilage mutant phenotypes was observed. Generally, *dlx1*MO injected larval arch cartilage elements were shorter, and wider than normal. Ceratobranchial elements stained poorly with alcian blue (Fig. 3.1F, J). The dissected out palatoquadrate of the first arch, and hyosymplectic of the second arch elements were smaller and malformed compared to controls (Fig. 3.1N). The *dlx1a* morphant phenotype of flat facies and thicker cartilage elements indicates unique features in growth and maturation of elements not compensated for by *dlx2a*.

Dysmorphogenesis of *dlx2a* morphant arch cartilage elements was more severe than in *dlx1a* morphants. Mandibular, palatoquadrate, hyosymplectic and ceratohyal elements were typically smaller than normal and misshapen. Ceratobranchials and accompanying medial basihyal and basibranchials stained poorly with alcian blue (Fig. 3.1G, K, and O). Chondrocyte stacking within the elements was not arrayed in an orderly manner as normally

Figure 3.1. *dlx1a* and *dlx2a* are required for proper head outgrowth and arch cartilage formation. (A, E, I and M) Control MO (0.5 mM) injected 5 dpf larvae head morphology. (B, F, J and N) Dlx1MO (0.5 mM) injected larvae. (C, G, K and O) Dlx2MO (0.5 mM) injected larvae. (D, H, L and P) Dlx1/2MO (0.5 mM) injected larvae. (E-P) Alcian blue staining of 5 dpf larvae. (A-H) Lateral view. (I-L) Ventral view. (M-P) Flat-mounts of dissected anterior arches. Anterior is to the left. cb, ceratobranchials; e, eye; eth, ethmoid plate; hy, hyoid; hyo, hyosymplectic; m, mandible; pq, palatoquadrate. Bar is 100 μ m.



observed in wild-type elements. The mesodermally-derived neurocranium always formed. However, the ethmoid plate and trabeculae, comprised of both mesoderm and neural crest, tended to not extend fully anteriorly. Presently, it is unclear as to what contribution, if any, the zebrafish arch neural crest participates in forming the trabeculae. This defect may be a result of growth retardation. *dlx2a* is expressed earlier than *dlx1a* which correlates with the more severe abnormal phenotype observed in the former mutant. Disruption of cartilage formation indicates that both *dlx1a* and *dlx2a* contribute to aspects of chondrogenesis not completely compensated by coexpression.

Dlx1/2MO injected larvae revealed the severest malformed phenotypes, having shortened and malformed palatoquadrate and hyosymplectic elements (Fig. 3.1H and L). In some cases, the dorsal elements, the palatoquadrate and hyosymplectic, in addition to the ceratobranchials lacked alcian blue staining (Fig. 3.1P). These results indicate that *dlx1a* and *dlx2a* function in arch cartilage formation, and that reduction of their function leads to smaller and dysmorphic elements.

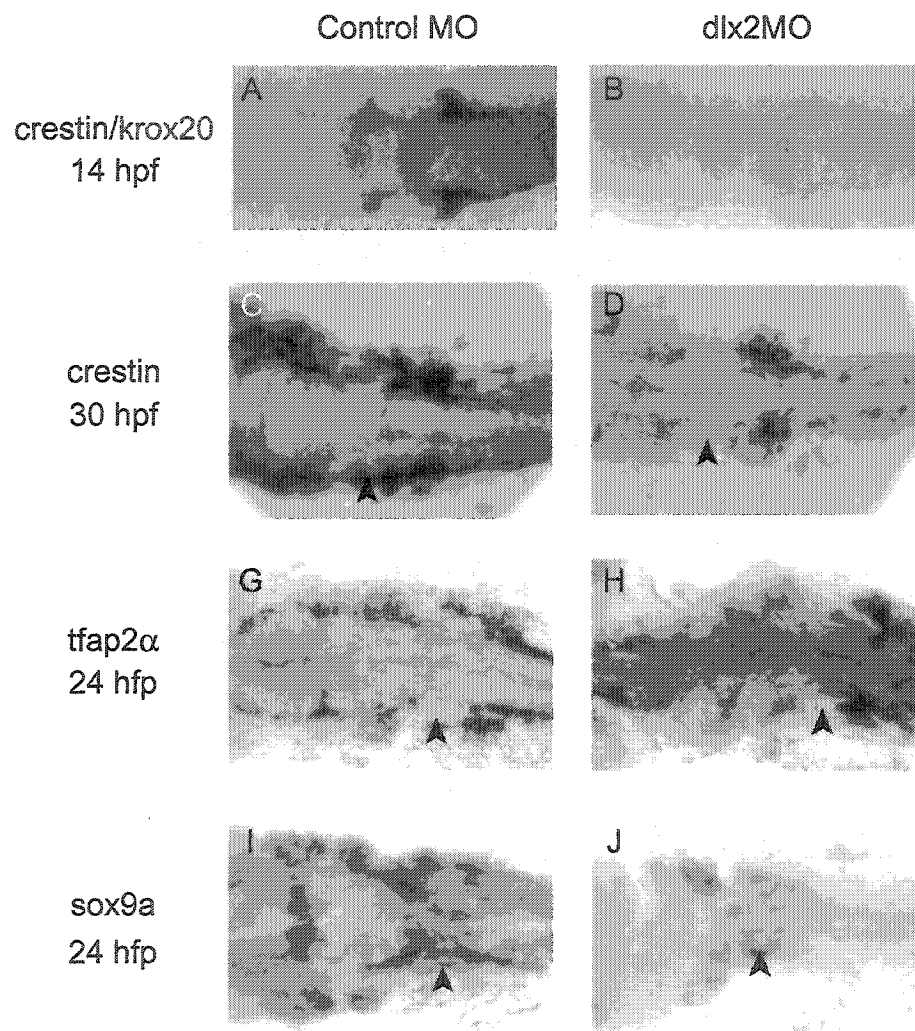
In the mouse, *Dlx2* is necessary for the differentiation of a subset of cranial neural crest cells. *Dlx2* homozygous null mutants have defects in proximal hard tissues of the mandible and hyoid, as well as in the forebrain, and gastrointestinal tract, and die shortly after birth (Qiu *et al.*, 1995). Loss of function of *Dlx1* results in a less severe mutant phenotype and death within one month by unknown causes (Qiu *et al.*, 1997). Loss of both *Dlx1* and *Dlx2* results in novel defects not found in the individual mutants, which include the absence of maxillary molars. Combined loss of zebrafish *dlx1/2a* suggests that the

paralogues work together for proper morphogenesis and maturation of the arch cartilage elements. These results are reminiscent of the mouse *Dlx1* and *Dlx2* single and double null mutants (Qiu *et al.*, 1995; Qiu *et al.*, 1997). The similarity and relative severity of defects between the zebrafish *dlx* morphants are comparable to the mouse *Dlx* mutants suggesting their functional role is conserved.

3.3.3 *dlx2a* specifies cranial neural crest destined for the arches

dlx2a is expressed in the subset of hindbrain migrating cranial neural crest cells destined for the pharyngeal arches. The malformation severity of *dlx2*MO-induced mutant phenotypes, compared to the *dlx1a* loss-of-function, suggests that *dlx2a* plays an important role in the arch neural crest cells. To examine the role of *dlx2a* in the rhombencephalic subset of cells, we examined early stages of migration and pharyngogenesis. *dlx2a* expression begins at approximately 12 hour post fertilization (hpf; 10 som) (Akimenko *et al.*, 1994). To determine the role of *dlx2a* in the migrating cranial neural crest cells, we examined MO-injected embryo that were stained for the pan-neural crest marker, *crestin*, a multi-copy retro-element (Rubinstein *et al.*, 2000; Luo *et al.*, 2001a), *tfap2a*, a neural crest progenitor marker expressed in premigratory and migrating neural crest (Mitchell *et al.*, 1991; Knight *et al.*, 2003; Barrallo-Gimeno *et al.*, 2004; Knight *et al.*, 2004), and *sox9a* a prechondrogenic marker of cranial neural crest (Yan *et al.*, 2002). Compared to the control embryos at 14 hpf (Fig. 3.2A), *dlx2*MO-injected embryos showed a decrease or loss in *crestin* staining in the head region (Fig. 3.2B). Embryos examined at several time points during pharyngogenesis showed similar reduction or loss of *crestin* staining anterior to the otic vesicle (Fig. 3.2C, D; arrowhead). The loss in *crestin* staining suggests that cranial neural crest specification is

Figure 3.2. *dlx2a* is necessary for arch neural crest cell specification. Dorsal flat-mount views with anterior to the left in all panels. Expression of cranial neural crest marker *crestin* (dark purple) and *krox20* (red) demarcating rhombomeres three and five at 14 hpf in control MO injected (A) and *dlx2*MO injected embryos (B). (C,D) *Crestin* expression at 30 hpf. *tfap2a* expression at 24 hpf in control MO (G) and *dlx2*MO (H) injected embryos. 24 hpf *sox9a* expression in control MO (I) and *dlx2*MO (J) injected embryos. Arrowheads indicate position of the otic vesicle.

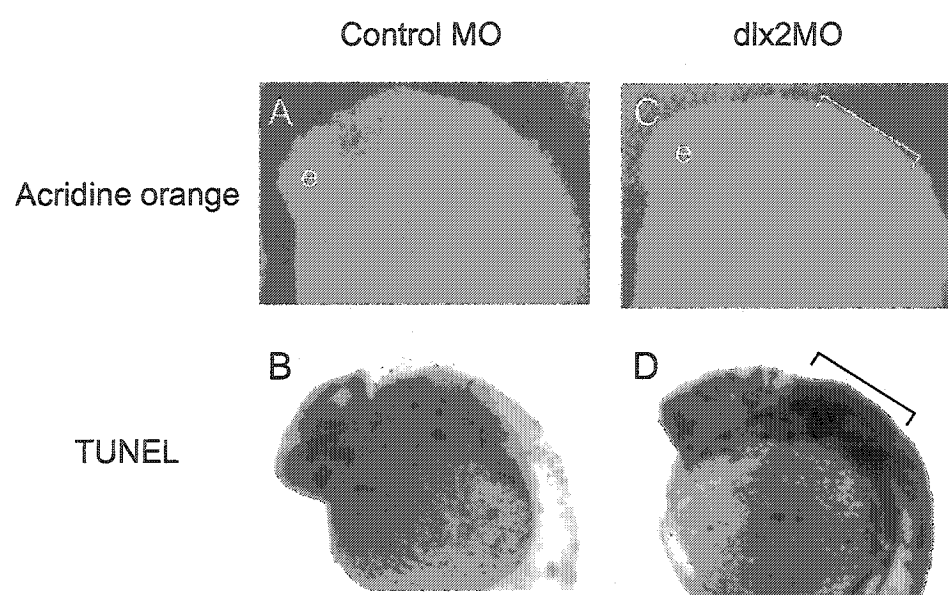


disrupted in *dlx2*MO-injected embryos. Loss of expression is reminiscent of similar reductions in *tfap2a* zebrafish mutant alleles, *mont blanc* (*mob*^{m819}), and *lockjaw* (*low*^{ts213}) (Knight *et al.*, 2003; Barrallo-Gimeno *et al.*, 2004). *tfap2a* expression was reduced in the hindbrain, yet maintained posterior to the otic vesicle (Fig. 3.2G, H). Similarly, *sox9a* expression showed reduced staining in the arches (Fig. 3.2I, J). These results suggest that the loss of pharyngogenesis capability is restricted to the hindbrain *dlx2a*-expressing neural crest. Therefore, *dlx2a* is necessary for the specification of a subset of the neural crest bound for the arches.

3.3.4 *dlx2a* is necessary for arch neural crest maintenance

To address the reductions in cranial neural crest specification, control MO and *dlx2*MO injected embryos were examined for apoptosis. Acridine orange (AO) staining and TUNEL labeling were used to assess cell death (Fig 3.3). Prior to neural crest migration, no difference in apoptotic levels were observed between wild-type non-injected, control MO and *dlx2*MO-injected embryos. During the initiation of neural crest cell migration, control embryos displayed only punctate TUNEL labeling and AO staining similar to wild-type non-injected embryos (Fig. 3.3A, B). *Dlx2*MO embryos, however, showed increases in TUNEL and AO (bracket) staining in the hindbrain. (Fig. 3.3C, D). The observed apoptosis is similar to the pattern of cell death previously characterized in the *mob/tfap2a* mutant (Knight *et al.*, 2003; Barrallo-Gimeno *et al.*, 2004). Many pharyngeal arch mutants have increases in mid- and hindbrain cell death (Schilling *et al.*, 1996). The numerous factors involved indicate the complexity of neural crest specification, and epitheliomesenchymal transition

Figure 3.3. *dlx2a* participates in arch cranial neural crest survival. 14 hpf acridine orange stained and 24 hpf TUNEL labeled (A,B) control embryos, and (C,D) *dlx2*MO injected embryos. Anterior is to the left. e, eye; bracket, indicates apoptotic hindbrain region.



necessary for successful migration. We conclude that *dlx2a* participates in the maintenance and survival of the arch subset of cranial neural crest.

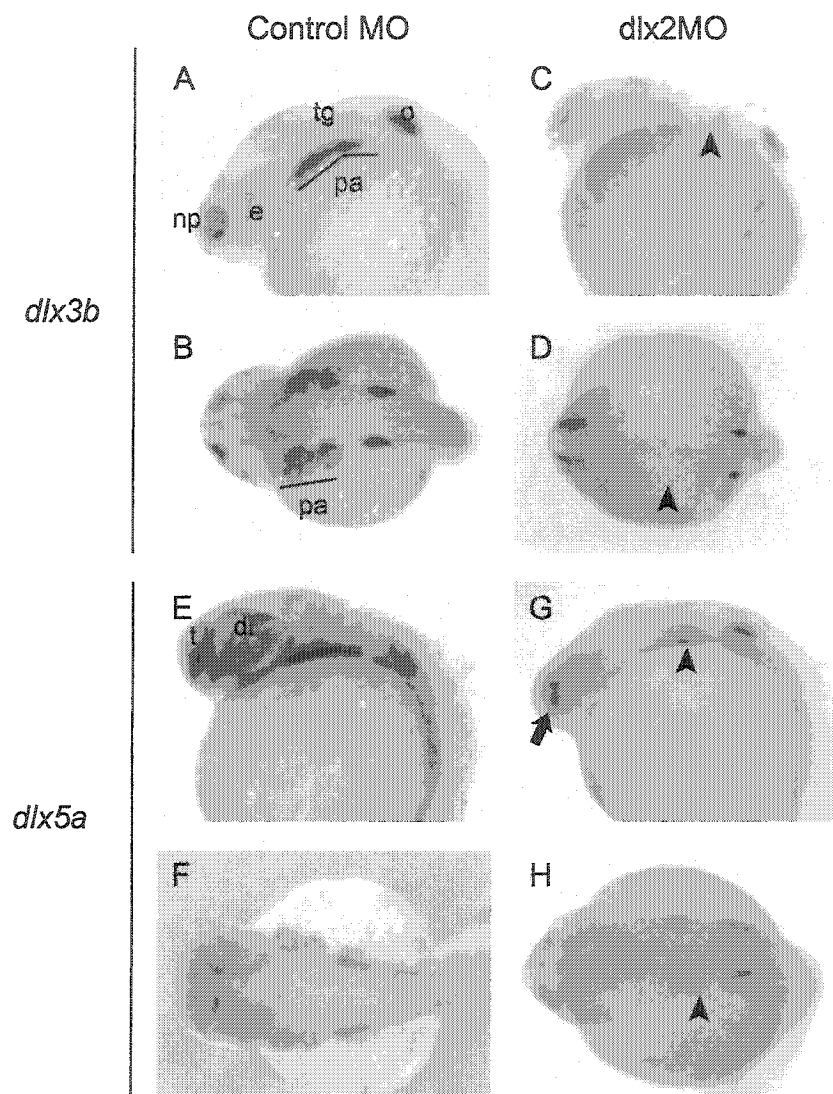
3.3.5 *dlx2a* is necessary for *dlx3b* and *dlx5a* visceral arch expression

Previous studies show *Dlx2* positively regulates other *Dlx* family members (Zerucha *et al.*, 2000; Stuhmer *et al.*, 2002a). To examine whether *dlx2a* function is necessary for subsequently expressed *dlx* gene expression in the pharyngeal arches, we examined *dlx3b* and *dlx5a* expression during early pharyngogenesis. In 24 hpf control MO-injected embryos, *dlx3b* is expressed in the nasal placodes, arches, trigeminal ganglia, and otic vesicle (Fig. 3.4A, B). *dlx2a* morphants retained *dlx3b* expression in the nasal placodes and otic vesicle, but lost expression in the trigeminal ganglia and the pharyngeal arches (arrowhead; Fig. 3.4C, D). Control MO-injected embryos expressed *dlx5b* in the forebrain, arches, otic vesicle and olfactory placode (Fig. 3.4E, F). *dlx2a* morphants maintained *dlx5a* expression in the telencephalon (arrow) and otic vesicle and olfactory placode, however, expression was lost in the visceral arches (arrowhead; Fig. 3.4G, H). Reduction in *dlx5a* staining was also observed in the diencephalon of the forebrain (Fig. 3.4G, H). These results indicate that *dlx2a* is necessary for subsequent *dlx3a* and *dlx5b* arch expression. Therefore *dlx2a* may be functioning as a regulator of family members within the visceral arches and in the *dlx* expressing cells of the diencephalon.

3.3.6 *dlx2a* is necessary for specification of the dorsal aspect of the anterior arches

Mouse *Dlx2*^{-/-} and *Dlx1/2*^{-/-} mutants have proximal (dorsal) patterning defects in the mandible and hyoid arches (Qiu *et al.*, 1995; Qiu *et al.*, 1997). To investigate possible

Figure 3.4. Loss of *dlx2a* function disrupts *dlx3b*, *dlx5a* expression in the pharyngeal arches. 24 hpf expression of *dlx3b* (A-D), *dlx5a* (E-H) in control MO injected (A, B, E, and F) and *dlx2*MO injected embryos (C, D, G and H). Anterior is to the left, (A, C, E and G) lateral views, (B, D, F and H) ventral views. e, eye; di, diencephalon; np, nasal placodes; o, otic vesicle; pa, pharyngeal arches; t, telencephalon; tg, trigeminal ganglia. Arrowheads indicate position of pharyngeal arches; arrow indicates telencephalon.

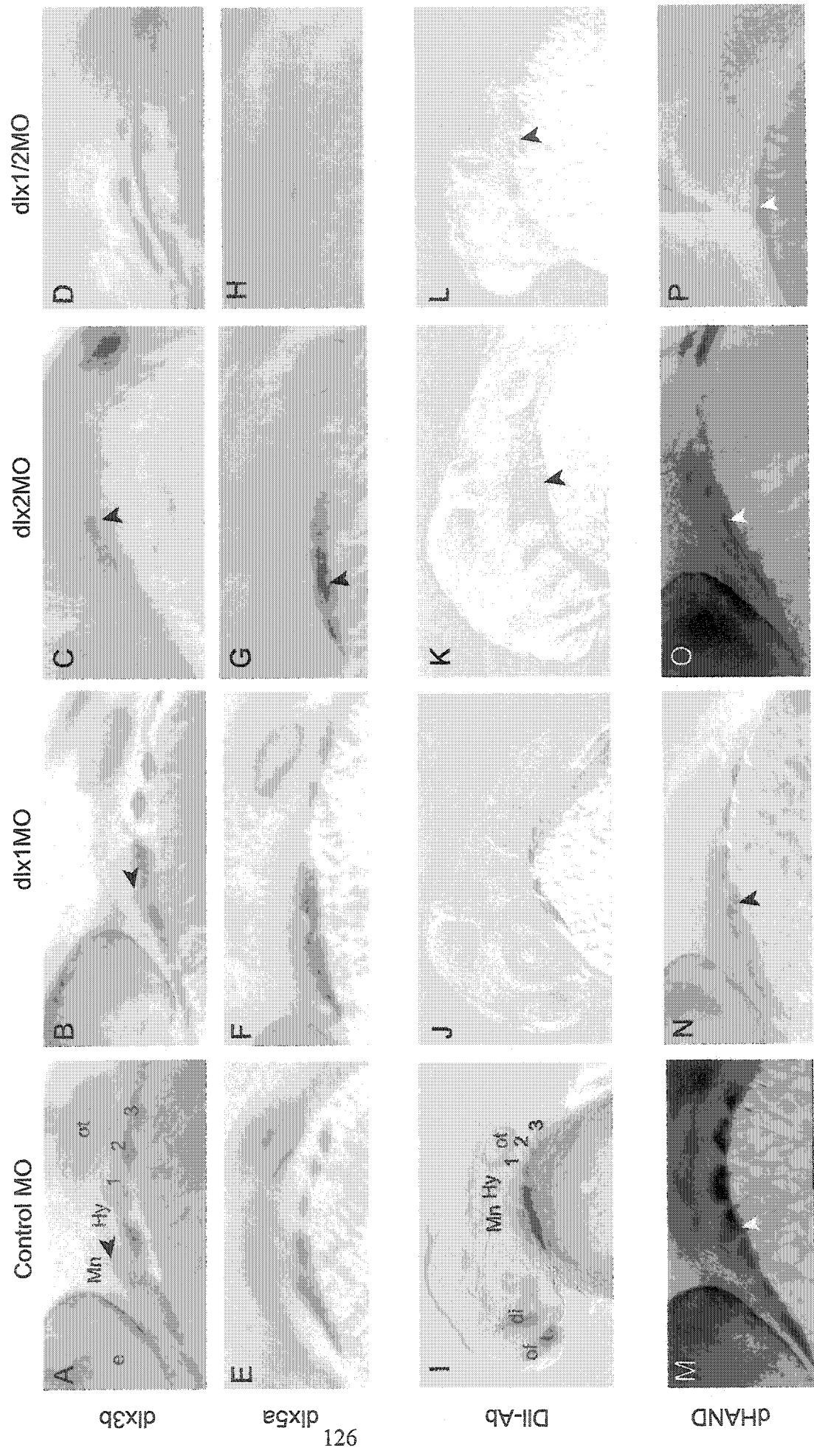


patterning defects of the dorsal aspects of the arches in zebrafish, we examined markers in *dlx1a*, *dlx2a* as well as *dlx1/2a* morphants at 34 hpf. In control MO-injected embryos, *dlx3b* transcripts were detected in the developing otic and olfactory sense organs, and in the pharyngeal arches (Fig. 3.5A). *dlx1a* morphants appear to have normal expression in the anterior mandibular and hyoid arches, as well as in the posterior gill arches, in the otic vesicles and in the olfactory organs. However, a mild shortening of the mandibular arch is apparent (arrowhead; Fig. 3.5B). As previously indicated for 24 hpf, *dlx2a* morphants at 34 hpf continue to have a loss of *dlx3b* expression in the gill arches, and a shortening of expression or tentatively interpreted as a reduction in the dorsal aspect of the mandibular and hyoid arches, but maintain expression in the ventral aspect of the anterior arches (arrowhead; Fig. 3.5C). A similar loss of expression is shown in *dlx1/2a* morphants (Fig. 3.5D). Control MO-injected embryos show normal *dlx5a* expression in the forebrain, arches and otic vesicle (Fig. 3.5E). *dlx1a* morphants reveal an apparent mild shortening of the *dlx5a* anterior arch expression domain, and a reduction of expression in the gill arches (Fig. 3.5F). *dlx2a* morphants show a loss of expression in the dorsal aspects of the first and second arches and complete loss of expression in the gill arches. Similar to *dlx3b*, however, the ventral aspects of the anterior arches maintain some *dlx5a* expression (arrowhead; Fig. 3.5G). *dlx2a* morphants further show a loss of *dlx5a* expression in the diencephalon, and a reduction in the telencephalon (not shown). *dlx1/2a* morphants have a loss of arch expression similar to *dlx2a* morphants, with additional absence of expression in the diencephalon (forebrain expression not shown; Fig. 3.5H). These results suggest that *dlx2a* functions in dorsal arch patterning of *dlx* arch and telencephalon expression. *dlx2a* and *dlx1/2a* morphants are more severely dysmorphic than *dlx1a* morphants, which correlates with the earlier expression of

dlx2a in the pharyngeal arches. We conclude that *dlx2a* is necessary for the expression of downstream *dlx* genes in the most dorsal (proximal) aspects of the first and second arches, and the gill arches. *dlx1a* appears to function in a lesser role at this particular period of development, possibly compensated by *dlx2a*.

Other family members, such as, *dlx6a* (formerly *dlx6*), *dlx4b* (*dlx7*), and *dlx4a* (*dlx8*) are also expressed in the pharyngeal arches. To examine if the reduction of *dlx1a* or *dlx2a* function resulted in loss of Dlx protein, we performed whole-mount immunostaining using a pan-Dlx/Dlx-antibody (Fig. 3.5I-L). In control embryos, the Dlx-Ab stained all the sites of Dlx activity, that include the telencephalon and diencephalon of the forebrain, the olfactory and otic vesicles, the pharyngeal arches, fin bud and the median fin fold (Fig. 3.5I, fins not shown). An increasing loss of Dlx immunoreactivity was observed in embryos that were injected with *dlx1*MO, *dlx2*MO and *dlx1/2*MO (Fig 3.5J-L). *dlx1a* morphants showed staining within the arches, as well as in the forebrain, and fin folds indistinguishable from control embryos (Fig. 3.5J, fin fold not shown). *dlx2*MO injected embryos revealed a decrease of the dorsal portion of arch immunoreactivity in the anterior arches (arrowhead), and a loss of staining in the gill arches, as well as a loss of staining in the diencephalon at 24 hpf (not shown) and later at 34 hpf (Fig. 3.5K). This result corroborates the previously mentioned reduction of *dlx5a* expression in the telencephalon and loss in the diencephalon. Reductions in Dlx-Ab staining were also observed in the olfactory placodes. Staining in the medial fin fold was present although reduced (not shown). *dlx1/2a* morphants exhibited loss of immunoreactivity in the diencephalon and telencephalon, olfactory placodes, and in the more posterior gill arches similar to that in the *dlx2a* morphants (Fig. 3.5L). The ventral

Figure 3.5. *dlx1a* and *dlx2a* pattern dorsal arch expression. Expression in 34 hpf embryos of *dlx3b* (A-D), *dlx5a* (E-H), Dlx immunostaining (I-L), and *dhand* expression (M-P) are compared in control MO injected embryos (A, E, I and M) to *dlx1*MO (B, F, J and N), *dlx2*MO (C, G, K and O) and *dlx1/2*MO co-injected embryos (D, H, L and P). Anterior is to the left; all panels are lateral views. di, diencephalon; e, eye; Hy, hyoid; Mn, mandible; of, olfactory placode; ot, otic placode; t, telencephalon; gill arches are numbered.



aspects of the anterior arches (arrowhead), as well the otic vesicle, however, still stained for the presence of Dlx protein. The continued presence of Dlx protein suggests that expression of family members, such as *dlx3/4b* is not adversely affected by the loss of *dlx1/2a* in the otic vesicle. Compared to *dlx1a* morphants, the loss of *dlx2a* has a greater impact on reduction of Dlx protein and dorsal expression of arch markers, suggesting its important role in influencing family member expression.

In addition to the *dlx* expression, *dhand* (*hand2*) was examined (Angelo *et al.*, 2000). *dhand* is a transcription factor downstream of endothelin-1 signaling and has been shown to be regulated by Dlx6 (Charite *et al.*, 2001). *dhand* transcripts are detected in the ventral portion of the pharyngeal arch mesenchyme, surrounding a mesodermally derived core that overlaps with *dlx2a* expression (Miller *et al.*, 2000). Compared to control embryos (Fig. 3.5M), *dlx1a* morphants showed an apparent mild reduction in the dorsal region of the expression domain (Fig. 3.5N). *dlx2a* morphants (Fig. 3.5O) and *dlx1/2a* morphants (Fig. 3.5P) showed losses of expression in the gill arches, and in dorsal aspects of the anterior arches, yet still retained ventral expression (arrowheads) in the mandible and hyoid arch.

The persistence of *dlx3b*, *-5a* transcripts, as well as Dll-Ab staining indicates that other factors are involved in positively influencing ventral expression. It is possible that *dlx* members such as *dlx5a* and *-3b* are influenced, similar to *dhand* expression, by *et-1* signaling (Miller *et al.*, 2000). *et-1* expression is observed in the arch mesodermal cores beginning at approximately 18 hpf (Miller *et al.*, 2000), a time that corresponds to arch neural crest migration, and just prior to subsequent *dlx* arch expression (Akimenko *et al.*, 1994). The mouse *Dlx6* mandible expression domain includes a ventral or most distal

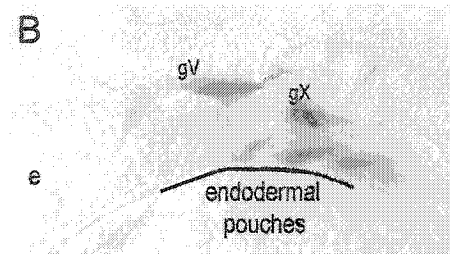
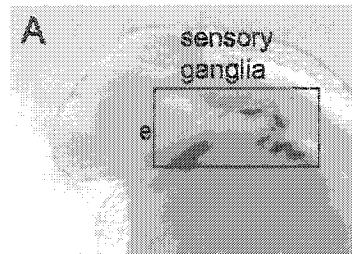
portion that is dependent on ET-1 signaling (Charite *et al.*, 2001). Similarly, zebrafish mutant for *et-1* (sucker) lose ventral *dlx3b* and *dlx2a* arch expression (Miller *et al.*, 2000). It is possible that the posterior midbrain neural crest that lack *dlx2a* expression contribute to the arches and are sufficient to maintain responsiveness to the endodermally derived signal. Therefore, *et-1* signaling is likely positively influencing anterior arch *dlx5a*, and *dlx3b* expression.

3.3.7 *dlx2a* is necessary for derivation of sensory ganglia

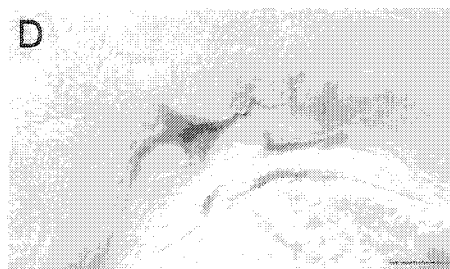
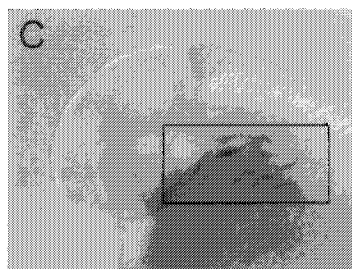
To further explore the effects of *dlx2a* and *dlx1a* loss of function, we examined sensory ganglia and endodermal pouch formation using the zn-5 antibody. Zn-5 recognizes DM-GRASP, an adhesion molecule on the surface of a subset of growing motoneurons, and early endodermal cell-types (Fashena and Westerfield, 1999). Compared to control embryos (Fig. 3.6A, B), *dlx1a* morphants showed only an apparently mild reduction in endodermal staining (Fig. 3.6C, D). Staining in the sensory ganglia appeared normal. However, in *dlx2a* morphants, zn-5 showed a decrease in immunoreactivity in the sensory ganglia and endodermal pouches (Fig. 3.6E, F). Similar results were observed for *dlx1/2a* morphants (Fig. 3.6G, H). Diminished staining of the ganglia in *dlx2*MO-injected embryos suggests neural crest cells are not differentiating into neurons and the endodermal pouches are not being properly patterned. Therefore, the loss of zn-5 staining in zebrafish suggests that *dlx2a* is necessary for either the maintenance or differentiation of the sensory ganglia.

Figure 3.6. Comparison of zn-5 staining in control and *dlx1a* and *dlx2a* and *dlx1/2a* morphants. Whole-mount immunohistochemical staining of 34 hpf embryos with zn-5 antibody. Wild-type embryos (A, B) were compared with *dlx1*MO (C, D), *dlx2*MO (E, F), and *dlx1/2*MO co-injected embryos (G, H). Anterior is left; all panels are lateral views. Box indicates magnified area (B, D, F and H). e, eye; gV, trigeminal ganglia; gX, vagus ganglia; ot, otic placode.

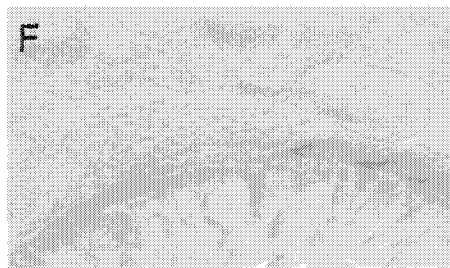
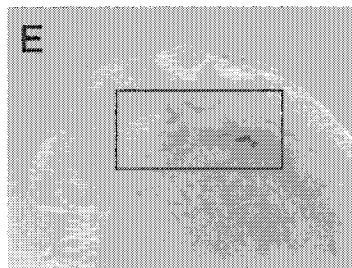
WT



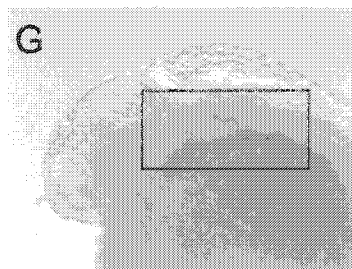
dlx1MO



dlx2MO



dlx1/2MO



3.4 Discussion

The aim of the present study was to determine the roles of the *dlx1a* and *dlx2a* genes in the embryogenesis of the zebrafish pharyngeal arches. It has been established that *Dlx1/2* linked paralogues share overlapping expression patterns in the pharyngeal arches that appear indistinguishable from each other. One exception is that *Dlx2* is expressed in the migrating cranial neural crest uncoupled from *Dlx1*. To further explore the role of the *Dlx2* in the migrating neural crest cells, and its paralogue *Dlx1* in pharyngeal arch patterning, we have investigated the zebrafish orthologues *dlx2a* and *dlx1a*, by performing targeted morpholino-mediated functional reduction experiments.

*Dlx1*MO and *-2*MO injected embryos exhibit a hammer-head like phenotype (Piotrowski *et al.*, 1996); reduced head length and the eyes are set anteriorly, with the mouth located ventrally. This facial malformation is likely the result of a failure of rostral head growth due to early perturbation of the developmental plan. Therefore, *dlx1a* and *-2a* participate in patterning the facial form.

3.4.1 The role of *dlx2a* in cranial neural crest specification and lineage determination

Loss of *dlx2a* function results in reduction of the neural crest markers *crestin*, *tfap2a* and *sox9a* within the arches, indicating a role in specifying the subset of cells destined for the pharyngeal arches. Increased hindbrain apoptosis was observed, suggesting that the rhombencephalic neural crest requires *dlx2a* for maintenance of identity and survival. Facial malformations are known to be a result of increased cell-death. Apoptosis of the neural crest cells is the underlying reason behind the autosomal-dominant human disorder

Treacher Collins syndrome, which adversely affects first and second arch morphology (Dixon *et al.*, 2000). Additionally, null mutants for the *tfap-2a* mouse ortholog (Schorle *et al.*, 1996; Zhang *et al.*, 1996; Brewer *et al.*, 2004), as well as the zebrafish *tfap2a* mutants (Knight *et al.*, 2003; Barrallo-Gimeno *et al.*, 2004), exhibit apoptosis in the pharyngeal arches. It has previously been suggested that cell differentiation and apoptosis are coupled events, and cells incapable of differentiating or that do not attain the correct stage of development at a particular place or time undergo programmed cell-death (Raff, 1992; Raff *et al.*, 1993). Therefore, after the epitheliomesenchymal transition stage, *tfap2a* and *dlx2a* may be participating in the same pathway for maintaining the arch cranial neural crest cells.

dlx2a morphants also showed a reduction in *sox9a* expression in the cranial neural crest. Previous work, characterizing the zebrafish *sox9a* mutant, *jellyfish* (*jef*), reveals a phenotype that lacks cartilage differentiation, including disruption of chondrocyte arrays and shaping of elements (Yan *et al.*, 2002). This phenotype is similar to our milder morphants, suggesting that *dlx2a* may modulate *sox9a* expression in the arches and consequently influence chondrogenesis through this interaction.

In all the morphants, *dlx3b* and *-5a* expression persisted in the ventrodorsal aspect of the anterior arches, but was diminished in the proximodorsal aspects of the anterior arches and the gill arches. Neural crest cells from both the mid- and hindbrain are thought to contribute to the mandibular arch (Langille and Hall, 1988; Schilling and Kimmel, 1994). *dlx2a* expression is restricted to the rhombencephalic neural crest (Ellies *et al.*, 1997a),

therefore, ventral *dlx* expression in the anterior arches may arise from the mesencephalic neural crest contribution.

Clonal analysis indicates that arch segments, and cell-types arise from specific locations. Segmental restrictions are coordinated in an anteroposterior manner in the rhombomeres, while lineage restrictions are observed in a dorsoventral coordinate manner within the premigratory neural crest cells. Generally, more laterally originating neural crest cells contribute to the neural fate. Medial cells, tend to form the cartilage and connective tissues (Schilling and Kimmel, 1994). As seen in the morphants, *dlx2a* influences rhombencephalon-derived neural crest cell survival. However, some neural crest cells do migrate, and contribute to the cartilage elements. Detailed fate maps have yet to characterize the relative contributions of the ectomesenchymal cells and their fates within the individual branchiomeres (Kimmel *et al.*, 2001). Therefore, it is difficult to interpret the mesencephalic cranial neural crest contribution to the anterior arches. Presumably, ventral cartilage elements of the mandible and hyoid are derived from the ventral aspects of the arches, while dorsal elements, the palatoquadrate and hyosymplectic, form from the dorsal specified cells. Supporting genetic evidence is revealed in the ventral arch mutants *sucker* (*suc*), *schmerle* (*she*) and *sturgeon* (*stu*). These mutants have smaller ventral arch cartilage elements fused with dorsal arch elements, and segment defects in the gill arches (Miller *et al.*, 2000; Kimmel *et al.*, 2001). It is conceivable that reduced cell numbers attempt to contribute to all tissues resulting in smaller elements. Consequently, reduced cell numbers cannot create the critical mass thought to be required for maturation of prechondrogenic condensations (reviewed in Hall and Miyake, 2000). Reduction in cartilage extracellular matrix is

manifested by reductions and loss of alcian blue staining. Here we propose that *dlx1a* and -*2a* are necessary for specifying aspects of the mandibular and hyoid arches as well as the gill arches.

Our *dlx2*MO injected embryos showed deficiencies in neuronal as well as cartilage tissues. Lineage analysis indicates that more laterally located neural crest cells differentiate into neuronal or Schwann cell fates, while more medially located neural crest takes on cartilage, pigment and connective tissue fates (Schilling and Kimmel, 1994; Yelick and Schilling, 2002). In chick, midbrain and cranial rhombomeres 1, 2, 3 neural crest cells contribute to the trigeminal ganglia formation (Lumsden *et al.*, 1991). In mouse, *Dlx2* is expressed in the trigeminal ganglia, and null mutants exhibit aberrant arborization of the mandibular branches of the sensory cells (Qiu *et al.*, 1995). In zebrafish *dlx2a* morphants, sensory ganglia are diminished or lost, indicating that the crestal cells have not differentiated. At 24 hpf, *dlx3b/4b* are expressed in the trigeminal ganglia, suggesting that *dlx2a* may influence their expression in the neurogenic cells.

Unlike *Dlx2*^{-/-} mouse mutants (Qiu *et al.*, 1995), no cartilage elements were completely abolished in zebrafish morphants but were instead smaller and malformed. This may be due to incomplete translational silencing or functional redundancy may also minimize the mutant phenotype. Therefore, we speculate that *dlx2a* is involved in specifying both the medial and lateral subsets of the hindbrain cranial neural crest destined for the arches, that are necessary in the differentiation of sensory ganglia and cartilage cell lineages. Pigmented melanocytes are another neural crest derivative, whose development

was delayed but not abolished in the *dlx2a* morphants (data not shown). Therefore, *dlx2a* may only be involved in some arch neural crest fate specification. Alternatively, the effects of the morpholino gene silencing may be limited, and therefore allow some delayed differentiation as development progresses and the antisense oligonucleotide is diluted.

dlx1a morphants showed a milder mutant phenotype compared to *dlx2*MO injected embryos suggesting partial compensation by *dlx2a* and other family members. It is likely that *dlx2a* functions in a more primary role than *dlx1a* in patterning the elements. Functional differences between *Dlx2* and *Dlx1* have previously been identified. Primary cultures of embryonic telencephalon require *Dlx2* for maturation of neurons to an intermediate differentiated phenotype. *Dlx1* is not able to substitute for *Dlx2*, indicating unique functions for each of the paired genes (Ding *et al.*, 1997). Therefore, qualitative, as well as heterochronic differences have evolved between linked members of this bigene cluster.

3.4.2 Arch dorsoventral patterning

The nested expression pattern of the *Dlx* genes in the arches correlate with their role in determining proximodistal identity. Mouse *Dlx2*^{-/-} and *Dlx1/2* null mutants exhibit dysmorphology of proximal (dorsal) features (Qiu *et al.*, 1995; Qiu *et al.*, 1997). We observed in zebrafish *dlx2a* and *dlx1/2a* morphants, a loss of *dlx3b* and *-5a* expression in the dorsal aspects of the anterior mandible and hyoid arches. Similar to the mouse *Dlx1* null mutants (Qiu *et al.*, 1997), the zebrafish *dlx1a* morphant phenotype was much less severely afflicted than *dlx2a* mutant phenotypes. Developmental perturbations in *dlx1a* morphants were not observed until after pharyngogenesis in zebrafish (Qiu *et al.*, 1997). Therefore, it

appears that *dlx1a* and *dlx2a* relative contributions to proximal-dorsal arch patterning are conserved, and function in an analogous manner to *Dlx1* and -2 in the mouse.

3.4.3 *The role of dlx2a and dlx1a in chondrogenesis*

The role of the *Dlx* genes during chondro- and skeletogenesis has been examined (Ferrari *et al.*, 1995; Depew *et al.*, 1999; Xu *et al.*, 2001; Ferrari and Kosher, 2002; Robledo *et al.*, 2002; Holleville *et al.*, 2003). In zebrafish, *dlx* expression has been correlated with chondrocyte condensation (Ellies *et al.*, 1997a). *Dlx1/2* null mice are lethal at birth, and are characterized by a phenotype that includes defects in the proximal skeletal elements of the cranial neural crest-derived bones of the first and second arches. Defects in the zebrafish palatoquadrate and hyosymplectic dorsal cartilages are reminiscent of the abnormal morphogenesis of proximal skeletal elements seen in mouse *Dlx1/2*^{-/-} mutants. Maxillary derivatives, such as the palatopterygoquadrate, are abnormal in the mutant mice while the ventral (distal) elements always formed (Qiu *et al.*, 1997), suggesting compensation by other *Dlx* family members. These results appear similar to the *dlx1/2a* morphants, suggesting a role in patterning cartilage elements in the anterior arches and the gill arch elements. Therefore, the role of *dlx1a* and *dlx2a* in proximodorsal patterning of the pharyngeal arches appears to be conserved in jawed vertebrates as divergent as zebrafish and mice.

3.4.4 *Cross-regulation of dlx genes*

Dlx family members are expressed, subsequent to migration of the arch neural crest cells, in overlapping patterns suggesting possible regulatory interactions (Ellies *et al.*, 1997b; Qiu *et al.*, 1997). *Dlx* genes have previously been implicated in auto- and cross-regulation of

family members (Liu *et al.*, 1997; Zerucha *et al.*, 1997; Zerucha and Ekker, 2000; Solomon and Fritz, 2002; Stuhmer *et al.*, 2002a). Cross-regulation has been demonstrated by Dlx induction of reporter constructs harbouring conserved *Dlx cis*-acting elements (Zerucha *et al.*, 2000), and ectopic expression in the forebrain (Stuhmer *et al.*, 2002a). Zebrafish *dlx3b* has been shown to regulate *dlx5a* and *dlx4b* expression in the arches and otic vesicles (Zerucha *et al.*, 1997; Solomon and Fritz, 2002). Corroborating genetic evidence for a cross-regulation is illustrated in *Dlx1/2^{-/-}* mice exhibiting a loss of Dlx5 forebrain immunoreactivity (Eisenstat *et al.*, 1999). Conserved enhancer elements capable of targetting reporter expression to sites in the arches and forebrain recapitulating endogenous *Dlx* expression patterns, contain putative Dlx homeodomain binding sites suggesting a mechanism of cross-regulation (Ghanem *et al.*, 2003; Sumiyama and Ruddle, 2003b; Park *et al.*, 2004).

In *dlx2*MO injected embryos, we observed reductions in *dlx5a* expression and Dll/Dlx-antibody staining in the diencephalon. *Dlx1/2*MO injected embryos revealed a loss of *dlx5a* expression in both the diencephalon and telencephalon. It appears that *dlx1a* and *dlx2a* cooperate in regulating *dlx* expression in the telencephalon. Corroborating MO injection experiments performed on a transgenic zebrafish expressing *gfp* targeted to the forebrain, through the *dlx6a* promoter and intergenic enhancer elements (Zerucha *et al.*, 2000), show similar loss of reporter gene expression in the forebrain of *dlx2a* and *dlx1/2a* morphants (data not shown). Our results suggest that *dlx5/6a* expression in the diencephalon is dependent on *dlx2a*, while *dlx1/2a* function is necessary for telencephalic expression. Interestingly, in *Dlx2^{-/-}* mice, no difference was observed in forebrain expression of *Dlx1* and

Dlx5 (Qiu *et al.*, 1995), suggesting some species-specific differences exist in regulating of the *Dlx* forebrain expression.

3.4.5 *The evolutionary role of dlx coordinate expression in jaw formation*

Dlx genes are involved in morphological innovations. One innovation particular to modern jawed vertebrates so far studied is the nested pattern of *Dlx* expression in the developing pharyngeal arches. A gnathostome characteristic, the regionalization of the jaw is thought to be a prerequisite for the formation of the jaws. Qiu and colleagues (1995) proposed that *Dlx2* null mutants are reminiscent of more primitive reptilian jaws, suggesting an atavistic morphological change had occurred. *Dlx5/6* null mutants reflect the symmetrical nature of the stomatognathic apparatus as seen in the fossil record of paleozoic fishes (Beverdam *et al.*, 2002). Present-day gnathostomes, thus far seem to have a single *Dlx* gene expressed in the migrating cranial neural crest, while the jawless lamprey purportedly has three out of its four *Dlx* genes expressed in the migrating cells (Neidert *et al.*, 2001). Lamprey *Dlx* arch expression is patterned in a non-nested overlapping manner in the mouth and arches (Neidert *et al.*, 2001). It is possible to speculate that reduction of *Dlx* expression in cranial neural crest cells, perhaps by loss of *cis*-acting regulatory elements, aided in the regionalization observed in present day jawed animals. Therefore, discrete changes in the genetic pathway can lead to morphological innovations. As more species are studied with different jaw morphology, it will be of interest to see how the role of *Dlx* genes in patterning is adapted and maintained.

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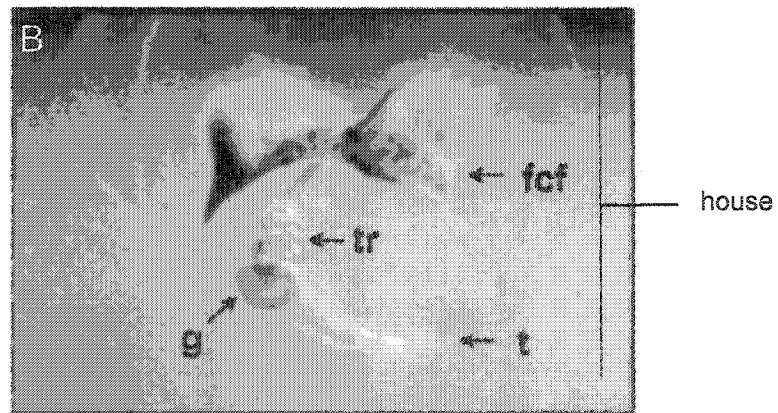
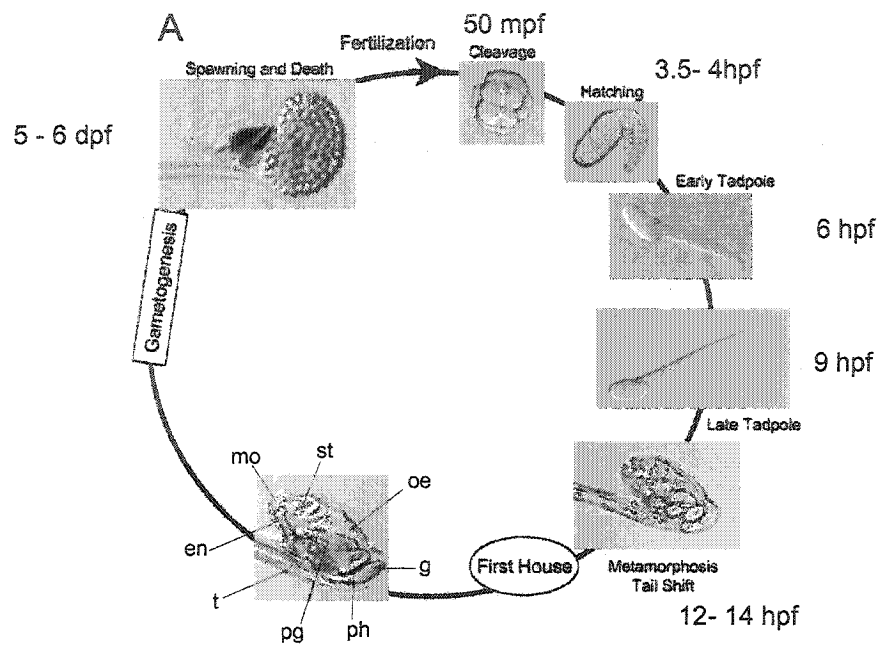
Preface

The following paper, “*OdiDll*, a unique *distal-less* homeobox gene of the tunicate *Oikopleura dioica*, and the origin of vertebrate *Dlx* bigene clusters,” uses a model organism that the reader may not be familiar with. Briefly, the animal, *Oikopleura dioica*, is a planktonic urochordate species distantly related to the more familiar ascidian, *Ciona intestinalis*. *O. dioica* is a member of the larvacean class of urochordates that differ from the other classes in that they retain their neotic form through to sexual maturity. Other urochordate larvae have a free-swimming stage and undergo a metamorphosis to become sessile filter-feeders.

Urochordata represents the most basal level of the chordata phylum. An important question of chordate evolution is whether cephalochordates and vertebrates evolved from a free-swimming or a sessile common ancestor have undergone paedomorphosis (form derived from the child). Molecular data suggests a monophyletic group for deuterostomes, and therefore a free living common ancestor (Wada and Satoh, 1994; Wada, 1998).

O. dioica has recently been advocated as a proxy organism for a protochordate model, because of its retention of a pelagic free-swimming form, as well as its remarkably small genome (~70 Mb), short 5 day life-cycle, translucent body, and high fecundity (Fig. 4.0) (Seo *et al.*, 2001). To date, very little molecular characterization has been performed on the organism.

Figure. 4.0. The *Oikopleura dioica* life cycle. (A) Development is temperature sensitive, therefore, times of stages are approximations. Fertilized embryos develop rapidly, undergoing gastrulation within the first 2.5 hpf, and hatch at tail bud stage at 6-7 hpf. 12-14 hpf, tail-shift stage orientates the head in a more orthogonal position aligning the mouth with the tail. Filter feeding is facilitated through the secretion of a net-like house from the epithelium. When clogged with particulate the house is abandoned to begin anew. Old houses contribute a large portion of the sea snow observed upon diving in costal waters. The animal increases rapidly in size mostly by cellular enlargement. Sexual maturity occurs on the 5-6th day, whereby, the head lethally ruptures and the gonads are released. (B) Adult *O. dioica* in house. en, endostyle; fcf, food concentrating filter; g, gonad; mo, mouth; oe, oesophagus; pg, pharyngeal gill slit; ph, pharynx; st, statolith; t, tail; trunk (Adapted from Thompson *et al.*, 2001).



4. *OdiDll*, a unique *distal-less* homeobox gene of the tunicate *Oikopleura dioica*, and the origin of vertebrate *Dlx* bigene clusters.

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Daniel Chourrout^{c**}

Abbreviated title: *Oikopleura distal-less*

Word Count: *at this time approx 5,200 words*

Key Words: *Dlx*, Urochordate, larvacean, urochordate, gene duplication, genome evolution, forebrain, branchial arches.

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Abstract

Distal-less (*Dll/Dlx*) related genes are homeodomain containing transcription factors involved in the patterning of a variety of different tissue types and are associated with many chordate and vertebrate evolutionary innovations. Vertebrates have at least six *Dlx* genes, generally arranged in convergently transcribed bigene clusters, with conserved enhancers often found in the intergenic region. Whereas only one *Dll* gene has been found in the cephalochordate amphioxus, three have been identified in the urochordate *Ciona intestinalis*, with two in a vertebrate-like bigene configuration. We present an extensive genome analysis that strongly suggests the existence of a single *Dll* gene in a larvacean urochordate, *Oikopleura dioica*. We suggest that none of the known chordate *Dll* genes shows a particular relationship to the vertebrate *Dlx* genes, based on the lack of sequence conservation outside the homeodomain, the lack of conserved enhancer sequences in the *Dll* loci of *Oikopleura/Ciona*/amphioxus, and the absence of conserved synteny. *OdiDll* is expressed principally in a variety of ectoderm-derived tissues, starting at the tailbud stage. Thus, *OdiDll* expression could be considered ancestral in chordates as it is similar to that of amphioxus *Dll* and to one of the three *Dll* genes of *Ciona*, *Dll-A*. Our findings tend to question the hypothesis that *Dll* bigene clusters were formed before the emergence of vertebrates, with a gene loss in the amphioxus lineage. Instead, a unique *Dll* gene may be typical of invertebrates, and the three genes of *Ciona* including the *DIIA/DIIB* bigene cluster may be the result of a lineage-specific amplification.

4.1 Introduction

The *Dll/Dlx*-related genes encode transcription factors that play an important role in the specification of proximodistal patterning. In a variety of jawed vertebrates, *Dlx* genes are expressed in the forebrain, cephalic neural crest, otic vesicle, olfactory placodes, pharyngeal arches, limb buds, and teeth. Gene-knockout experiments in mice indeed support a critical role for *Dlx* genes in the development of many of these characteristics (reviewed in Panganiban and Rubenstein; 2002). Examination of *Dlx* gene expression in urochordates should give an invaluable insight into vertebrate developmental evolution, because these sites of *Dlx* expression and function represent chordate or vertebrate innovations. In vertebrates, the *Dlx* gene family is comprised of at least six members that are generally arranged as convergently transcribed pairs separated by a short intergenic region (Stock *et al.*, 1996; Ellies *et al.*, 1997b). Phylogenetic analysis indicates that a single ancestral *Dll/Dlx* gene was duplicated to form a pair that in turn was replicated along with subsequent genomic duplications or large-scale duplications of chromosomal regions (Stock *et al.*, 1996). Although gene number augmented along with genome duplications at the origin of vertebrates, the formation of the first *Dll/Dlx* bigene cluster is thought to have happened earlier, based on the existence of one such bigene in the urochordate *Ciona intestinalis*, together with a third gene found in another region of the genome (Di Gregorio *et al.*, 1995; Caracciolo *et al.*, 2000; Harada *et al.*, 2001). All other invertebrates studied thus far, have single *Dll* genes. This includes deuterostomes such as the cephalochordate amphioxus and the enteropneust hemichordate *Ptychodera flava* for which genome data remain limited (Holland *et al.*, 1996; Williams and Holland, 1998; Harada *et al.*, 2001; Mittmann and Scholtz, 2001). Bigene clusters have not been found in any invertebrate protostome

including in the fully sequenced genomes of the fruitfly, of the mosquito, and of the nematode *Caenorhabditis elegans* (Cohen *et al.*, 1989; Panganiban *et al.*, 1994; Gonzalez-Crespo and Morata, 1996; Burglin and Aspöck, 1999; Beermann *et al.*, 2001; Lee *et al.*, 2001a; Schoppmeier and Damen, 2001; Beldade *et al.*, 2002).

Extensive work by us and others has revealed the presence of enhancer sequences in the intergenic regions of all three vertebrate bigene clusters (Zerucha *et al.*, 2000; Ghanem *et al.*, 2003; Sumiyama and Ruddle, 2003b), some of which are involved in the regulation of *Dlx* expression in the forebrain, a conserved characteristic of the *Dlx1/Dlx2* and *Dlx5/Dlx6*, but not of the *Dlx3/Dlx4* bigene clusters. Expression of *Dll* genes in amphioxus and the ascidian *Ciona intestinalis* has been detected in the anterior part of the central nervous system which may be homologous to the vertebrate forebrain. However, the conserved intergenic regulatory elements driving forebrain expression in vertebrates are not detectable in the *Ciona* bigene cluster. This may be because they were gained in the vertebrate lineage, or lost in the urochordate lineage. Alternatively, these elements may be present in both chordate groups but have strongly diverged after their separation.

To assess further the evolutionary conservation of the *Dll/Dlx* gene family we have searched for *distal-less*-related genes in the free-swimming planktonic filter feeder, *Oikopleura dioica*, belonging to the larvaceans, another class of urochordates. Larvaceans are thought to have diverged the earliest of the urochordate classes based on 18S ribosomal DNA phylogenetic analysis as well as their retention of a free-swimming form, which is considered to be an ancestral trait (Wada and Satoh, 1994; Wada, 1998; Swalla *et al.*, 2000). The present

report brings strong evidence for a single *Dll* gene in *O. dioica* (*OdiDll*). This gene is expressed in a variety of ectoderm-derived areas, which may correspond to the ancestral *Dll* expression pattern. The characterization of a single *Dll* gene in *Oikopleura* and in the amphioxus challenges the hypothesis that *Dll* bigene clusters appeared before the emergence of vertebrates.

4.2 Materials and Methods

4.2.1 *Oikopleura* culture and reproduction

O. dioica collected in the vicinity of Bergen, Norway were raised at 15°C in 6 liter beakers of constantly stirred seawater and maintained on a diet of algal strains, *Isochrysis galbana*, *Chaetoceros calcitrans*, and *Synechococcus* sp. supplemented after 3 dpf with *Rhodomonas* sp.,. The culture was maintained in the laboratory either by natural spawning or *in vitro* fertilization, after a 5-6 day long life cycle. Synchronized *in vitro* fertilizations were performed by rupturing and mixing the content of mature male and female gonads with subsequent development in watchglasses and then plastic beakers. Staged animals were fixed overnight at 4°C in 4% PFA, 0.1 M MOPS (pH 7.5), 500 mM NaCl for *in situ* hybridizations.

4.2.2 Isolation of the *OdiDll* gene

The *OdiDll* gene was identified during a whole genome shotgun analysis of clones from a plasmid library made in pUC19 (Seo *et al.*, 2001). The same gene was also found on BAC clones 005-B5, insert size: 125 kb and 007-G9, insert size: 170 kb. A cDNA *OdiDll* was cloned by RT-PCR from tailshift and 4 dpf RNA samples. The full-length nucleotide and predicted amino acid sequences will be available under EMBL/GenBank. Sequence analysis

and alignments were performed using the Genetics Computer Group (GCG) Wisconsin software package (Web Ver. 1.1).

4.2.3 Phylogenetic analysis

Aligned amino acid sequences of the *Dlx* homeodomain from the following genes were used to create trees with several methods including minimum evolution and neighbor-joining, maximum parsimony and maximum likelihood analysis in the PAUP program (version 4.0b8): *Amphioxus* *AmphiDll* (U47058) (Holland *et al.*, 1996); *C. intestinalis* *Dll-A* (AJ278696), *Dll-B* (AJ278697), *Dll-C* (AJ278698) (Di Gregorio *et al.*, 1995; Caracciolo *et al.*, 2000); *Drosophila* *Dll* (NM 166689); (Cohen *et al.*, 1989); mouse *Dlx1* (NM 010053), *Dlx2* (NM 010054), *Msx1* (NM 010835) (Holland, 1991); *Ptychodera flava* (AB028221) (Harada *et al.*, 2001); zebrafish *Dlx1a* (NM 13105) (Stock *et al.*, 1996). Mouse *Brx1* was used as an outgroup to root the trees.

4.2.4 In situ hybridization

The *OdiDll* cDNA, cloned in pGEM-T Easy (Promega) was used as template to prepare sense and antisense DIG-labeled RNA probes approximately 2.5 kb in length (T7, SP6, RNA polymerases, Roche) for whole-mount *in situ* hybridizations. A whole-mount *in situ* protocol was adapted from Spada *et al.* (2001) with the following modifications: embryos were fixed overnight in 4% paraformaldehyde in 0.1M MOPS, pH 7.5, and 0.5M NaCl at 4°C, transferred to 1M MOPS, 0.5M NaCl, washed in Tris/HCl 0.05M pH 8, treated for 1 minute with proteinase K 20 µg/ml in Tris/HCl 0.05M pH 8 at 37°C, followed by incubation in MOPS 1M, NaCl 0.5M at room temperature for 20 minutes. Embryos were prehybridized

for 1 hour and hybridized in 50% deionized formamide, 5XSSC 0.1% Triton X-100, 1% blocking reagent at 60°C overnight. Following the washing procedure, the hybridized embryos were treated with 1% blocking reagent (Roche) and 1% 0.45 µm filtered lamb sera (Invitrogen/Gibco) in PBS for 1 hour at room temperature. They were then incubated for another hour at room temperature in alkaline-phosphatase coupled anti-digoxigenin Fab fragments (Roche) in PBS 1X, Triton X-100 0.1%, before the standard signal detection. Embryos hybridized with the sense probe showed no staining.

4.3 Results

4.3.1 Identification and molecular characterization of a single *OdiDll* gene

To search for *Dll* genes in *Oikopleura*, we examined an extensive shotgun dataset containing 44,797 contigs and covering 40-98 megabases of genome sequence. This represents a substantial part of the total genome size estimated to be 50-72 Mb by several methods (Seo *et al.*, 2001). Alignments of the shotgun dataset with sequences of multiple randomly-cloned genes, as well as with 1155 non-redundant EST sequences, confirmed that at least two-thirds of the genome were covered (D.C., unpublished). A fairly dense and even distribution of the shotgun reads across the genome was also revealed by alignments with the sequences of nine distinct BAC inserts.

In this shotgun dataset, BLASTX revealed the presence of numerous candidate homeobox sequences, among which only one showed a particular similarity with vertebrate and invertebrate *Dll/Dlx* genes. This sequence, which was part of a 1.5 kb plasmid insert produced after sperm DNA sonication, was used to isolate cDNAs from late tadpole stage

Figure 4.1. DNA and encoded amino acid sequence and genomic structure of the *OdiDll* gene. (A) Sequence of an *OdiDll* cDNA. Deduced amino acid sequence. Start codon is bolded. The homeodomain is boxed. A star indicates the stop codon. The polyA site is underlined. Vertical lines in the nucleotide sequence demarcate position of intron splice sites. (B) Alignment of homeodomain sequences from *O. dioica*, *OdiDll*; *C. intestinalis* *Dll-A*, *Dll-B*, *Dll-C* (Caracciolo *et al.*, 2000); hemichordate *Ptychodera flava* (Harada *et al.*, 2001); *Drosophila*, *Dll* (Cohen *et al.*, 1989); amphioxus, *AmphiDll* (Holland *et al.*, 1996); zebrafish *dlx1a* (Stock *et al.*, 1996); and mouse *Dlx1* (Price *et al.*, 1991).

A

1 CTGATAGAGAATGGTAGGTAGCACCCACCAGCCGCATATAAACCCATAATTTACACCCCATATAATTTACACCAATTTTTCGCTGATTGTA
91 TCAGCCCCCTCCCTACTAATAACCCCTTAACCTCTGGAGAAGAGAAGCGCCCGCTCCTAAGAGATGTGTGTAAATTATGGTTGTTTCAGCTT
M V V Q L
181 GGTTCAAACGAAACAGCTGGAATGAGCTACGGTTACACTGGTTACCCAGACACGGCTTATGGTGTGCATCGAGAGGAAGCAACTACTAC
G S N E T A G M S Y G Y T G Y P D T A Y G A A S R G S N Y Y
271 TCCGGAGTCTACAATAATTTCTTCTCTCTGATCTCTGCTCTAGCCTTATCAATTCCTCGCCGACAGGAGGTCTCGCAGCTTCGTCGCAG
S G V Y N N S S S S Y P G S S L I N S S P T G G L A A S S Q
361 TATGAGCTTTTCGCGCAGTCTGGATACCCACCACTCGGATTCGAGACAATCCTTTCCGCTGTCTACGAGCTCGAGGGGGAAGAAGAAA
Y A A F A O S G Y P P L G F A D N P F G L L P D S K G K K K
451 AAGATGCGAAAGCTCGGACGATTTACAGTTCTGCTTCAGCTACAGGAACCAACAAACGCTTCAATCAAACTCAATATCTGGCAATTACCA
K M R K P R T I Y S S L Q L Q E L N K R F N Q T Q Y L A L P
541 GAACGGGCCGAACTTGTCTGCTTGGCTTCAGCAGACTCAGTCAAGATTTGGTTCCAAAACAGACGAAGCAAATACAAGAAGATC
E R A E L A A S L G L T O T Q V K I W F Q N R R S K Y K K I
631 ATCAAACAGGAGGCGTTTCTGTGAAATCGAAAACGCGGTGGAAGTCCAGCTGGATCGGTCAGGAGGAGCTAATGCTGAGGAC
M K O G G V S V K I E N D G G S P A G S G D E A G A N G E D
721 GATGAGGAAAACAGTCCGAACACTTTCTCCAAACGACTCTTAATCAATCAACGGAATGCGACGCTCGCCAACTGCTG
D E E N Q S E H S S P N A S S I L N Q S N G M M P R S P S S
811 ATTCGCGGTCTAATCTTGGATCTAATCTCCCTTCGATGCGCTTCTGCTCGCCACAGCTGTCTTCCGCTTTGAGCGCTTCCACTTCC
I P G S N L G S N L P S M P S G S P T A V L P G L T A S T S
901 GCCCACTATCCCGTCTGGACCAAGCCAGCTGCTGCAAGCTCAGCTCGAAGCAGCAGCAAGCATCGCAGCAGCAAGTGCGGTA
A H S S R L D Q A T L L H Q A Q L E A A A S I A A A S A G V
991 CTCGCAACCGGATCCAGTTCGACCGTGCAGTTCAGAAAGCAAGCTCTTCGCGCCAGCAGGATACTACTCCAGTCTACAGTCGACCGCG
L A N G I Q S H G A V P E A S S S A S T D T T P V Y T S T A
1081 CTTTCTACAGCTCCCAACGCTCACTGGCAGCTCACTACCGAGCGGACTACCAATGGCCTGACAGACTCAGCAATCGACTCG
P T S L S P T L T G S L T H T D S G L T N G L T D S A I D S
1171 AAGTCGTTTATCTCGACCTCGGCTCGCTGAATCCTTCTAATCTCCTTCACAGTTCGTACTCGTCTGACTTCTCCACACAGCTGGTTTG
K S F I S T S A A L N P S N L L H S S Y S S D F S T H A G L
1261 CCGACTAGTTTGTCTGGCAGCTGGGTGGCCCCAGCAAGCTTATTACAAACATTAAACCAATCAGCTAAATATGTATCTACATAAAAC
P T S L S W A A G W P Q Q A Y Y K H *
1351 ATCCACCACGACATCAAGCACACACGCAATGAAAATGCAATTGTCTAGTGAATTATCTAGAATCTGTATTTCTACACACTCAATTTGC
1441 TCAACTCGCTGTACATACCTGAAAACACATTTTTTCTTCTGTTTATTAAACTCCGGTAAACTCAAAAAACGTCGAGAAAAATAAA
1531 CGGGCACTCAGGTCGATGGTTCTTTAACTTGCCCGGACCAATGGAATGGAAAGATGCAATGAAACCATCGTCGAGTGGAATGCTTGC
1621 CTCCTTCCGATGATTGCGTGGATGGTGGAGACTTCTGCCAAATGACCGATGAAGATATTGCTCAGAAGTGTGAACGCTATCGAAGATT
1711 GTTGCAAAACATGCACAGACTTTAGACTTAAGCTCGAAGAAGCCATCAAGAAACAGGCTGAGGAAACAACTGCAACTGCCGATCCTCCAG
1801 CAGAAAAAGACATCCAGAATGACCCGACAGAAGACACAGACGAAACGGAGTCAGTTGAAGAAGAACTGATCCCGAGACTGATCCAAGTG
1891 CTGAGCCATCCCCAGAGCCTGAGACAAGCTCAACGAGACTGAGCCAACTGAAGAAAAGGTTGACCCCTGAACCCGAAACGAGGTTGGAT
1981 CTGCAAGTGAGCTAGTTCTTGGTTTCGGACTTATTTTAATTTCACTTTTTCGCTGTAATATTGAAATTATACAAACCTCTTTTATTT
2071 CTGTCAAAAAAGTACAATCTGAAGTATCAATCCGCCGCTCTTTTGTACTGCACCTTTAAACAACACTGCCCTAAGCCACTCAGTACAAA
2161 TAACACAATATATATCTTCTTCCACTAATCTATTGAATAATTCTTAATAAACTTCAATAAAATGCGAAAAAATAAAAAA

B

<i>O. dioica</i> <i>Odid1l</i>	MRKPRTIYSSLQQLQELNKRFNQTOYLLALPERAELAASLGLTQTQVKIWFQNNRRSKYKKIM
<i>C. intestinalis</i> <i>Dll-A</i>	TRKPRTIYTSYQLQQLVRRFQRTQYLLALPERAELAASLGLTQTQVKIWFQNNRRSNYKKLL
<i>C. intestinalis</i> <i>Dll-B</i>	KRNPRTVYSNYQLQELHMYFKKVQYLLALPERARLAAALGLTQTQVKVWFQNNRRSKIKKLL
<i>C. intestinalis</i> <i>Dll-C</i>	MRKPRTIYSSLQQLQALNRRFQQTQYLLALPERAELAATLGLTQTQVKIWFQNNRRSKCKKLM
<i>P. flava</i> <i>Pf-dlx</i>	MRKPRTIYSSLQQLQALNRRFQRTQYLLALPERAELAASLGLTQTQVKIWFQNNRRSKYKKVL
<i>Drosophila</i> <i>Dll</i>	MRKPRTIYSSLQQLQALNRRFQRTQYLLALPERAELAASLGLTQTQVKIWFQNNRRSKYKKMM
<i>Amphioxus</i> <i>Amphid1l</i>	MRKPRTIYTSFQLQQLNRRFQRTQYLLALPERAELAAQLGLTQTQVKIWFQNNRRSKYKKLM
<i>Zebrafish</i> <i>dlx1a</i>	IRKPRTIYSSLQQLQALNRRFQQTQYLLALPERAELAASLGLTQTQVKIWFQNNRRSKFKKLM
<i>Mouse</i> <i>Dlx1</i>	IRKPRTIYSSLQQLQALNRRFQQTQYLLALPERAELAASLGLTQTQVKIWFQNNRRSKFKKLM

and 4 dpf libraries, using RT-PCR followed by 5' and 3' RACE. The 4 dpf cDNA obtained by RACE predicts a polypeptide of 383 amino acids (Fig. 4.1A). However, a second cDNA, isolated from the young tadpole library predicted another *OdiDll* protein of 371 amino acids expressed from the same gene, suggesting that distinct juvenile and adult expression products may exist as a result of alternative splicing (not shown). A third possible transcript which would encode for a protein of 386 amino acids (not shown) was also predicted based on possible alternative start site and splicing events. The lack of other *Dll*-related members in this large shotgun dataset was itself a strong indication for the existence of a single gene in *Oikopleura dioica*. We also performed a systematic cloning of homeobox genes and cDNAs by PCR with degenerate primers from the first and third helices and obtained a variety of products (not shown). Only one *Distal-less*-related gene was present in the collection and proved to be the same gene *OdiDll*.

Comparative analysis of the predicted homeodomain sequence from *OdiDll* showed a high percentage of identity with the homeodomains of mouse *Dlx1* (87%), zebrafish *dlx1a* (89%), *Drosophila Dll* (90%), the hemichordate *Ptychodera flava Dll* (acorn worm; 89%), and *C. intestinalis Dll* proteins (*Dll-A*, 81%; *Dll-B*, 73%; *Dll-C*, 89%), suggesting that *OdiDll* is a *Oikopleura dioica Dll*-related gene (Fig 4.1B). The predicted amino acid sequence outside the homeodomain showed no significant similarity to known *distal-less* proteins from invertebrates or vertebrates.

The vertebrate *Dlx* genes have been classified into six orthologous groups (*Dlx1-6*), regrouped into two clades, *Dlx1, 4, 6* and *Dlx2, 3, 5*, based on sequence similarities (Stock *et*

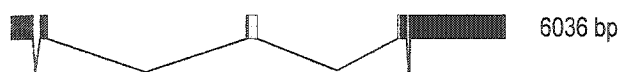
al., 1996). Each vertebrate *Dlx* bigene cluster contains one gene from each clade. Alignments of homeodomains, alone, are generally not sufficient to infer phylogenetic relationships with a good statistical basis, and our own analyses of vertebrate and invertebrate *Dll/Dlx* homeodomains, including *OdiDll*, with three methods (minimum evolution including neighbor-joining, maximum likelihood and parsimony) indeed produced poorly resolved trees. Both vertebrate clades were generally recognized, but the respective position of invertebrate genes was variable and statistically uncertain in every case (data not shown). There was, overall, no clear tendency that would place *OdiDll*, nor any of the other invertebrate *Dll* homeodomain, next to one of the two vertebrate clades.

4.3.2 Genomic organization and environment of *OdiDll*

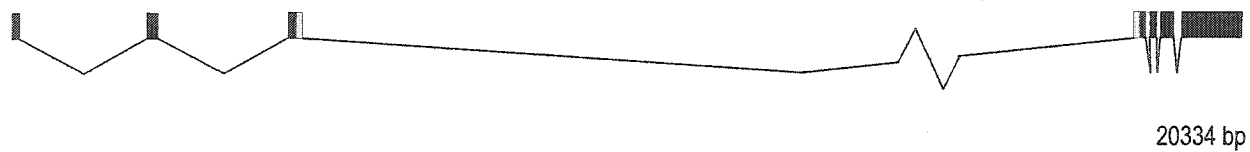
A BAC library comprising 9,216 clones was screened using a cDNA probe. This library covers the genome 18 times, and indeed, a total of 20 positive hybridization signals were unambiguously counted. Three positive clones were confirmed by PCR and one of them (B5-5) has been sequenced for *Dll* containing region by primer walking on subclones. The sequence of the B5-5 clone showed the *OdiDll* gene is organized into 5 exons with the predicted homeodomain sequence spanning the third and fourth exons (Fig. 4.2). The highly conserved splice site dividing the homeodomain, between exons three and four is similar to the intron-exon boundary found in *Drosophila*, *C. intestinalis*, and all vertebrate *Dll/Dlx* homologues (Vachon *et al.*, 1992; Di Gregorio *et al.*, 1995; McGuinness *et al.*, 1996; Ellies *et al.*, 1997a). The other intron-exon boundaries of *OdiDll* do not show any particular similarities to those of the other *Dll* genes from invertebrates, chordates, or vertebrates, as most frequently observed for *Oikopleura* genes in general (Edvardsen *et al.*, in press). The

Figure 4.2. Divergent exon-intron organization of *Dll/Dlx* genes. Schematic diagram of exon size comparison and number between *Dll/Dlx* genes of *O. dioica*, *C. intestinalis*, *Drosophila* and zebrafish. All characterized *Dlx* genes of vertebrates have an exon-intron organization similar to that of zebrafish *dlx1a*. Black boxes represent exons with the homeodomain outlined.

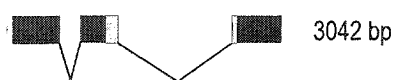
Odidi



Drosophila Dll



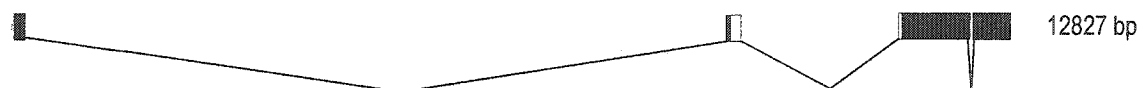
Ciona Dll-A



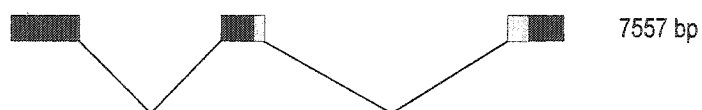
Ciona Dll-B



Ciona Dll-C



dlx1a



introns of *OdiDll* range in size from 98 bp to 2283 bp. Thus some of the introns of *OdiDll* are exceptionally large compared to most introns in *Oikopleura*, which do not exceed 50 base pairs (Seo *et al.*, 2001). We fully sequenced and annotated 53 kb of genomic DNA surrounding *OdiDll* (Fig. 4.3), using both BLASTX and GENSCAN, which when combined have been shown to provide reliable predictions. The sequence confirmed the absence of other *Distal-less*-related genes around *OdiDll*. Not less than five and ten genes were predicted upstream and downstream of *OdiDll*, respectively, and six of them showed very strong matches with known coding sequences in public databases (e-value < 10^{-34}). Candidate orthologs of these six genes were identified and mapped in the finished genomes of *C. elegans*, *D. melanogaster*, *C. intestinalis* and *H. sapiens* using Locuslink (<http://www.ncbi.nlm.nih.gov/LocusLink/>) and TBLASTN on the *Ciona* genome (<http://www.jgi.doe.gov/ciona>), but in no case these were located in the vicinity of *Dll/Dlx* genes (data not shown). Also, the *OdiDll* locus was not in the neighborhood of *Oikopleura Hox* genes (H.C.S. *et al.*, unpublished), in contrast to vertebrate *Dlx* genes that are linked to *Hox* clusters (Stock *et al.*, 1996; Amores *et al.*, 1998).

A remarkable feature of vertebrate *Dlx* bigene clusters is the exceptionally high-level conservation of intergenic sequences between species as distant as fish and mammals (Zerucha *et al.*, 2000; Ghanem *et al.*, 2003; Sumiyama and Ruddle, 2003b). A more limited sequence conservation exists between the 5'-flanking regions of orthologous *Dlx* genes. We retrieved non-coding sequences of *Dll/Dlx* genes, including 5kb of 5'-flanking regions for all genes, the entire intergenic region of bigenes, 5 kb of the 3'-flanking region of each "monogene", as well as the introns of all genes, from mouse/human, *Ciona intestinalis* and

Figure 4.3. The *OdiDll* genomic environment. A 52,924 bp contiguous sequence including the *O. dioica Dll* gene has been annotated using the NIX software package (<http://www.hgmp.mrc.ac.uk>) and mainly BLASTX and GENSCAN. Exons showing BLAST similarity with known genes are colored in red. Exons predicted by other tools are in blue. Exons identified by alignment with cDNA or EST sequence are in black. Genes numbered from 1 to 15 are: (1) GENSCAN prediction, (2) Integrin alpha 3, (3) related to Homo sapiens hypothetical protein Q8NDM7, (4) fragment, weakly related to Regulator of G-protein signaling 10, (5) *Distal-less* aligning with the full-length cDNA, (6) DNA repair protein RAD51, (7) GENSCAN prediction, (8) Peptidyl-prolyl *cis-trans* isomerase, (9) GENSCAN prediction, (10) GENSCAN prediction, (11) Ras-related protein RAP1, (12) Calcium transporter 2 aligning with one EST, (13) WD40 repeats containing protein (intron-exon organization is unknown, region containing eight WD-repeats detected by SMART is shown), (14) Polyadenylate binding protein aligning with one EST, (15) weakly related to EMSY protein.

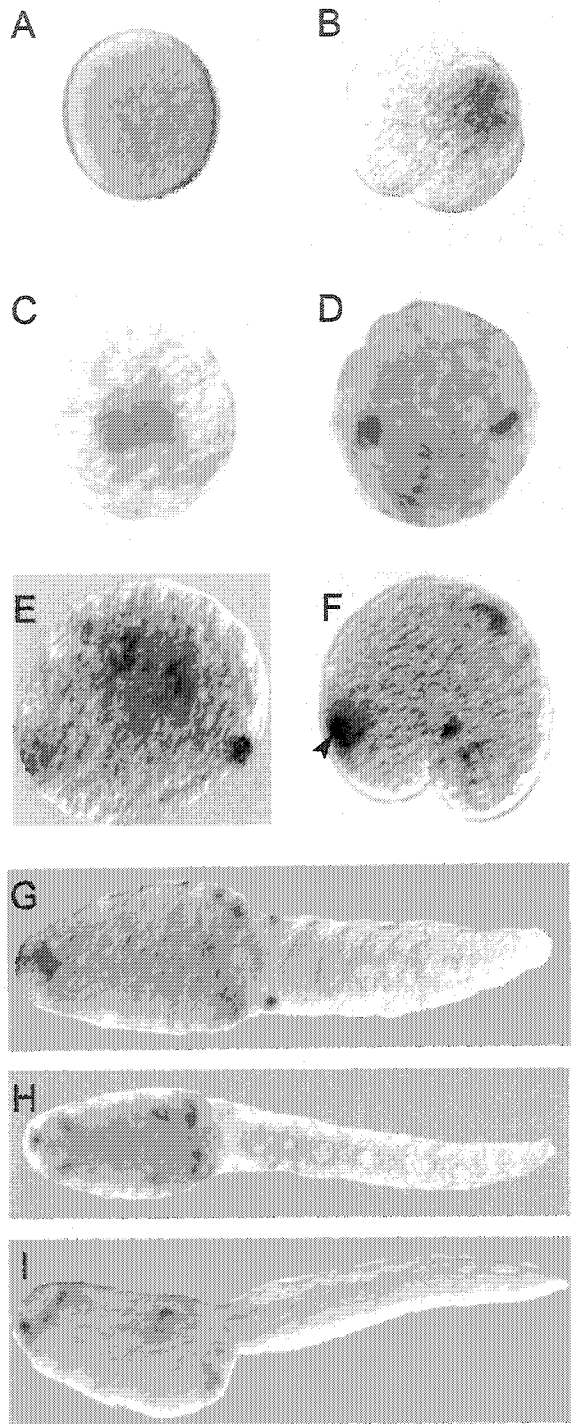


Oikopleura dioica. Attempts to align all these sequences two by two using either BLASTN or PipMaker (Schwartz *et al.*, 2000) confirmed the conservation already known between human and mouse non-coding sequences in orthologous *Dlx* genes, but failed to reveal any sequence similarities between sequences of genes from *Oikopleura* and other species, including *Ciona*. Interestingly, well conserved motifs of 50-200 bp were found in the promoters and introns of all three *Ciona Dll* genes, and were also present in the downstream region of *CiDlIC* (not shown). However, these motifs proved to be highly repeated in the entire *Ciona* genome and are therefore not specific to the *Dll* loci.

4.3.3 *OdiDll* expression during *Oikopleura dioica* development

A series of embryonic and larval stages from unfertilized egg to 12 hours post-fertilization (hpf) were examined for *OdiDll* expression. No expression of *OdiDll* transcripts was observed in unfertilized embryos (Fig. 4.4A). Embryos started showing hybridization signals after several cleavages, first in one spot which seemed to be a single blastomere (Fig. 4.4B), and then soon after in two spots which may be its two daughter cells (Fig. 4.4C). These two spots then moved laterally apart (Fig. 4.4D), and additional signals appeared in the embryo (Fig. 4.4E). From the tailbud stage onwards, the embryo could be easily oriented due to the clear tail-trunk division, and seven main signals could be counted (Fig. 4.4F). The most intense one was located dorsally in the anterior region of the trunk, just beneath the epidermal layer (arrowhead). The other six signals were located on the surface of the posterior part of the trunk, in three locations with clear left-right symmetry. This pattern remained unchanged after hatching (Fig. 4.4G). During organogenesis, the anterior signal

Figure 4.4. Spatiotemporal expression pattern of the *OdiDll* gene. Localization of *OdiDll* expression by whole-mount *in situ* hybridizations with an antisense *OdiDll* riboprobe. (A) Unfertilized egg (B) early cleavage stage, (C) blastula stage, (D) early gastrulation, (E) late gastrulation, (F) lateral view of tailbud embryo, anterior to the left, arrowhead indicates site of most intense staining, (G) dorsal view hatching stage, anterior to the left, (H) dorsal view of early tadpole stage, anterior to the left (I) lateral view of early tadpole stage, anterior to the left.



gradually differentiated into a more complex pattern including a ring-shaped set of multiple cells surrounding the developing mouth (Fig. 4.4H, I).

4.4 Discussion

The presence of three *Dll* genes in *Ciona intestinalis*, another urochordate, and, particularly, the linkage of two of them in convergent orientation has led us to postulate that *Dll* bigene clusters predated the emergence of vertebrates. This observation was somewhat surprising since an earlier study had revealed a single *Dll* gene in the cephalochordate amphioxus (Holland *et al.*, 1996), although conclusions were drawn uniquely from Southern hybridizations to genomic DNA. Since cephalochordates are considered to be the last chordate group having diverged from vertebrates, a plausible explanation was that one or several *Dll* genes had been lost in the amphioxus lineage. This assumption is challenged by the present report, which brings arguments for the presence of a single *Distal-less*-related gene in *Oikopleura dioica*. First, the genome shotgun dataset examined here contains a single *Dll* gene. Second, our efforts to clone homeobox genes and cDNAs provided sequences of a variety of genes, among which *OdiDll* was the only *Distal-less*-related product. Finally, the full sequencing of 53 kilobases around the *OdiDll* gene did not reveal the presence of a second *Dll*-related gene.

To reconcile these observations with the time of appearance of vertebrate-like *Dll* bigene clusters, one would have to postulate losses of *Dll* genes in cephalochordates and in at least some of the urochordate lineages. This hypothesis appears a little parsimonious, and

apart from the linkage between two *Dll* genes in *Ciona*, it does not receive support from other genomic information (synteny conservation, conservation of non-coding sequences) or from phylogenetic analysis.

Instead, the possibility that *Dll* genes were independently amplified in the *Ciona* lineage is given more strength by the present study. In the absence of whole genome duplication, tandem duplication is a prevalent mechanism for gene amplification, and a local chromosome inversion can have coincidentally produced a vertebrate-like bigene cluster in the *Ciona* genome. Interestingly, we found a number of repeats around the three *Ciona Dll* genes, which could either result from such duplications and/or have mediated a local inversion. Another line of evidence, albeit indirect, for the independent formation of a bigene cluster in *Ciona* is the absence of overlap of expression patterns of the two linked genes, *Dll-A* and *Dll-B* (Caracciolo *et al.*, 2000). This contrasts markedly with the situation in vertebrates where the expression of the two *Dlx* genes from a bigene cluster overlaps extensively (Ellies *et al.*, 1997b; Quint *et al.*, 2000).

One may argue that forces driving the exceptional genome compaction in *Oikopleura* (Seo *et al.*, 2001) could have induced the loss of many genes, but this does not seem to be the case: we have indeed found quite a few situations where the number of homeobox genes from a given family exceeded the expectations for an invertebrate. Examples include the occurrence of two *Otx* genes, at least three *Pax3/7* genes and at least five *Six* genes (D.C. unpublished observations). Duplicated homeobox genes, with or without a tandem arrangement, have also been found in a variety of invertebrates, including *Emx* and *Evx*

genes in amphioxus (Minguillon *et al.*, 2002). The latter study showed that one of the duplicates displays the putative ancestral expression, whereas the other duplicate has a divergent, and most likely derived expression pattern. Our observations of duplicated gene expression in *Oikopleura* (*Otx* and *Pax3/7*, unpublished) are in full accordance with this scheme. Interestingly, the comparison of *Dll* gene expression patterns in invertebrate chordates also gives some support for the lineage-specific duplication of *Ciona* genes, even though their interpretation remains imprecise due to the lack of co-localization with other markers. In amphioxus, the expression of the single *Dll* gene occurs exclusively in ectoderm-derived tissues. These include: 1) extended regions of the epidermis, including a population of cells possibly related to the vertebrate neural crest, 2) defined areas of the nervous system such as the anterior part of the cerebral vesicle (a candidate homolog of the vertebrate diencephalon), 3) putative photoreceptive cells outside the brain (Holland *et al.*, 1996). While the mesodermal expression of some vertebrate *Dlx* genes seems to be a vertebrate acquisition, their ectodermal expression seems fairly well “summed up” in the *AmphiDll* expression pattern, which may therefore represent an ancestral chordate pattern. In the *Oikopleura* tadpole, *OdiDll* expression seems confined to ectodermal tissues, in several superficial spots on the trunk, as well as in an intensely marked area placed more anteriorly beneath the epidermis. This latter signal gradually evolves into a group of putative sensory cells that surround the mouth. A very similar anterior signal, identified as the anterior-most region of the neurogenic ectoderm, is also found in the expression pattern of *Ciona Dll-A*, together with additional signals on the trunk surface (Caracciolo *et al.*, 2000). In contrast, the other two genes of *Ciona* have distinct expression patterns which are

not reminiscent of *Dlx* expression in vertebrates. This divergent expression may be typical of a lineage-specific amplification.

In conclusion, the finding of a single *Dll* gene in *Oikopleura* and the presence of a single gene in amphioxus, both of which are expressed in ectoderm-derived tissues, including the presumptive anterior CNS, leads us to question the hypothesis according to which *Distal-less* bigene clusters similar to those of vertebrates appeared in invertebrate chordates. The *Dll* gene complement of *Ciona intestinalis* may instead be the result of a lineage-specific amplification.

Acknowledgments

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5. Conclusions

The main focus of this thesis was the investigation of the *Distal-less/Dlx* genes in the formation of the pharyngeal arches. The pharyngeal arches provide an interesting model to study the conserved *Dlx* genes as they show differential regulation and different patterns of expression in the arches. Three aspects of the *Dlx* genes were studied. First, how the *Dlx* genes, organized as bigene clusters, are coordinately patterned in a nested manner for specifying proximodistal identity in jaw formation. Secondly, I investigated the functional roles of the zebrafish *dlx1a* and *dlx2a* genes during cranial neural crest migration, and pharyngogenesis. Lastly, the tunicate *distal-less*-related gene, *OdiDll*, in the protochordate *Oikopleura dioica*, was characterized to examine evolutionary aspects of *Dlx* organization, and its expression in an extant chordate species prior to advanced evolutionary morphological innovations.

The *Dlx* genes are a conserved family of transcriptional regulators involved in patterning morphological innovations. Of particular interest is the role the *Dlx* genes play in patterning the vertebrate jaws – a highly derived morphological innovation from the first pharyngeal arch. It has been suggested that the jaws evolved from preexisting developmental mechanisms in the anterior pharyngeal arch (Shigetani *et al.*, 2002). The paradoxical question as to how morphological innovations arise when genes are conserved is often raised. Homeobox-containing genes tend to be highly conserved within the homeodomain, implying conservation of function (Arthur, 2002). Conservation, however, is relative and as genes evolve so do morphological innovations. Morphological innovations, however, may arise with simple alterations. Alterations may include changes in expression

patterns (heterotopy), timing of expression (heterochrony), gene product concentrations (heterometry), and quality (heterotypy), all of which are constrained within the developmental mechanism through which the genes function (Arthur, 2002). Some examples of these alterations are seen in the patterning of the *Dlx* genes in the pharyngeal arches. Heterotopic shifts in *Dlx* patterns are evident when comparing agnathan arch expression to gnathostome patterning (Shigetani *et al.*, 2002). A topographical shift in patterning was facilitated by proposed changes in the distribution of signaling cues, such as FGF8 and BMP4 growth factors. We have shown that these signaling factors influence *Dlx* gene expression through unrelated but conserved intergenic *cis*-acting regulatory elements, demonstrating a mechanism for the establishment of the pharyngeal arch nested pattern of expression. The *Dlx* nested pattern of expression, which defines proximodistal identity, is thought to be necessary for the formation of modern vertebrate jaws. Our experimental evidence shows that the I12a and I56i elements target reporter gene expression to specific mesenchymal populations of cells within the mandible. These cell populations were responsive to ectopic growth factors within their endogenous domain of expression in a time dependent manner. Reporter gene expression, however, was not expandable beyond the initial endogenous domain, indicating that only particular cells were responsive to the signals. This suggests that specific cell populations within the mandible exist, and are subject to their own intrinsic interpretation of the external cues, and therefore confers a second mechanism for regionalization of the mandibular tissues. The I12a and I56i enhancers, as well as I37-2 situated between *Dlx3/4* (Sumiyama and Ruddle, 2003b), all target expression to the mandibular mesenchyme, but show no overall sequence similarity, suggesting that they may be regulated differently. However, I56i and I37-2 elements contain

similarly arranged *Dlx* homeodomain binding sites suggesting a common cross-regulatory mechanism. *Dlx1/2*^{-/-} mice exhibit a loss of *I56i-LacZ* expression in forebrain tissues similar to the loss of endogenous *Dlx5/6* expression indicating that *I56i* is a downstream target of *Dlx1* or *-2* (Zerucha *et al.*, 2000a; Anderson *et al.*, 1997b). As well, we showed that *I56i*, but not *I12a*, was influenced by ET-1 signaling, further indicating additional methods for differential regulation between *Dlx* genes. It is possible that different cell populations within the mandible use combinations of the regulatory mechanism. Future investigations of the *Dlx cis*-acting enhancers will likely focus on finding the tissue specific factors that directly regulate *Dlx* nested expression.

A conserved feature of the *Dlx* genes is their bigene organization. The tandem duplication arrangement reflects how they are regulated and expressed. *Dlx* genes of a bigene cluster have near indistinguishable overlapping expression patterns. The intergenic enhancers are presumably shared between linked paralogues, which suggests a means of generating expression patterns that overlap. Moreover, overlapping patterns of expression suggests genetic redundancy between linked *Dlx* genes. Single *Dlx* mouse mutant's exhibit distinct yet milder abnormal phenotypes than what would reasonably be expected based on the expression patterns. Double null mutants of a bigene cluster reveal a more severe phenotype, with novel defects previously not observed for the single mutants indicating some functional redundancy. Such is the case in the comparison of mouse single *Dlx1* and *-2*, and double *Dlx1/2* null mutants within a cluster (Qiu *et al.*, 1995; Qiu *et al.*, 1997), as well as the zebrafish *dlx1a* and *dlx2a* morphants described in chapter 3, suggesting that subtle but essential differences exist between *cis*-paralogues.

Possible functional similarities or differences between *Dlx* paralogues may be clade specific. The *Dlx* genes are organized into six orthologous groups based on sequence similarity in the conserved homeodomain (Stock *et al.*, 1996). As well, the *Dlx* genes are grouped into two clades based on sequence similarity in the homeodomain, as well as conserved motifs in the protein (reviewed in Zerucha and Ekker, 2000b). The clades are composed of *Dlx1/4/6* and *Dlx2/3/5* (Fig. 1.3). The clades arose from the initial tandem duplication event, and subsequent large or whole genome duplications creating the three bigene clusters observed in mammals (Stock *et al.*, 1996). Intriguingly, *Dlx* genes within a bigene cluster, have more closely overlapping patterns of expression than their clade related *trans*-paralogues; however, *trans*-paralogues within the same clade share greater amino acid identity, suggesting at possible closer functional similarities. Performing reciprocal experiments between *Dlx trans*- and *cis*-paralogues would be informative for determining any functional differences.

The evolutionary retention of the bigene cluster in vertebrates indicates its importance, and may be due to the necessity of enhancer sharing. The sharing of enhancers would account for overlapping expression patterns between linked genes, but not for the retention of redundant genes. Without adapted functions, redundant genes would be lost. Alterations of the intergenic enhancers may impair both genes in a cluster, and therefore selective pressure likely maintains the enhancers and closely linked genes (Sumiyama *et al.*, 2003a). Distinct functions between *cis*-paralogues may have arisen, from unshared enhancers perhaps located in the 5'-flanking region (Thomas *et al.*, 2000). Alternatively,

subtle differences in expression patterns and heterometry may be a result of the shared enhancer's differential activity on each of the two linked genes. It has previously been noted that one member of a bigene cluster is generally more strongly expressed, than the other, suggesting possible regulatory differences (Ellies *et al.*, 1997b). Further investigation to determine the relative regulatory contributions of the enhancers for each member of a cluster could entail exchanging linked paralogues with reporter genes within the same construct and close examination of the resulting expression patterns in transgenic animals.

The *Dlx* ancestral functions are considered to be involved in patterning neural tissue, and defining rudimentary outgrowth as seen in analogous structures that include insect limbs, and facial appendages, echinoderm tube feet, the amphioxus cerebral vesicle (Holland *et al.*, 1996) and vertebrate forebrain, limbs and pharyngeal arches (reviewed in Panganiban *et al.*, 1997; Panganiban and Rubenstein, 2002). Therefore, the mechanism, and its application as a unit of morphogenesis is conserved in analogous structures. In addition to their ancestral functions, the *Dlx* genes participate in the patterning of novel features. Of particular interest is the role the *Dlx* genes play in patterning the pharyngeal arches. In chapter three, characterization of *dlx2a* function in the migrating cranial neural crest cells, revealed a role in maintaining a subset of hindbrain neural crest cell identity, as well as fate determination of neurogenic and chondrogenic cranial neural crest cell lineages. Additionally, both *dlx1a* and *dlx2a* participate in the patterning and maturation of the cartilage arch elements of the viscerocranium, and other pharyngeal arch derivatives, reminiscent of the functional roles of the mouse *Dlx1* and *Dlx2* orthologues (Qiu *et al.*, 1995; 1997). Furthermore, the *Dlx* genes have been also proposed to participate in the odontogenic

homeodomain code that specifies the organization of the dentition; incisors in the distal portion of the mandible and maxilla, molars located more proximally (Sharpe, 1995). The *Dlx1/2*^{-/-} null mutations in mice result in the loss of maxillary molars and produces cartilage tissue in their place (Qiu *et al.*, 1997). Thus, the *Dlx* genes are adapting their regulatory role to novel tissues, such as tooth and cranial neural crest specification while maintaining their ancestral functions.

Distal aspects of the pharyngeal arches express all the *Dlx* family members, while proximal aspects only express *Dlx1/2* suggesting a heterometric mechanism for distal designation. It would be informative to alter the nested arch pattern of the *Dlx* genes by overexpressing more distally expressed family members such as *Dlx5/6* more proximally, possibly by utilizing *Dlx1/2* regulatory elements in a transgenic mouse. These experiments would presumably cause a reverse homeotic transformation of the maxilla into a mandibular phenotype. Future investigations will likely include genetic replacement experiments (knock-in) and overexpression studies in specific tissues and developmental stages. These experiments will help to elucidate functional versus possible quantitative differences between *Dlx* paralogues.

In tracing back the evolutionary history of the *Dll/Dlx* genes extant chordate species and basal level organisms have been studied. The characterization of a single *Dll*-related gene in the hemichordate *Ptychodera flava* (Harada *et al.*, 2001), and a *Ci-Dll-A/B* bigene cluster in the tunicate, *Ciona intestinalis* (Di Gregorio *et al.*, 1995; Caracciolo *et al.*, 2000) homologous to the vertebrate *Dlx* clusters, suggested that the ancestral duplication event

occurred in a common ancestor prior to the radiation of the extant chordates. An inverted tandem repeat is considered to be a rare event because it requires a double crossover of looped unequally paired homologs (Sumiyama *et al.*, 2003a), suggesting that it likely occurred only once for *Dll/Dlx* homologues in a common ancestor. A single *OdiDll* gene suggests that the organism may have either diverged prior to polyploidization, or subsequently lost a linked paralogue. Furthermore, the *Odidll* expression pattern proposes possible ancestral roles in ectodermally derived cell-types, as well as possible co-option into novel aspects of morphogenesis. Interestingly, *Odidll* is expressed in a complex pattern suggesting an intricate regulatory mechanism has evolved in *O. dioica*, a relatively simple organism with a small number of cells.

Oikopleura has the smallest chordate genome characterized to date and is relatively easy to culture in the laboratory, therefore making it a convenient basal level model for investigating genome evolution. The importance of examining genomic organization in diverse organisms, such as *Oikopleura*, allows for the following: (1) examination of a condensed genome (Seo *et al.*, 2001), (2) greater understanding of chordate evolution, (3) exploring the adaptation of homologous gene function in diverse organisms, (4) calculation of a more accurate statistical model of when chordate gene duplications occurred, utilizing the molecular clock (McLysaght *et al.*, 2002), (5) refine models of phylogenetic evolution, the more species and taxa characterized, the more comparisons can be made and subtleties explored, (6) observe conservation and/or exaptation of the developmental pathway (Chipman, 2001). (7) Lastly, examination of genomes from divergent organisms will aid in

the understanding of general morphological mechanisms in diversely-shaped animals (reviewed in Arthur, 2002).

To what extent the *Dlx* genes play a role in modulating potential downstream cellular responses, such as proliferation, migration, differentiation, and tissue condensation is currently unknown. Additionally, how the *Dlx* genes perform in a hierarchy of upstream and downstream interactions, some involved in possible feed-back and cross-regulation, remains unclear. Further investigation of these interactions will be necessary to appreciate the subtleties of these morphogenetic pathways. My research has contributed to a greater understanding of the transcriptional regulation of the *Dlx* genes, their contribution to pharyngeal arch patterning, and the continued characterization of homologues in diverse organisms as part of the newly emerging “evo-devo” discipline. It will be interesting to follow the progress of future research, and see what new avenues are uncovered and explored.

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