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Dlx genes pattern mammalian jaw primordium by regulating both lower jaw-specific and upper jaw-specific genetic programs

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Abstract

Dlx transcription factors are implicated in patterning the mammalian jaw, based on their nested expression patterns in the first branchial arch (primordium for jaw) and mutant phenotypes; inactivation of *Dlx1* and *Dlx2* (*Dlx1/2*$^{-/-}$) causes defects in the upper jaw, whereas *Dlx5/6*$^{-/-}$ results in homeotic transformation of the lower jaw into upper jaw. Therefore, the 'Dlx codes' appear to regionalize the jaw primordium such that *Dlx1/2* regulate upper jaw development, while *Dlx5/6* confer the lower jaw fate. Towards identifying the genetic pathways downstream of *Dlx5/6*, we compared the gene expression profiles of the wild-type and *Dlx5/6*$^{-/-}$ mouse mandibular arch (prospective lower jaw). We identified 20 previously unrecognized *Dlx5/6*-downstream genes, of which 12 were downregulated and 8 upregulated in the mutant. We found a Dlx-regulated transcriptional enhancer in close proximity to *Gbx2*, one of the *Dlx5/6*-downstream genes, strongly suggesting that *Gbx2* is a direct target of *Dlx5/6*. We also showed that *Pou3f3* is normally expressed in the maxillary (prospective upper jaw) but not mandibular arch, is upregulated in the mandibular arch of *Dlx5/6*$^{-/-}$, and is essential for formation of some of the maxillary arch-derived skeleton. A comparative analysis of the morphological and molecular phenotypes of various Dlx single and double mutants revealed that *Dlx1*, *2*, *5* and *6* act both partially redundantly and antagonistically to direct differential expression of downstream genes in each domain of the first branchial arch. We propose a new model for Dlx-mediated mammalian jaw patterning.

Keywords

Dlx; Gbx2; Pou3f3; Craniofacial; Branchial arch; Jaw; Mouse

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Supplementary material

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INTRODUCTION

Craniofacial development begins when the cranial neural crest cells (CNCCs), migratory multipotent precursors that contribute to most of the face, delaminate from the dorsal brain and migrate ventrolaterally to form the ectomesenchyme of facial primordia known as the frontonasal prominence (FNP) and branchial arches (BAs) (see Fig. S1A in the supplementary material) (Noden, 1978; Couly et al., 1993; Osumi-Yamashita et al., 1994; Köntges and Lumsden, 1996; Chai et al., 2000). Subsequently, the FNP becomes the mid- and upper face, while the first branchial arch (BA1) develops into most of the jaw, the lateral skull, palate and the middle ear (Köntges and Lumsden, 1996). BA1 is further divided into maxillary arch (mxBA1, prospective upper jaw) on the proximal half, and mandibular arch (mdBA1, prospective lower jaw) on the distal half (see Fig. S1A,B in the supplementary material). The second branchial arch (BA2) mainly contributes to the ear and neck skeleton.

How CNCCs recognize their positional information and develop accordingly is beginning to be understood (reviewed by Depew et al., 2002a; Santagati and Rijli, 2003; Chai and Maxon, 2006). Interactions of CNCCs with the neighboring tissues result in the expression of a diverse set of transcription factors in CNCCs, and the specific combination of transcription factors provides a positional identity to the cells.

The vertebrate Dlx genes are homologs of *Drosophila Distal-less*; they encode homeodomain transcription factors (Panganiban and Rubenstein, 2002). Mice have six Dlx genes, which are organized as three linked pairs in the genome (*Dlx1/2*, *Dlx3/4* and *Dlx5/6*) (Porteus et al., 1991; Price et al., 1991; Robinson and Mahon, 1994; Simeone et al., 1994; McGuinness et al., 1996; Nakamura et al., 1996; Liu et al., 1997).

During craniofacial development, mouse Dlx genes are regionally expressed within BAs as well as in olfactory and otic placodes (see Fig. S1A,C in the supplementary material) (Dolle et al., 1992; Bulfone et al., 1993; Robinson and Mahon, 1994; Simeone et al., 1994; Qiu et al., 1997; Depew et al., 2002b). In the ectomesenchyme of BA1 in mid-gestation stage embryos, *Dlx1/2* are expressed in both mxBA1 and mdBA1, whereas *Dlx5/6* are expressed in mdBA1 only. *Dlx3/4* expression is further restricted to a narrow domain within mdBA1. The same proximodistal arrangement is also found in BA2. Since *Dlx3/4* expression is dependent on *Dlx5/6* (Depew et al., 2002b) (this study), essentially two different combinations of Dlx partition much of BA1: *Dlx1/2* for mxBA1 and *Dlx1/2+5/6* for mdBA1. The functional importance of this ‘Dlx code’ in BA patterning has been investigated using mouse loss-of-function mutants. Owing to the tight linkage in the genome, the double mutations of *Dlx1/2* and *Dlx5/6* pairs were achieved by deleting both genes in one allele (Qiu et al., 1997; Merlo et al., 2002; Depew et al., 2002b). Inactivation of *Dlx1* and/or *Dlx2* (*Dlx1*$^{-/-}$, *Dlx2*$^{-/-}$ and *Dlx1/2*$^{-/-}$) caused abnormalities in upper jaw skeleton with little effect on the lower jaw (Qiu et al., 1995; Qiu et al., 1997; Depew et al., 2005). By contrast, *Dlx5*$^{-/-}$ exhibited defects in lower jaw development (Depew et al., 1999). Most strikingly, the simultaneous inactivation of *Dlx5* and *Dlx6* (*Dlx5/6*$^{-/-}$) resulted in homeotic transformation of the lower jaw into upper jaw (Beverdam et al., 2002; Depew et al., 2002b). Therefore, the differential expression of Dlx genes along the proximodistal

axis is important for the regional specification of BA1; *Dlx1/2* are necessary for the proper development of mxBA1, whereas *Dlx5/6* confer mdBA1 identity.

Our current work addresses three important issues on how the Dlx genes regulate BA patterning. First, the mechanism through which *Dlx5/6* specify lower jaw fate needs to be understood. To this end, we performed a genome-wide transcriptional profiling and obtained a comprehensive list of genes with altered expression in $Dlx5/6^{-/-}$ mdBA1. Second, we provide the first evidence of the upper jaw-specific genetic program and show that it is partly regulated by Dlx genes. Prior to this study, the abundance of mdBA1-specific markers but the lack of any known mxBA1-specific markers has been compatible with the idea that the upper jaw is the default state upon which the lower jaw fate is imposed. Finally, we investigated the functional relationship of different Dlx genes expressed in BA1 by comparing the morphological and molecular phenotypes of various combinations of Dlx single and double mutants. We found that *Dlx1, 2, 5* and *6* act both partially redundantly and antagonistically, depending on the context, to achieve differential expression of their downstream genes in mxBA1 and mdBA1.

MATERIALS AND METHODS

Animals

All experiments using mice were performed following UCSF institutional regulations on the care and use of laboratory animals.

Transcriptional profiling using DNA microarrays

$Dlx5/6^{-/-}$ and $Dlx5/6^{+/+}$ littermates were collected from $Dlx5/6^{+/-}$ intercrosses at E10.5. The mdBA1s were dissected, flash-frozen and stored in liquid nitrogen until the day of RNA extraction. The tissue was homogenized in Trizol reagent (Invitrogen) using Pellet Pestle (Kontes). RNA was extracted using chloroform and then concentrated by isopropanol precipitation. After a rinse with 80% ethanol, the RNA pellet was dissolved in water and purified using the RNeasy Mini Kit (Qiagen). From 13 $Dlx5/6^{-/-}$ and 13 $Dlx5/6^{+/+}$ E10.5 embryos, we recovered 14.7 μg and 13 μg of mdBA1 total RNA, respectively.

All subsequent steps of the microarray experiment were performed by the Translational Genomics Research Institute (TGen, Phoenix, AZ), through the NIH Neuroscience Microarray Consortium. The RNA sample from each genotype was hybridized onto GeneChip Mouse Genome 430 2.0 arrays (Affymetrix) in triplicate. Data acquisition and analysis employed GeneChip Operating Software (GCOS, Affymetrix) version 1.2.

In situ hybridization and skeletal preparation

Whole-mount and section in situ hybridizations were performed using digoxigenin-labeled RNA probes as described (Jeong et al., 2004; Jeong and McMahon, 2005), except that 20 μm sections were used for the section in situ hybridization. The control and mutant embryos were stage-matched using a combination of several morphological criteria, including the size and shape of the limb buds, morphogenesis of the eye, and somite numbers. Skeletons of E18.5 or P0 animals were stained with Alcian Blue and Alizarin Red as described (Jeong et

al., 2004). For the in situ hybridization and skeletal preparations shown in Figs 1–4, embryos of +/+ and *+/−* genotypes were used indiscriminately and are referred to as 'wild type'. For Figs 5 and 6, 'wild type' refers to +/+ for all the genes in question, or *Dlx1*$^{+/-}$.

DNA templates for in situ hybridization probes were obtained by PCR from a wild-type mouse E10.5 BA1 cDNA library or from adult tail genomic DNA, purchased from companies, or kindly provided by other investigators. Further information on the probes is available upon request.

Luciferase reporter activation assay

The 1 kb putative *Gbx2* enhancer (see Fig. 3A) was amplified by PCR from mouse tail genomic DNA using primers 5′-ACACCTCGAGAGAGGATGACAGCGAGCTTCG-3′and 5′-GTGTAAGCTTGAGCAAACATTCCAGTTTTAATGC-3′, and cloned into *Xho*I-*Hin*dIII sites of pGL4.23 (Promega). pGL4.23 contains a minimal promoter and the firefly luciferase coding sequence. pCAGGS-Dlx5 (Stuhmer et al., 2002) was used to express Dlx5 protein. pGL4.73 (Promega), a plasmid that constitutively expresses *Renilla* luciferase, was used as a control for variations in transfection efficiency. 3T3 cells were transfected with FuGene 6 (Roche), and 40 hours later the cells were lysed and analyzed for luciferase activity using the Dual Luciferase Reporter Assay System (Promega). The experiments were performed in triplicate and the results combined for statistical analysis.

Generation of *Dlx6* mutant allele

To generate the *Dlx6-lacZ* (*Dlx6*$^{-}$) allele, a 4.3 kb *Spe*I-*Sna*BI genomic fragment spanning *Dlx6* exon 3 (which encodes the homeodomain) was subcloned into the *Spe*I-*Sma*I sites of pBS KS+. Site-directed mutagenesis was performed to engineer a unique *Nru*I site immediately following the 219th amino acid from the N-terminus of the Dlx6 protein (F of the sequence VKIWFQNKRS). Flanking genomic sequences were added between the unique 5′ *Xho*I site (5.8 kb 5′ homology arm) and the 3′ *Not*I site (3.5 kb 3′ homology arm), and the reporter cassette IRES-lacZ-PGKneo (Robledo et al., 2002) was cloned into the unique *Nru*I site to generate the final targeting construct. The *Dlx6-lacZ* allele therefore interrupts the Dlx6 protein immediately following the amino acid F as described above. ES cell culture, screening, chimera generation and testing were as previously described (Robledo et al., 2002).

RESULTS

Genome-wide transcriptional profiling identifies changes in RNA expression in the mdBA1 of *Dlx5/6*$^{-/-}$ mutants

To understand the molecular changes underlying *Dlx5/6*$^{-/-}$ jaw phenotypes, we compared transcriptional profiles of the wild-type and mutant mdBA1 at E10.5 using Affymetrix GeneChip Mouse Genome 430 2.0 array. We chose E10.5 for our analysis because E9.0–10.5 is when the Dlx genes exhibit proximodistally nested expression in BA1 (Qiu et al., 1997; Acampora et al., 1999) (see Fig. S1 in the supplementary material), and the wild-type and mutant BA1 still appear grossly comparable in size and morphology at E10.5 (see also Fig. S2 in the supplementary material). The *Dlx5/6*$^{-/-}$ mutant exhibited downregulation of

39 genes (see Table S1 in the supplementary material) and upregulation of 24 genes (see Table S2 in the supplementary material) with greater than a 2-fold change. Our results included most, but not all, of the genes that were previously shown to be dysregulated in the *Dlx5/6*$^{-/-}$ mutant (Depew et al., 1999; Depew et al., 2002b). Thus, although it is a robust procedure, technical limitations exist (see Tables S1 and S2 in the supplementary material).

The complete set of raw data from our transcriptional profiling experiment is available at NIH Neuroscience Microarray Consortium data repository (http://arrayconsortium.tgen.org) under accession ruben-affy-mouse-187820, and at GEO (accession number GSE 4774).

Genes encoding transcription factors, non-coding RNAs and signaling molecules exhibit decreased expression in *Dlx5/6*$^{-/-}$ mdBA1

The Dlx3, Hand2 and Alx4 transcription factors were previously identified as being downstream of *Dlx5/6* (Depew et al., 2002b). Our screen found that their close relatives, Dlx4, Hand1 and Alx3, are also downregulated in *Dlx5/6*$^{-/-}$ mdBA1 and BA2 (Fig. 1A,B,M–P). In the otic vesicle, *Dlx5/6* function is required for the expression of Gbx2, a homeodomain transcription factor (Robledo and Lufkin, 2006). We confirmed the same regulatory relationship in mdBA1 (Fig. 1C,D). The transcription factors Cited1 (Msg1) (Shioda et al., 1996) and Zac1 (Lot1, Plagl1) (Abdollahi et al., 1997; Spengler et al., 1997) are expressed at high levels in the medial mdBA1 and BA2; their expression was downregulated in *Dlx5/6*$^{-/-}$ (Fig. 1Q–T).

Mouse *Dlx1* locus encodes several putative non-coding RNA (ncRNA) transcripts (*A/S Dlx1*; see Fig. S3A in the supplementary material) (McGuiness et al., 1996; Liu et al., 1997). In BA1 and BA2, the expression of *A/S Dlx1* was restricted to the distal region (Fig. 1E), and was completely abolished in *Dlx5/6*$^{-/-}$ (Fig. 1F). *Evf1/2*, the two splicing variants of a ncRNA gene (*Dlx6os1* – Mouse Genome Informatics), map adjacent to *Dlx5/6*; their brain expression depends on *Dlx1/2* function (see Fig. S3B in the supplementary material) (Faedo et al., 2004; Kohtz and Fishell, 2004). *Evf1/2* expression was greatly reduced in both brain and BAs of *Dlx5/6*$^{-/-}$ mutants (Fig. 1G,H). It should be noted that the promoter and the first exon of *Evf2* fall within the deletion in the *Dlx5/6* null allele (Robledo et al., 2002), and thus the decreased *Evf1/2* expression in *Dlx5/6*$^{-/-}$ could simply be due to the loss of the *Evf2* promoter. However, we found reduced expression of *Evf1/2* also in *Dlx5*$^{-/-}$ and *Dlx6*$^{-/-}$ mutants (see below), in which both *Evf1* and *Evf2* sequences are intact. This result, and the fact that *Dlx1/2* activity is necessary for the expression of *Evf1/2* in the brain, argue that *Dlx5/6* regulate *Evf1/2* transcription, directly or indirectly.

A secreted signaling molecule, hepatocyte growth factor (Hgf, also known as scatter factor, SF) (Stoker et al., 1987; Birchmeier and Gherardi, 1998), is expressed in BA1 and BA2; this expression was dependent on *Dlx5/6* function (Fig. 1I,J). *Unc5c* encodes one of the receptors for the axon guidance molecule netrin 1 (Ackerman et al., 1997). *Unc5c* was expressed in the medial domain of mdBA1 and BA2, and was severely downregulated in *Dlx5/6*$^{-/-}$ (Fig. 1U,V). BMP-binding endothelial regulator (Bmper, also known as crossveinless-2, Cv2) is a secreted molecule that binds to and enhances the signaling of bone morphogenetic proteins (BMPs) (Coffinier et al., 2002; Moser et al., 2003; Coles et al., 2004; Ikeya et al., 2006). *Bmper* expression in distal BA1 and BA2 was dependent on

Dlx5/6 (Fig. 1K,L). The regulator of G-protein signaling 5 (*Rgs5*) gene is expressed only at the rostromedial tip of mdBA1; this expression was lost in *Dlx5/6*$^{-/-}$ (Fig. 1W,X). Rgs proteins are GTPase-activating proteins that attenuate G-protein-coupled receptor signaling (Chen et al., 1997; Xie and Palmer, 2007).

Since *Dlx3/4* expression is lost in *Dlx5/6*$^{-/-}$ mdBA1 (Depew et al., 2002b) (Fig. 1A,B), it is possible that at least some of the gene expression changes in *Dlx5/6*$^{-/-}$ mutants are due to the loss of Dlx3/4 activity.

Pou3f3, *Foxl2* and uncharacterized transcripts linked to them, are strongly repressed by *Dlx5/6* in mdBA1

Pou3f3 (Brn1), a POU-domain transcription factor (Hara et al., 1992), was highly expressed in the entire mxBA1 and maxillary-mandibular junction, but was absent from most of mdBA1 (Fig. 2A). It was also confined to the proximal region in BA2. In *Dlx5/6*$^{-/-}$, *Pou3f3* expression expanded into mdBA1 and distal BA2 with the same intensity as in its normal expression domains (Fig. 2B).

We identified two Riken cDNA clones (*2610017I09Rik*, *2900092D14Rik*), both suspected to be non-coding, that are closely linked to and have very similar expression patterns as *Pou3f3*, in both wild-type and *Dlx5/6*$^{-/-}$ embryos (Fig. 2D,E,G,H; see Fig. S3C in the supplementary material). Because the proximal BA expression of *Pou3f3*, *2610017I09Rik* and *2900092D14Rik* overlaps with that of *Dlx1/2* (see Fig. S1C in the supplementary material), we tested whether the expression of these genes is dependent on the activity of *Dlx1/2* in the mxBA1. We found downregulation of all three genes in BA1 and BA2 of *Dlx1/2*$^{-/-}$, demonstrating that *Dlx1/2* are necessary for their normal expression (Fig. 2C,F,I).

Foxl2 encodes a winged helix/forkhead transcription factor (Crisponi et al., 2001). It was expressed strongly in the dorsal mxBA1 just below the eye, in addition to a small domain at the maxillary-mandibular junction (Fig. 2J). In *Dlx5/6*$^{-/-}$, the mxBA1 pattern of *Foxl2* expression was duplicated in mdBA1 (Fig. 2K). Riken cDNA *E330015D05Rik* is a poorly characterized gene closely linked to *Foxl2* (see Fig. S3D in the supplementary material). Its expression pattern, both in wild type and *Dlx5/6*$^{-/-}$, was identical to that of *Foxl2* (Fig. 2M,N).

Cyp26a1, a retinoic acid-metabolizing enzyme cytochrome P450 (White et al., 1996; Fujii et al., 1997; Ray et al., 1997), is normally expressed along the border of BA1 and BA2 with higher expression in mxBA1 than in mdBA1 (Fig. 2P). In BA2, *Cyp26a1* expression was also strong proximally and weak distally. Removal of *Dlx5/6* activity lead to upregulation of *Cyp26a1* in distal BA1 and BA2 to the same intensity as in the proximal domains (Fig. 2Q). *Irx5*, which encodes an Iroquios-related homeodomain transcription factor (Bosse et al., 2000; Cohen et al., 2000), was expressed strongly in the dorsal mxBA1 and weakly in the ventral mdBA1 (Fig. 2S). The latter expression was moderately increased in *Dlx5/6*$^{-/-}$ (Fig. 2T). Unlike *Pou3f3*, *2610017I09Rik* and *2900092D14Rik*, the mxBA1 expression of *Foxl2*, *E330015D05Rik*, *Cyp26a1* and *Irx5* was not dependent on *Dlx1/2* (Fig. 2L,O,R,U).

The genes described above in this section are normally expressed in mxBA1 and upregulated in the *Dlx5/6*$^{-/-}$ mdBA1, making the mutant mdBA1 molecularly similar to mxBA1. By contrast, transmembrane protein 30b (Tmem30b), a homolog of yeast endosomal protein Cdc50 (Katoh and Katoh, 2004), was barely detectable in either mxBA1 or mdBA1 in wild-type embryos (Fig. 2V), but was strongly upregulated in *Dlx5/6*$^{-/-}$ mdBA1, making the mutant mdBA1 molecularly different from mxBA1 (Fig. 2W).

Identification of a Dlx-regulated transcriptional enhancer upstream of *Gbx2*

An important question about the *Dlx5/6*-downstream genes identified by our microarray analysis is whether *Dlx5/6* directly regulate them. There is already evidence that three of the genes listed in Table S1 (see Table S1 in the supplementary material) are direct targets of *Dlx5/6.* Charite et al. (Charite et al., 2001) showed that Dlx6 binds to, and thus most likely directly regulates, a *Hand2* BA enhancer. Sumiyama and Ruddle (Sumiyama and Ruddle, 2003) showed that the BA enhancer for *Dlx3/4* contains a consensus Dlx-binding motif, and thus it is highly likely that Dlx5/6 directly regulate *Dlx3/4* expression in the BA. Thus, the fact that *Hand2*, *Dlx3* and *Dlx4* were identified by our microarray analysis supports the validity of our approach, and suggests that additional genes listed in Table S1 (see Table S1 in the supplementary material) are directly regulated by Dlx5 and Dlx6 proteins.

To identify direct targets of Dlx5/6, we performed in silico analyses of the genomic sequences surrounding *Dlx5/6*-downstream genes. Previously, a systematic in vitro binding assay determined that (A/C/G)TAATT(G/A)(C/G) is a consensus binding motif for Dlx proteins (Feledy et al., 1999). In addition, various researchers have analyzed cis-regulatory elements from several Dlx target genes, discovering 13 sequences to which Dlx proteins bind directly, including some that do not conform to (A/C/G)TAATT(G/A)(C/G) (see Table S3 in the supplementary material). Using rVISTA (Loots and Ovcharenko, 2004), we searched genomic sequences flanking several of the genes listed in Table S1 (see Table S1 in the supplementary material) for all the known Dlx-binding motifs that are conserved between mouse and human. This analysis identified ~0.6 kb of a highly conserved region (conserved down to chick) with a cluster of three putative Dlx-binding sites located ~10 kb upstream of *Gbx2* (Fig. 3A,B). We tested a 1 kb fragment containing this region (red bar in Fig. 3A, including the adjacent 0.25 kb sequence conserved down to opossum) for transcriptional enhancer activity in 3T3 cells. Co-transfection with a Dlx5 expression vector resulted in a >70-fold activation (Fig. 3C), demonstrating that the fragment contains a transcriptional enhancer regulated by Dlx proteins.

Pou3f3 is required for the formation of the zygomatic arch and the maxillary component of the jaw joint

To our knowledge, *Pou3f3*, *Foxl2*, *2610017I09Rik*, *2900092D14Rik* and *E330015D05* are the first examples of genes that are expressed specifically in the maxillary domain of BA1 at any stage of mouse development. Therefore, they provide evidence of an upper jaw-specific genetic program (see Discussion).

Among the five genes, *Pou3f3* has the strongest and broadest expression in mxBA1 (Fig. 2), and thus we performed further analysis on its expression and function during upper jaw

development (Fig. 4). Section in situ hybridization at E10.5 revealed that *Pou3f3* is expressed only in the mesenchyme (Fig. 4A,B). At E12.5 (data not shown) and E13.5 (Fig. 4C–E), *Pou3f3* expression was found in both upper and lower jaw; however, its expression in the condensed dental mesenchyme was restricted to upper molars (Fig. 4D). In addition, *Pou3f3* was expressed in the caudal, but not rostral, palatal shelves (PS) (Fig. 4C,E).

Pou3f3$^{-/-}$ (McEvilly et al., 2002) mutant skull revealed the essential role of this gene in the development of a part of the upper jaw; mxBA1-derived squamosal bone (SQ) normally articulates with mdBA1-derived dentary to make the functioning jaw joint in mammals. In *Pou3f3*$^{-/-}$, the squamosal bone is largely missing (except the retrotympanic process, rt), and thus the jaw joint does not exist (Fig. 4F–I,L,M). Jugal bone (JG), which forms the zygomatic arch on the lateral skull together with the maxilla and squamosal bone, was also lost in the mutant (Fig. 4H,I,L,M). In the mutant middle ear, the malleus (mdBA1-derived) appeared normal, whereas the incus (mxBA1-derived) had a slightly elongated short crus (Fig. 4J,K). In addition, the BA2-derived stapes was fused to the styloid process (Fig. 4J,K). Other bones in the skull, and the teeth and palate, appeared unaffected in the *Pou3f3*$^{-/-}$ mutant (data not shown).

Dlx6$^{-/-}$ exhibits BA1-associated phenotypes that are very similar to those of *Dlx5*$^{-/-}$ but far less severe than those of *Dlx5/6*$^{-/-}$

Simultaneous inactivation of *Dlx5/6* results in the homeotic transformation of the lower jaw into upper jaw, whereas *Dlx5* single mutants have relatively mild defects in lower jaw morphogenesis (Depew et al., 1999; Acampora et al., 1999; Beverdam et al., 2002; Depew et al., 2002b). Based on these results alone, it was not clear whether *Dlx5* and *Dlx6* are functionally redundant, or whether *Dlx6* has a more prominent role. To address this, we generated an allele that inactivated *Dlx6* alone, by inserting an IRES-lacZ-neomycin resistance cassette within the *Dlx6* homeobox coding sequence (Fig. 5A). This insertion prevents translation of one-third of the homeodomain and the entire C-terminal domain, including the nuclear localization signal [amino acids 220–228 (Cokol et al., 2000)]; thus, this allele is likely to be null.

Dlx6$^{-/-}$ mice were born alive but died within a day with aerophagia, as reported for *Dlx2*$^{-/-}$, *Dlx1/2*$^{-/-}$ and *Dlx5*$^{-/-}$ (Qiu et al., 1995; Qiu et al., 1997; Acampora et al., 1999; Depew et al., 1999). The head skeleton of *Dlx6*$^{-/-}$ neonates had several abnormalities that are also found in *Dlx5*$^{-/-}$ animals (Acampora et al., 1999; Depew et al., 1999). The mutant had a slightly reduced mandible (Fig. 5B,D), in which the dentary lacked the coronoid process and had a hypoplastic condylar process (Fig. 5E–G, arrows and arrowheads). The ectotympanic of the mutant ear was shortened (Fig. 5H–J, arrows), and the gonial bone was attached to an ectopic piece of bone [named os paradoxicum (Depew et al., 1999)] extending toward the ala temporalis on the skull base (Figs 5H–J; see S4A–C, arrowheads, in the supplementary material). The skeletal elements mentioned thus far are thought to be derivatives of BA1. Therefore, allowing for some individual variations in morphological details, *Dlx6*$^{-/-}$ mice have BA1-associated defects that are very similar to those of *Dlx5*$^{-/-}$, but are much less severe than the homeotic transformation observed in *Dlx5/6*$^{-/-}$. This result establishes that *Dlx5* and *Dlx6* are in large part functionally redundant in lower jaw development.

In addition to the BAs, *Dlx5/6* were expressed in otic and olfactory placodes (see Fig. S1A,C in the supplementary material) and, as a result, $Dlx5^{-/-}$ animals have dorsally deficient otic capsule (Fig. 5H,I, open arrowheads) and hypoplastic nasal cartilage (see Fig. S4D,E, arrowheads, in the supplementary material) (Acampora et al., 1999; Depew et al., 1999). However, both structures appeared normal in $Dlx6^{-/-}$ (Fig. 5J; see Fig. S4F in the supplementary material), which suggests that *Dlx6* is less important than *Dlx5* in otic and olfactory placode development.

Next, we examined the expression of the molecular markers that are affected in $Dlx5/6^{-/-}$ mdBA1 (Figs 1 and 2) in $Dlx5^{-/-}$ and $Dlx6^{-/-}$ single mutants. Most of the genes showed no, or moderate, changes in either mutant, indicating that they are directly or indirectly regulated by both *Dlx5* and *Dlx6* to similar degrees (see Fig. S5A–d in the supplementary material). Surprisingly, however, several genes were differentially changed in $Dlx5^{-/-}$ and $Dlx6^{-/-}$; *Gbx2*, *Bmper*, *A/S Dlx1*, *Dlx4* and *Evf1/2* were all severely downregulated in $Dlx5/6^{-/-}$ BAs (Fig. 1), but *Gbx2*, *Dlx4* and *Evf1/2* were more downregulated in $Dlx5^{-/-}$ than in $Dlx6^{-/-}$, whereas *Bmper* and *A/S Dlx1* were more affected in $Dlx6^{-/-}$ than in $Dlx5^{-/-}$ (Fig. 5K–P; see Fig. S4G–I and Fig. S5e–j in the supplementary material). Similarly, for the genes upregulated in $Dlx5/6^{-/-}$, *Pou3f3*, *Foxl2*, *2610017I09Rik* and *E330015D05Rik* showed greater changes in $Dlx5^{-/-}$ than in $Dlx6^{-/-}$, whereas *Tmem30b* was upregulated only in $Dlx6^{-/-}$ (Fig. 5Q–V; see Fig. S4J–L and Fig. S5k–p in the supplementary material). These results suggest that there are some differences between the transcriptional activities of *Dlx5* and *Dlx6*, even though inactivation of each gene results in similar morphological consequences.

Dlx6 activity in lower jaw development is shared by *Dlx1* and *Dlx2*

Although $Dlx1/2^{-/-}$ mice have no significant defects in the lower jaw (Qiu et al., 1995; Qiu et al., 1997; Depew et al., 2005), *Dlx1/2* can contribute to mdBA1 development, as the lower jaw phenotypes of $Dlx5^{-/-}$ are greatly exacerbated in $Dlx1^{-/-};5^{-/-}$ and $Dlx2^{-/-};5^{-/-}$ mutants (Depew et al., 2005).

We tested whether *Dlx6* is also at least in part functionally redundant with *Dlx1/2* by generating $Dlx1^{-/-};6^{-/-}$ and $Dlx2^{-/-};6^{-/-}$ double mutants. The compound mutants exhibited far greater craniofacial defects than those found in any of the single mutants. The dentaries of $Dlx1^{-/-};6^{-/-}$ and $Dlx2^{-/-};6^{-/-}$ were shortened, fragmented, and bifurcated to become bones resembling the maxilla, jugal and pterygoid of the upper jaw (Fig. 6A–L; see S6A–F in the supplementary material). The ventrolateral side of the skull of $Dlx1^{-/-};6^{-/-}$ and $Dlx2^{-/-};6^{-/-}$ had what appears to be a duplicate lamina obturans juxtaposed to the endogenous one (Fig. 6S–X; LO* in Fig. 6W,X). In the ear, the ectotympanic and middle ear ossicles were progressively reduced or lost in $Dlx1^{-/-};6^{-/-}$ and $Dlx2^{-/-};6^{-/-}$ (Fig. 6Y–d). The basihyoid and lesser horn of the hyoid were also progressively more affected in $Dlx1^{-/-};6^{-/-}$ and $Dlx2^{-/-};6^{-/-}$ (see Fig. S6G–L in the supplementary material).

The phenotypes of $Dlx1^{-/-};6^{-/-}$ and $Dlx2^{-/-};6^{-/-}$ are reminiscent of those reported for $Dlx1^{-/-};5^{-/-}$ and $Dlx2^{-/-};5^{-/-}$ (Depew et al., 2005). In all four cases, the mdBA1-derivatives are reduced in size and/or transformed into structures resembling upper jaw elements, although the extent of transformation is less than in $Dlx5/6^{-/-}$.

Dlx1/2 function partially redundantly with *Dlx5/6* in regulating mdBA1 gene expression

To elucidate the mechanisms underlying the morphological changes in *Dlx1*$^{-/-}$;*6*$^{-/-}$, *Dlx2*$^{-/-}$;*6*$^{-/-}$ and *Dlx2*$^{-/-}$;*5*$^{-/-}$, we examined the gene expression patterns in their mdBA1.

The expression of *Gbx2* and *Bmper* in mdBA1 was minimally or moderately downregulated in *Dlx5*$^{-/-}$ and *Dlx6*$^{-/-}$ (Fig. 5). However, their expression is severely reduced or completely abolished in *Dlx1*$^{-/-}$;*6*$^{-/-}$, *Dlx2*$^{-/-}$;*6*$^{-/-}$ and *Dlx2*$^{-/-}$;*5*$^{-/-}$ (Fig. 7A–H). *Pou3f3* was repressed by *Dlx5/6* in mdBA1, and was moderately upregulated in *Dlx5*$^{-/-}$ and *Dlx6*$^{-/-}$ (Fig. 5). Further removing *Dlx1* or *Dlx2* activity expanded and intensified *Pou3f3* overexpression in mdBA1 (compare Fig. 7J,K with Fig. 5S, and Fig. 7L with Fig. 5R). Therefore, even though *Dlx1/2* activity is required for the normal expression of *Pou3f3* in mxBA1 (Fig. 2A,C), *Dlx1/2* apparently share the repressive effects of *Dlx5/6* on *Pou3f3* in mdBA1. *Hand2* was severely downregulated in *Dlx5/6*$^{-/-}$, but unaffected in *Dlx5*$^{-/-}$ and *Dlx6*$^{-/-}$ (Depew et al., 2002b) (see Fig. S5A–D in the supplementary material). *Hand2* expression appeared normal in *Dlx1*$^{-/-}$;*6*$^{-/-}$, but was gradually reduced and became restricted to caudomedial mdBA1 in *Dlx2*$^{-/-}$;*6*$^{-/-}$ and *Dlx2*$^{-/-}$;*5*$^{-/-}$ (Fig. 7M–P).

The expression changes of *Gbx2*, *Bmper* and *Pou3f3* in *Dlx1*$^{-/-}$;*6*$^{-/-}$, *Dlx2*$^{-/-}$;*6*$^{-/-}$ and *Dlx2*$^{-/-}$;*5*$^{-/-}$ are either similar or identical to the changes in *Dlx5/6*$^{-/-}$ (Figs 1 and 2), in line with the overall similar morphological defects of these four mutants. By contrast, *Hand2* expression was more severely reduced in *Dlx5/6*$^{-/-}$ than in any of *Dlx1*$^{-/-}$;*6*$^{-/-}$, *Dlx2*$^{-/-}$;*6*$^{-/-}$ or *Dlx2*$^{-/-}$;*5*$^{-/-}$ (see Fig. S5D in the supplementary material). This could explain why the morphological transformations of lower jaw into upper jaw in the latter three mutants are incomplete compared with *Dlx5/6*$^{-/-}$.

DISCUSSION

Genes downstream of *Dlx5/6* in mouse jaw development

Our genome-scale expression profiling experiment greatly expanded the number of potential targets of *Dlx5/6* in mdBA1 (see Tables S1 and S2 in the supplementary material), and we confirmed 20 novel *Dlx5/6*-downstream genes by in situ hybridization (Figs 1 and 2). They include genes encoding transcription factors, signaling molecules, ncRNAs or unclassifiable products. Some of the *Dlx5/6*-downstream genes have been implicated in craniofacial development based on their mutant analysis in mice (summarized in Table 1); our study added *Pou3f3* to this list (Fig. 4). However, the reported mutant phenotypes of several genes identified from our screen do not provide an obvious connection to craniofacial development (Table 1). The possibilities are: (1) BA1 expression of these genes has no biological function; (2) the mutants of these genes do have craniofacial phenotypes but the previous studies did not examine/detect them; or (3) these genes contribute to BA1 development redundantly with others, so that the individual mutation does not result in defects. Finally, the rest of the genes from our screen await mutant generation and analysis to reveal their role in facial development.

A recent study showed that *Evf2* ncRNA and Dlx2 protein form a complex in embryonic tissues and that Evf2 functions as a transcriptional coactivator of Dlx2 in tissue culture cells (Feng et al., 2006). Therefore, it is possible that Evf2 similarly controls the activities of Dlx

proteins in mdBA1 and thus the downregulation of *Evf1/2* expression in *Dlx5/6*$^{-/-}$ (Fig. 1) contributes to the gene expression changes observed in *Dlx5/6*$^{-/-}$ mutants.

To date, none of the *Dlx5/6*-downstream genes has been shown to recapitulate the phenotypes of *Dlx5/6*$^{-/-}$ when mutated in mice. Given that *Dlx5/6* directly or indirectly regulate the expression of dozens of genes, it is likely that *Dlx5/6* achieve their function through the combined efforts of many genes.

Among the *Dlx5/6*-downstream genes listed in Tables S1 and S2 (see Tables S1 and S2 in the supplementary material), BA enhancers have been characterized for only three (*Hand2* and *Dlx3/4*), all of which showed some evidence of direct regulation by Dlx proteins (Charite et al., 2001; Sumiyama and Ruddle, 2003). Our identification of a Dlx5-regulated enhancer near *Gbx2* suggests that *Gbx2* might also be a direct target of *Dlx5/6*. Since *Gbx2* is downstream of *Dlx5/6* in BA1 and the otic vesicle, this enhancer is likely to function in one or both of these tissues.

Upper jaw-specific developmental program

Our screen discovered several genes, including *Pou3f3* and *Foxl2*, the expression of which is largely restricted to mxBA1. Furthermore, we demonstrated that *Pou3f3* is essential for the normal development of a part of the upper jaw (Fig. 4). These results provide the first evidence for the genetic program that specifically regulates upper jaw development, and argue against the idea that upper jaw fate is the default state of BA1, whereas lower jaw fate requires specification.

We found that the normal level of *Pou3f3* expression in mxBA1 requires *Dlx1/2* activity, whereas *Foxl2* expression does not (Fig. 2). Therefore, the upper jaw-specific program in mxBA1 has both Dlx-dependent and Dlx-independent components. In addition, the mxBA1-specific genes that we identified show altered expression in *Dlx5/6*$^{-/-}$ mdBA1. It is possible that there are genes, the expression of which is restricted to mxBA1 but which is not upregulated in *Dlx5/6*$^{-/-}$ mdBA1; our screen was not designed to identify these.

Functional comparison of different Dlx genes in jaw patterning

Morphological analysis of the *Dlx5* and *Dlx6* single mutants indicates that they have very similar roles in mdBA1 development, despite the intriguing differences in their transcriptional activities (Fig 5; see Fig. S4 in the supplementary material). In addition, the analysis of *Dlx1*$^{-/-}$*;5*$^{-/-}$, *Dlx2*$^{-/-}$*;5*$^{-/-}$ (Depew et al., 2005), *Dlx1*$^{-/-}$*;6*$^{-/-}$ and *Dlx2*$^{-/-}$*;6*$^{-/-}$ (Figs 6 and 7) establishes that the activity of *Dlx5/6* to specify lower jaw fate is shared by *Dlx1/2*. However, *Dlx1/2* are clearly less important than *Dlx5/6* in this process because lower jaw development is essentially normal in *Dlx1/2*$^{-/-}$ mutants (Qiu et al., 1997; Depew et al., 2005). Also, among *Dlx1* and *Dlx2*, *Dlx2* appears to have a greater influence on lower jaw development because the defects seen in *Dlx2*$^{-/-}$*;5*$^{-/-}$ and *Dlx2*$^{-/-}$*;6*$^{-/-}$ are more severe than those in *Dlx1*$^{-/-}$*;5*$^{-/-}$ and *Dlx1*$^{-/-}$*;6*$^{-/-}$, respectively (Depew et al., 2005) (this study). These differences could be due to the molecular properties of each Dlx protein (as determined by amino acid sequence), but they could also be owing to differences in expression level, pattern or timing.

Another important conclusion from our analysis of *Dlx1*$^{-/-}$;*6*$^{-/-}$ and *Dlx2*$^{-/-}$;*6*$^{-/-}$ mutants concerns the functional relevance of the previous classification of Dlx genes based on their sequence homology (Stock et al., 1996) into type A (*Dlx2*, *3*, *5*) and type B (*Dlx1*, *4*, *6*). If two Dlx genes of the same type were functionally closer than those of different types, then removing one gene of each type would result in milder phenotypes than removing two genes of the same type, owing to compensation. Whereas *Dlx1*$^{-/-}$;*5*$^{-/-}$ has milder phenotypes than *Dlx2*$^{-/-}$;*5*$^{-/-}$ (Depew et al., 2005), *Dlx2*$^{-/-}$;*6*$^{-/-}$ has more severe phenotypes than *Dlx1*$^{-/-}$;*6*$^{-/-}$ (Figs 6 and 7), contrary to the prediction. Therefore, it appears that the greater sequence divergence between Dlx genes of different types does not result in more-dissimilar functions.

Proximodistal patterning of the mammalian jaw by the Dlx code

The Dlx code model proposed in previous studies (Qiu et al., 1995; Qiu et al., 1997; Depew et al., 2002b) is compatible with two different hypotheses on the nature of the codes: the qualitative hypothesis invokes the unique activities of Dlx1/2 versus Dlx5/6 proteins in specifying each domain of BA1, whereas the quantitative hypothesis proposes that the higher level of total Dlx protein in mdBA1 differentiates it from mxBA1. Although we do not have positive proof for the quantitative theory, a growing body of data has accumulated that cannot be explained by the qualitative theory, at least on its own. First of all, a recent study (Depew et al., 2005) and the present work (Fig. 6) showed that both *Dlx1/2* and *Dlx5/6* can regulate lower jaw development, and that *Dlx1/2* and *Dlx5/6* co-regulate several genes in mdBA1, directly or indirectly (Fig. 7). More importantly, *Dlx1/2* perform opposing roles in mxBA1 versus in mdBA1: in mxBA1, *Dlx1/2* positively regulate *Pou3f3* and other mxBA1-specific genes to guide upper jaw development (Fig. 2); whereas in mdBA1, *Dlx1/2* act partially redundantly with *Dlx5/6* to repress *Pou3f3* (and mxBA1 fate), and upregulate mdBA1-specific genes to promote lower jaw fate (Fig. 7). These results suggest that the patterning activity of a particular Dlx protein is context-dependent. For example, the activities of Dlx1/2 proteins might be modulated by some factors that are unevenly distributed between mxBA1 and mdBA1. Alternatively, *Pou3f3* might be induced by the moderate level of Dlx proteins found in mxBA1, but repressed by the high level of Dlx proteins in mdBA1.

Fig. 8 summarizes our hypothesis on the regionalization of BA1 by Dlx genes. During early stages of craniofacial development, the BA1 ectomesenchyme forms without its regional identity. Subsequently, through a poorly understood mechanism that involves endothelin signaling and *Mef2c* (Ozeki et al., 2004; Ruest et al., 2004; Miller et al., 2007; Verzi et al., 2007), Dlx genes are expressed such that *Dlx5/6* are restricted to mdBA1, while *Dlx1/2* are in both mdBA1 and mxBA1. *Dlx5/6* induce and/or maintain expression of the genes that promote the development of the lower jaw (Group A; see Fig. 8 legend). At the same time, *Dlx5/6* repress other sets of genes (Group B and Group C) so that their expression is mostly confined to mxBA1. Here, Group B and Group C genes promote upper jaw development. *Dlx1/2* participate in BA1 patterning by inducing and/or maintaining Group B genes in mxBA1. By contrast, in mdBA1, *Dlx1/2* positively regulate Group A genes and repress Group B genes, directly or indirectly, to specify lower jaw fate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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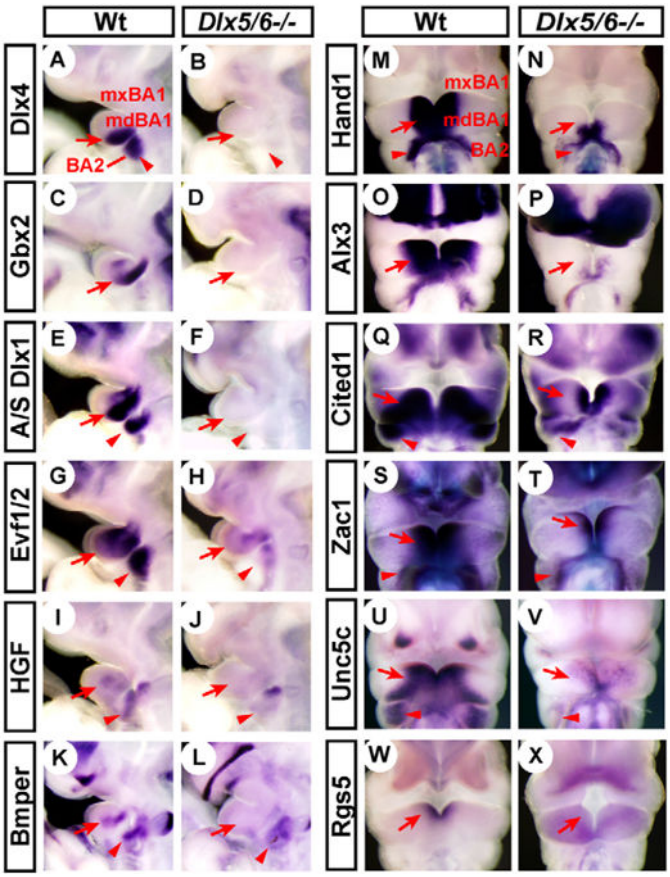

Fig. 1. Branchial arch expression patterns of the genes downregulated in *Dlx5/6*$^{-/-}$

Lateral views (**A–L**) or frontal views (**M–X**) of wild-type and *Dlx5/6*$^{-/-}$ E10.5 mouse embryos processed by whole-mount in situ hybridization. Arrows and arrowheads indicate changes in gene expression in mdBA1 and BA2, respectively.

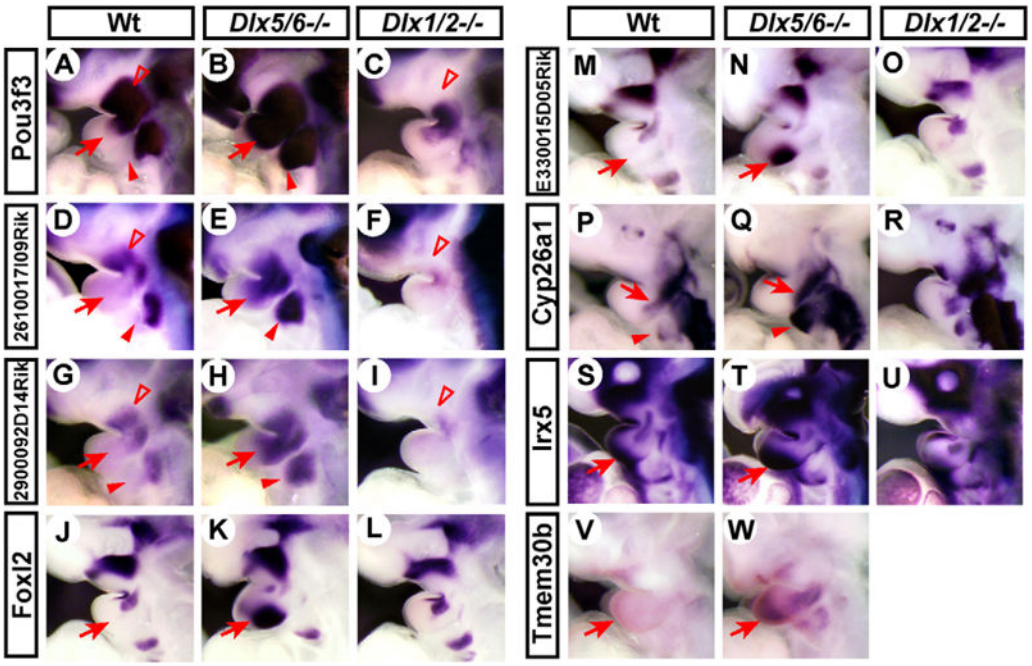

Fig. 2. Branchial arch expression patterns of the genes upregulated in *Dlx5/6*$^{-/-}$

(**A–W**) Lateral views of wild-type, *Dlx5/6*$^{-/-}$ and *Dlx1/2*$^{-/-}$ E10.5 mouse embryos processed by whole-mount in situ hybridization. Arrows and solid arrowheads indicate upregulation of expression in *Dlx5/6*$^{-/-}$ mdBA1 and distal BA2, respectively; open arrowheads indicate downregulation of expression in *Dlx1/2*$^{-/-}$ mxBA1.

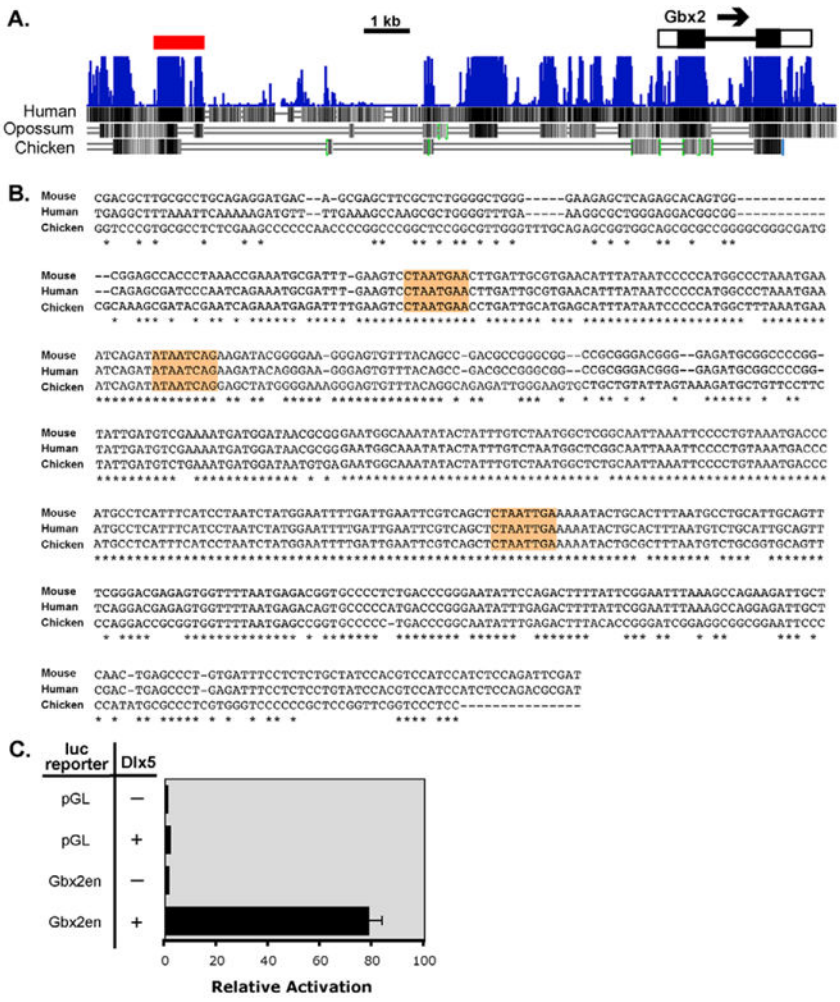

Fig. 3. Identification of a Dlx-regulated enhancer upstream of *Gbx2*

(**A**) Evolutionary conservation of the genomic sequence upstream of *Gbx2* analyzed using 30-way multiz alignment (Blanchette et al., 2004). Image generated using University of California Santa Cruz Genome Browser. Blue, degree of conservation among mammals; black vertical bars, conservation in each species as indicated; red bar, the *Gbx2* enhancer used for the reporter assay in C; black box, coding region of an exon; white box, untranslated region. (**B**) ClustalW alignment (Larkin et al., 2007) of the 0.6 kb region of the *Gbx2* enhancer conserved down to chicken. *, conserved nucleotides; putative Dlx-binding sites are highlighted in orange. (**C**) Results of the luciferase reporter activation assay. pGL, minimal promoter-reporter plasmid without an enhancer; Gbx2en, pGL plasmid with mouse *Gbx2* enhancer; − Dlx5, co-transfected with empty expression vector; + Dlx5, co-transfected with Dlx5 expression vector.

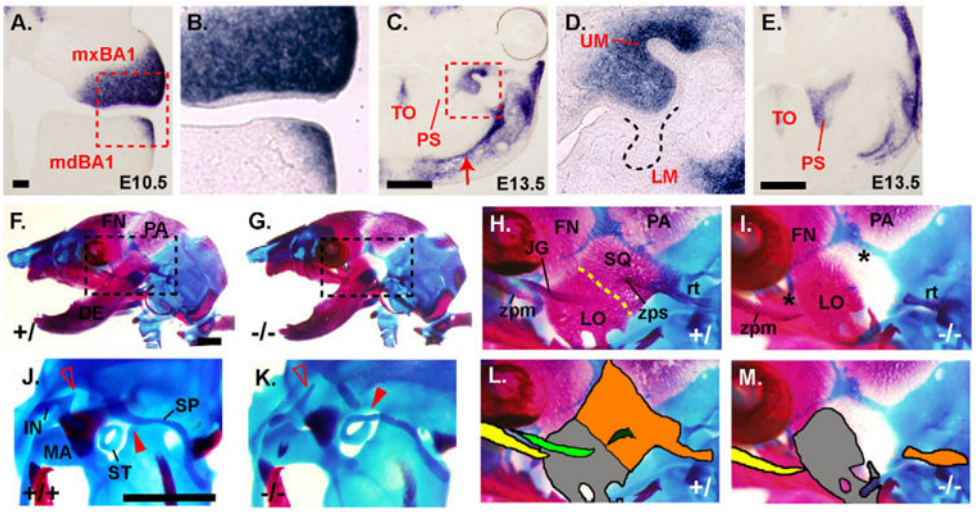

Fig. 4. Expression of *Pou3f3* RNA during jaw development and craniofacial skeletal defects in *Pou3f3*$^{-/-}$ mutants

(**A–E**) In situ hybridization for *Pou3f3* on the coronal sections of E10.5 (A,B) and E13.5 (C–E) wild-type heads. B and D are high-magnification views of the boxed areas in A and C, respectively. C and E are from the same head, but C is rostral to E. Arrow in C, mdBA1 expression of *Pou3f3*. (**F–M**) Head skeleton of E18.5 wild-type (F,H,J,L) and *Pou3f3*$^{-/-}$ (G,I,K,M) mice stained with Alcian Blue (cartilage) and Alizarin Red (bone). H and I are enlargements of the boxed areas in F and G, respectively. Asterisks in I indicate the absence of jugal and squamosal bone in the mutant. (J,K) Otic capsule and middle ear ossicles. Open arrowheads, incus phenotype of the mutant; solid arrowheads, abnormal attachment of stapes and styloid process in the mutant. L and M are the same pictures as H and I, but with individual skeletal elements highlighted by color: yellow, zygomatic process of maxilla; green, jugal; dark green, zygomatic process of squamosal; orange, squamosal; gray, lamina obturans. DE, dentary; FN, frontal bone; IN, incus; JG, jugal; LM, lower molar; LO, lamina obturans; MA, maleus; PA, parietal bone; PS, palatal shelf; rt, retrotympanic process of squamosal bone; SP, styloid process; SQ, squamosal bone; ST, stapes; TO, tongue; UM, upper molar; zpm, zygomatic process of maxilla; zps, zygomatic process of squamosal bone. Scale bars: 0.1 mm in A; 0.5 mm in C,E; 1 mm in F,J.

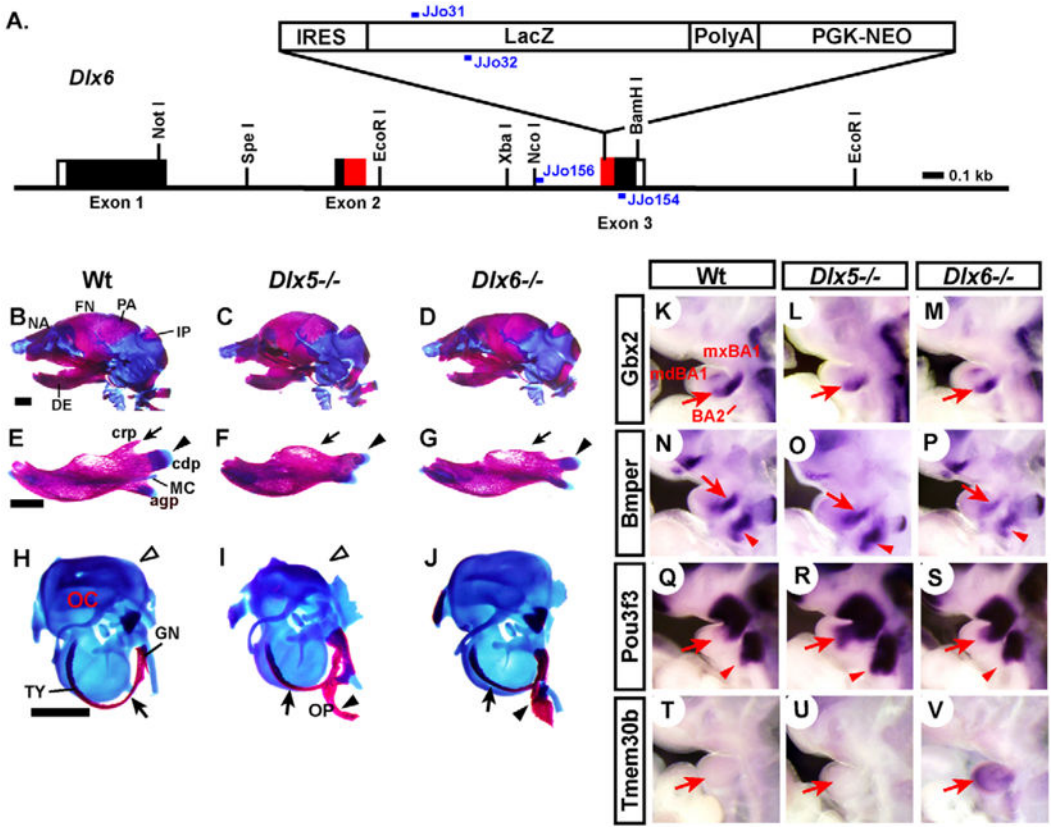

Fig. 5. Comparison of *Dlx5*$^{-/-}$ and *Dlx6*$^{-/-}$ head skeletal phenotypes and branchial arch gene expression changes

(**A**) Structure of the *Dlx6-lacZ* (*Dlx6*$^{-}$) allele. Black boxes, exon coding region; white boxes, untranslated region; red boxes, homeodomain; blue bars, PCR primers for genotyping. (**B–J**) Head skeleton and skeletal elements of E18.5-P0 mice stained with Alcian Blue (cartilage) and Alizarin Red (bone). (B–D) Lateral views of the whole head. (E–G) Dentaries. (H–J) Otic capsules and associated skeletal elements. Arrows and arrowheads indicate the skeletal abnormalities of the mutants; see text for details. (**K–V**) Lateral views of E10.5 mouse embryos processed by whole-mount in situ hybridization. Arrows and arrowheads, downregulation (K–P) or upregulation (Q–V) of expression in the mutant mdBA1 and BA2, respectively. agp, angular process; cdp, condylar process; crp, coronoid process; GN, gonial; IP, interparietal; MC, Meckel's cartilage; NA, nasal bone; OC, otic capsule; OP, os paradoxicum; TY, ectotympanic; for remainder, see legend to Fig. 4. Scale bar: 1 mm.

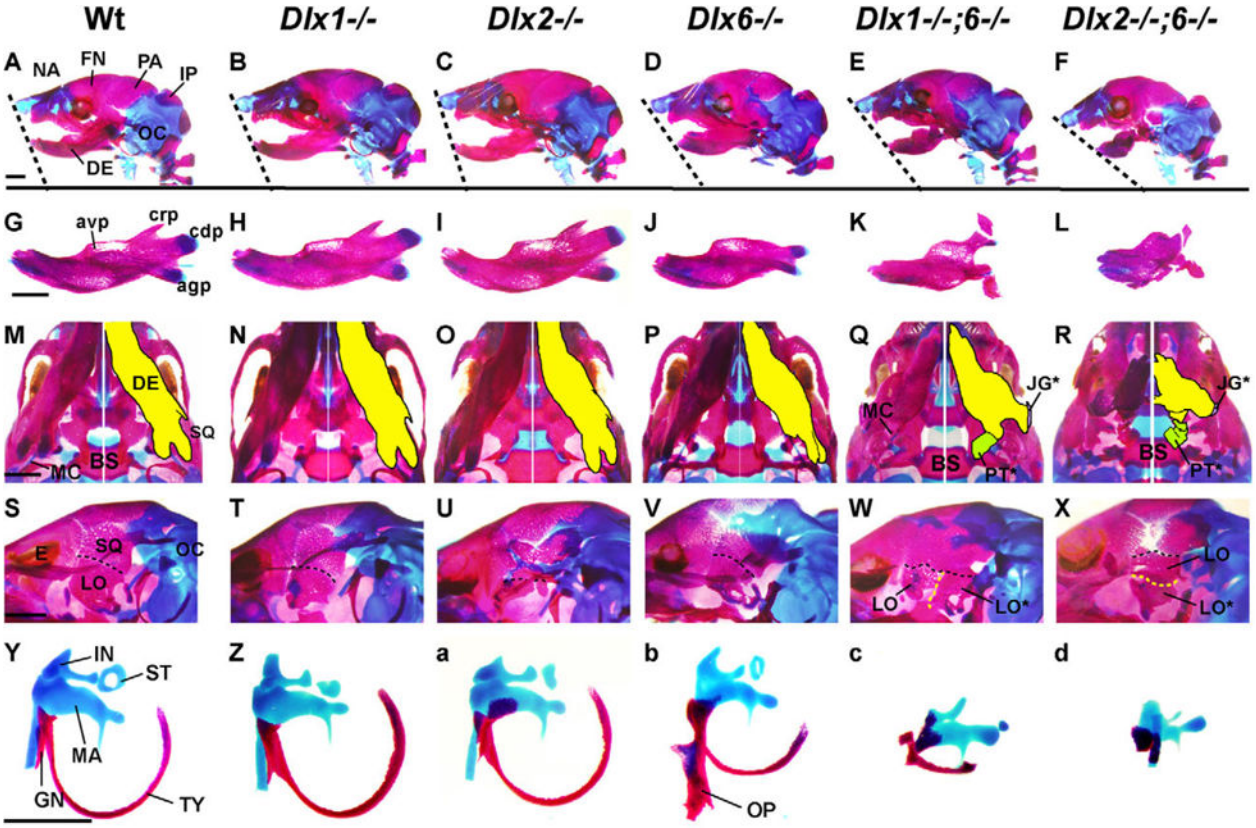

Fig. 6. *Dlx1*$^{-/-}$;*6*$^{-/-}$ and *Dlx2*$^{-/-}$;*6*$^{-/-}$ head skeleton phenotypes

Head skeleton and skeletal elements of E18.5-P0 mice stained with Alcian Blue (cartilage) and Alizarin Red (bone). (**A–F**) Lateral views of the whole head. (**G–L**) Dentaries. (**M–R**) Skull base views; the right half is a mirror image of the left half with individual skeletal components highlighted by color. (**S–X**) Oblique lateral views of the head after removing dentary. (**Y–d**) Middle ear ossicles, ectotympanic and gonial. Note that os paradoxicum (OP) of *Dlx1*$^{-/-}$;*6*$^{-/-}$ and *Dlx2*$^{-/-}$;*6*$^{-/-}$ has been integrated into the skull base (see Fig. S6E,F in the supplementary material), and thus excluded from c and d. avp, alveolar process; BS, basisphenoid; E, eye; PT, pterygoid; for remainder, see legends to Figs 4 and 5. *, duplicates found in the mutants. Scale bars: 1 mm.

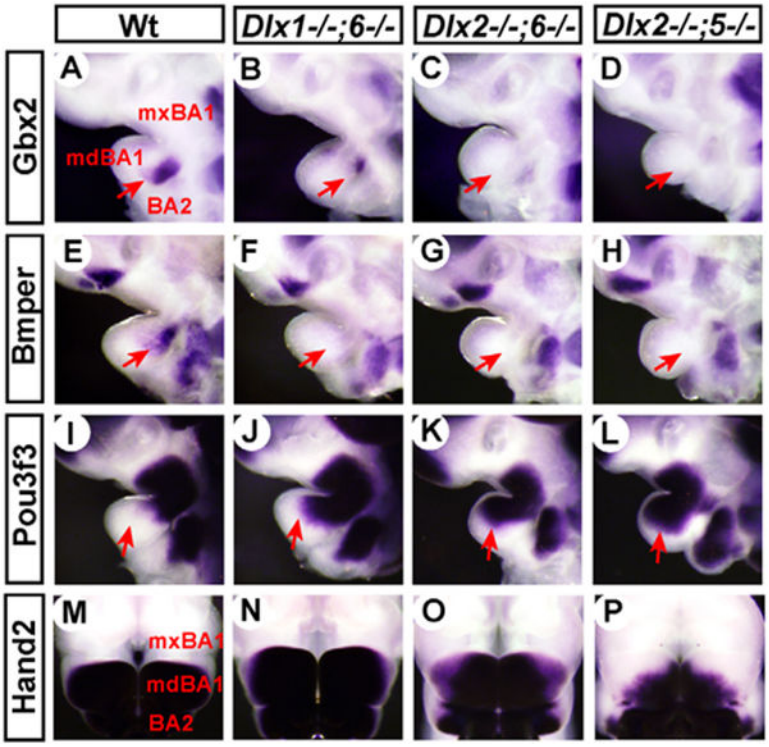

Fig. 7. Gene expression phenotypes in *Dlx1*$^{-/-}$;*6*$^{-/-}$, *Dlx2*$^{-/-}$;*6*$^{-/-}$ and *Dlx2*$^{-/-}$;*5*$^{-/-}$ mdBA1

Lateral views (**A–L**) and frontal views (**M–P**) of E10.5 mouse embryos processed by whole-mount in situ hybridization. Note that the samples in M–P are each one half of a hemisected face, but are digitally modified into a full face to help visualization. Arrows, expression changes in mdBA1.

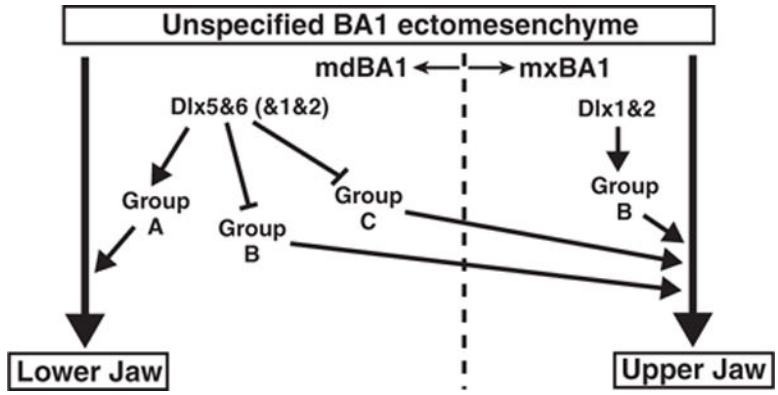

Fig. 8. A model for the mechanism of jaw patterning by Dlx genes

In BA1 mesenchyme, *Dlx5/6* are only expressed in mdBA1, whereas *Dlx1/2* are expressed in both mdBA1 and mxBA1. *Dlx5/6* induce and/or maintain expression of Group A genes (*Dlx3*, *Dlx4*, *Hand1*, *Hand2*, *Gbx2*, *Gsc*, *Alx3*, *Alx4*, *Bmper*, *Cited1*, *Zac1*, *Unc5c*, *Hgf*, *Rgs5*, *A/S Dlx1* and *Evf1/2*) in mdBA1, while repressing Group B (*Pou3f3*, *2610016I09Rik* and *2900092D14Rik*) and Group C (*Foxl2*, *E330015D05Rik*, *Cyp26a1* and *Irx5*) genes so that their expression is mostly confined to mxBA1. *Dlx1/2* induce and/or maintain Group B genes in mxBA1. By contrast, in mdBA1, *Dlx1/2* induce and/or maintain Group A genes and repress Group B genes. Presumably, Group A genes promote lower jaw development, whereas Group B and Group C genes promote upper jaw development.

Table 1

Mouse mutant phenotypes for the genes downstream of *Dlx5/6*

Gene	Mouse mutant phenotype	References
	Those with known craniofacial defects †	
Alx3, *Alx4**	Cleft nose, distal truncation and midline fusion of the dentary, hypoplastic skull vault	(Beverdam et al., 2001)
*Gsc**	Malformed nose and ear, hypoplastic dentary and malleus, absence of ectotympanic, abnormal tongue musculature	(Rivera-Perez et al., 1995; Yamada et al., 1995)
Hand1, *Hand2**	Reduced mandible, ectotympanic and gonial, cleft palate. Distal truncation and midline fusion of the dentary, fusion of the lower incisors	(Yanagisawa et al., 2003; Barbosa et al., 2007)
*Pitx1**	Reduced mandible, bifurcate tongue, cleft palate, reduced ectotympanic and missing gonial	(Lanctot et al., 1999)
Gbx2	Hypoplastic otic capsule and middle ear ossicles. Reduced mandible (*Gbx2*$^{-/-}$;*Fgf8*$^{+/-}$)	(Byrd and Meyers, 2005)
*Dlx3**	Hypoplasia of proximal dentary, dysmorphic incus and Meckel's cartilage, truncated ectotympanic (*Dlx3*$^{+/-}$;*Dlx5*$^{-/-}$)	(Depew et al., 2005)
Hgf	Impaired ingression of muscle precursors and motor axons into the tongue	(Bladt et al., 1995; Dietrich et al., 1999; Caton et al., 2000)
Bmper	Reduced or missing laryngeal cartilages, hypoplastic skull vault, a cavity in basisphenoid, reduced squamosal bone	(Ikeya et al., 2006)
Foxl2	Eye lid malformation	(Crisponi et al., 2001; Uda et al., 2004)
Pou3f3	Loss of jugal and most of squamosal bone, fusion of stapes and styloid process	This study
	Those without known craniofacial defects	
Cited1	Placenta defects and embryonic growth restriction, neonatal lethality	(Rodriguez et al., 2004)
Zac1	Embryonic growth restriction, reduced ossification in vertebrae and limb, neonatal lethality	(Varrault et al., 2006)
Unc5c	Abnormal neuronal migration in cerebellum	(Ackerman et al., 1997)
Irx5	Defects in retina development and cardiac function	(Cheng et al., 2005; Costantini et al., 2005)
Cyp26a1	Posterior axis truncation, spina bifida, internal organ defects, abnormalities in vertebrae and hindbrain, mid-late gestation lethality	(Abu-Abed et al., 2001)

* Genes identified to be downstream of *Dlx5/6* in previous studies (Depew et al., 1999; Depew et al., 2002b).

† Only craniofacial phenotypes are listed for these genes.