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Concerted action of two *dlx* paralogs in sensory placode formation

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SUMMARY

Sensory placodes are ectodermal thickenings that give rise to elements of the vertebrate cranial sensory nervous system, including the inner ear and nose. Although mutations have been described in humans, mice and zebrafish that perturb ear and nose development, no mutation is known to prevent sensory placode formation. Thus, it has been postulated that a functional redundancy exists in the genetic mechanisms that govern sensory placode development. We describe a zebrafish deletion mutation, *b380*, which results in a lack of both otic and olfactory placodes.

The *b380* deletion removes several known genes and expressed sequence tags, including *dlx3* and *dlx7*, two transcription factors that share a homoeobox domain similar in sequence to the *Drosophila Distal-less* gene. *dlx3* and *dlx7* are expressed in an overlapping pattern in the regions that produce the otic and olfactory placodes in zebrafish. We present evidence suggesting that it is specifically the removal of these two genes that leads to the otic and olfactory phenotype of *b380* mutants. Using morpholinos, antisense oligonucleotides that effectively block translation of target genes, we find that functional reduction of both *dlx* genes contributes to placode loss. Expression patterns of the otic marker *pax2.1*, olfactory marker *anxV* and *eya1*, a marker of both placodes, in morpholino-injected embryos recapitulate the reduced expression of these genes seen in *b380* mutants. We also examine expression of *dlx3* and *dlx7* in the morpholino-injected embryos and present evidence for existence of auto- and cross-regulatory control of expression among these genes.

We demonstrate that *dlx3* is necessary and sufficient for proper otic and olfactory placode development. However, our results indicate that *dlx3* and *dlx7* act in concert and their importance in placode formation is only revealed by inactivating both paralogs.

Key words: Zebrafish, *dlx3*, *dlx7*, Otic placode, Olfactory placode

INTRODUCTION

The vertebrate ear and nose arise from sensory placodes, which are morphologically visible thickenings of embryonic ectoderm. Early grafting studies in salamander (Jacobson, 1966; Yntema, 1933; Yntema, 1950) and chick (Waddington, 1937) have led to a model for placodal development (Torres and Giraldez, 1998). Sensory placodes develop from a stripe of ectoderm that lies laterally to the neural plate during gastrulation. Early in development, this stripe of tissue shows multiplacodal competence, such that any region of this stripe can develop into any of the sensory placodes, i.e. otic, olfactory or lens placode, given the proper inductive environment. As development proceeds, the domains within this stripe that are competent to form placodes become progressively restricted, ultimately producing localized regions committed to their respective placodal fates (Jacobson, 1963).

Several spontaneous and gene-targeted mutations have been described in mouse that produce ear defects (reviewed by Baker and Bronner-Fraser, 2001; Torres and Giraldez, 1998). In addition, genetic studies in zebrafish have identified over 20 different mutations that affect various aspects of otic placode development, including induction of the placode and morphogenesis and differentiation of the inner ear (Malicki et al., 1996; Whitfield et al., 1996). Nevertheless, the molecular mechanisms underlying the initial steps of sensory placode formation remain to be elucidated.

Recently, a large number of genes expressed in the placodes during development have been identified, in particular for the otic placode (reviewed by Baker and Bronner-Fraser, 2001; Torres and Giraldez, 1998). *dlx3* and *dlx7* are two homeobox-containing genes that are among some of the earliest genes expressed in the otic and olfactory primordia in zebrafish (Akimenko et al., 1994; Ekker et al., 1992; Ellies et al., 1997). The expression pattern of these two genes during development is strikingly coincident with the model of progressive restriction for placodal development. In mid-to-late gastrulation, *dlx3* and *dlx7* are expressed in a continuous stripe around the lateral edge of the neural plate that appears to be coincident with the region of multiplacodal competence. Expression then becomes downregulated everywhere in the stripe, except in the presumptive otic and olfactory primordia. *dlx3* and *dlx7* expression is restricted to the otic and olfactory primordia by the end of gastrulation when these regions have become committed to their placodal fates.

The vertebrate Dlx family consists of genes with homeoboxes similar in sequence to that of the *Drosophila Distal-less* gene (Cohen et al., 1989). Dlx genes have been

identified and cloned in several vertebrate species. Dlx genes are organized as linked pairs that lie in a convergently transcribed configuration and are separated by a few kilobases (kb) of intergenic sequence (Ellies et al., 1997; McGuinness et al., 1996; Nakamura et al., 1996; Simeone et al., 1994), and are thought to have originated from the duplication of an ancestral gene pair. Six Dlx genes constituting three linked pairs have been identified in mice (Nakamura et al., 1996; Porteus et al., 1991; Price et al., 1991; Robinson and Mahon, 1994; Robinson et al., 1991; Simeone et al., 1994; Stock et al., 1996; Weiss et al., 1995) and humans (Nakamura et al., 1996; Scherer et al., 1995; Simeone et al., 1994), and eight Dlx genes have been identified in zebrafish (Akimenko et al., 1994; Ekker et al., 1992; Stock et al., 1996), six of which constitute three pairs, and *dlx5* and *dlx8*, which are not linked to one another (Ellies et al., 1997; Stock et al., 1996). Expression of the different Dlx genes overlaps in many structures during early development, including the forebrain, migrating neural crest, branchial arches, otic and olfactory placodes, and fins/limbs (Akimenko et al., 1994; Ekker et al., 1992; Ellies et al., 1997; Morasso et al., 1995; Acampora et al., 1999; Bulfone et al., 1993; Depew et al., 1999; Dolle et al., 1992; Eisenstat et al., 1999; Fernandez et al., 1998; Neidert et al., 2001; Papalopulu and Kintner, 1993; Porteus et al., 1991; Price et al., 1991; Qiu et al., 1997; Robinson and Mahon, 1994; Robinson et al., 1991; Simeone et al., 1994; Yang et al., 1998). However, there are exceptions to this pattern, such as the differential expression of linked Dlx genes during mouse tooth (Zhao et al., 2000) and placental development (Morasso et al., 1999).

In vitro studies have demonstrated that Dlx proteins share similar DNA-binding properties, and they are thought to act as transcriptional regulators (Feledy et al., 1999; Liu et al., 1997; Zerucha et al., 1997; Zerucha et al., 2000; Zhang et al., 1997). Much of the proposed function of Dlx genes is based on studies of their expression patterns. The idea that a functional redundancy may exist among these genes is largely based on the overlapping nature of their expression patterns and on analyses of Dlx-targeted gene disruptions in mice. Single knockouts for mouse *Dlx1* (Qiu et al., 1997) and *Dlx2* (Qiu et al., 1995), which constitute a linked pair, have very similar craniofacial abnormalities. However, the double *Dlx1/Dlx2* mouse knockout has dentition abnormalities not seen in either of the single knockouts (Qiu et al., 1997), providing evidence for functional redundancy among these genes. *Dlx5* mouse knockouts also show craniofacial malformations and inner ear defects (Acampora et al., 1999; Depew et al., 1999). *Dlx3* mouse knockouts die of early placental failure (Morasso et al., 1999), and no other defects have been reported. In humans, a four base pair deletion in the *DLX3* gene is linked to Tricho-dento-osseus (TDO) syndrome, which is a hereditary autosomal dominant disorder characterized by abnormalities in the hair, teeth and cranial bones (Wright et al., 1997; Price et al., 1998). No known mutations affecting Dlx genes have been identified to date in zebrafish.

Based on their expression pattern, we test here the hypothesis that *dlx3* and/or *dlx7* may play a crucial role in determination and development of the otic and olfactory placodes. We describe a large, γ-ray induced deletion in zebrafish, *b380*, which removes both *dlx3* and *dlx7*. Homozygotes for the deletion show no evidence of otic and olfactory development. We also demonstrate that injection of *dlx3* and *dlx7* antisense morpholino oligonucleotides into zebrafish embryos produces an otic and olfactory phenotype similar to that of *b380* mutants. Together, these data indicate that *dlx3* and *dlx7* share an essential and partially redundant function in otic and olfactory placode development.

MATERIALS AND METHODS

Strains

Both the AB line (Eugene, OR) and a partially inbred line of store-bought fish were used as wild-type lines. The *b380* mutation was induced in the AB line with γ-rays (Fritz et al., 1996), and these mutants were hybridized with the line of store-bought fish.

In situ hybridization

Zebrafish (*Danio rerio*) embryos were raised at 28.5°C and staged as previously described (Kimmel et al., 1995). Embryos were fixed in phosphate-buffered solution containing 4% paraformaldehyde. Whole-mount in situ hybridization was performed as described (Thisse, 1998) using single-stranded RNA probes labeled with digoxigenin-UTP or fluorescein-UTP (Boehringer Mannheim). The zebrafish *dlx3* (Ekker et al., 1992), *dlx7* (Ellies et al., 1997), *eya1* (Sahly et al., 1999), *pax2.1* (Krauss et al., 1991) and *pax8* (Pfeffer et al., 1998) probes have been previously described.

Morpholino injections

Morpholinos were obtained from Gene Tools, LLC. The antisense oligonucleotide sequences were designed to bind to the 5′ UTR or flanking sequences including the initiation methionine. To minimize the possibility of nonspecific effects, we generated two morpholinos complementary to non-overlapping sequence for each gene. Sequences were as follows: *dlx3-1* MO, 5′-ATATGTCGGTCC-ACTCATCCTTAAT-3′; *dlx3-2* MO, 5′-TTTCCAAGGCAGACC-GAAGCAAGTC-3′; *dlx7-1* MO, 5′-TAACCGTCAAGTCCAT-AAAGCCCGA-3′; and *dlx7-2* MO, 5′-TCAGACATGAAACTCA-TAGACATCA-3′. The standard control morpholino (5′-CCTCTT-ACCTCAGTTACAATTTATA-3′) from Gene Tools, LLC was also injected in some experiments. Morpholino injections were essentially performed as described (Nasevicius and Ekker, 2000). One- to eight-cell embryos were injected with 8-22 ng of morpholino. No phenotypic differences were observed when injecting between 8-20 ng morpholino per embryo. Above 20 ng, some embryos showed nonspecific necrosis. In all subsequent experiments, embryos were injected with a total amount of 16 ng of morpholino oligonucleotide(s), i.e. 16 ng *dlx3* MO, 16 ng *dlx7* MO or (8 ng + 8 ng) *dlx3* MO and *dlx7* MO. Double injections were made with all possible combinations of *dlx3* MOs with *dlx7* MOs.

RNA Injections for MO rescue

We amplified a 1 kb *dlx7* fragment, including poly A sequence, from 17 h zebrafish cDNA. The following primer was used to amplify the 5′ end of the fragment: 5′-CGGTTAATGATGTCTATGAGTTTC-3′. This generated a fragment that begins with nucleotide position –6 with respect to the ATG start codon (A is +1) and does not contain the sequence complementary to *dlx7-1* MO. The PCR fragment was cloned into pCRII-TOPO vector (Invitrogen). A 925 bp *dlx3* fragment, beginning with nucleotide position –26 (with respect to the ATG start codon), was cloned into pT3TS vector (provided by Dr Steve Ekker). The vector contains a poly A sequence following the 3′ region of the insert fragment. The *dlx3* fragment did not contain the sequence complementary to *dlx3-2* MO. Capped sense RNA for both of these constructs was transcribed using Ambion mMessage mMachine in vitro transcription kits. *dlx3* and *dlx7* RNA were injected into one-cell stage zebrafish embryos at several different concentrations to determine a nontoxic concentration level for each RNA. The RNA was

then used in morpholino rescue experiments. *dlx7* RNA (6 pg) was co-injected with (9 ng + 9 ng) *dlx3-1* MO and *dlx7-1* MO, and 5 pg of *dlx3* RNA was co-injected with 18 ng of *dlx3-2* MO.

RESULTS

Co-expression of *dlx3* and *dlx7*

We examined *dlx3* and *dlx7* expression patterns in zebrafish embryos using single gene-specific probes in whole-mount RNA in situ hybridization. We find the expression patterns of these genes to be highly overlapping based on comparisons between these different in situ hybridization. At the late gastrula stage, *dlx3* and *dlx7* are expressed in a continuous stripe of presumptive ectoderm around the lateral edge of the neural plate (not shown). After initiation of somitogenesis, this expression becomes progressively restricted to the prospective otic and olfactory primordia. At the 18-somite stage, *dlx3* and *dlx7* expression is detectable in the olfactory placodes, otic vesicles, visceral arches (Fig. 1A,B) and the median fin fold (not shown). Our data confirms the patterns originally described by others using these single gene-specific probes (Ekker et al., 1992; Akimenko et al., 1994; Ellies et al., 1997).

To definitively determine whether these genes are co-expressed by the same cells, we examined the expression overlap in double in situ hybridization. We find that *dlx3* and *dlx7* are expressed in the same cells in the otic vesicles (Fig. 1D), olfactory placodes (Fig. 1C), visceral arches and median fin fold (not shown) at 18 somites. These results conclusively demonstrate that these two Dlx genes are expressed in many of the same cells during zebrafish embryonic development, and that the domains of co-expression include the otic and olfactory primordia. This high degree of expression overlap indicates a potential for functional redundancy among these genes.

b380 mutants completely lack otic and olfactory placodes

In a γ-ray mutagenesis screen for deletions that remove specific cloned genes in zebrafish, we have identified a mutation, *b380*, that removes both *dlx3* and *dlx7* (Fritz et al., 1996). The deletion is approximately 6 cM in size and, in addition to *dlx3* and *dlx7*, removes at least six other known genes and seven expressed sequence tags (ESTs). *b380* heterozygotes develop normally to adulthood and show no obvious defects. From early somitogenesis up to approximately 20 somites, embryos homozygous for the *b380* deletion show two predominant phenotypes; they appear to completely lack otic and olfactory placodes (Fig. 2E, olfactory phenotype not shown), and they also fail to form somites. The embryos begin to develop a severe compound phenotype during later stages of somitogenesis, presumably because of the removal of so many genes, and they typically die at ~56 hours of development (56 h).

dlx3 and *dlx7* morpholino injections disrupt otic development

The expression patterns of *dlx3* and *dlx7* suggested their specific removal in the large *b380* deletion was the cause of the placodal defects in this mutant. We therefore wanted to test whether the disruption of *dlx3* and *dlx7* could mimic the *b380* placodal phenotype. We also wanted to examine the individual functions of these genes to determine whether a functional redundancy existed, which was suggested by the overlapping nature of the expression patterns. To address these issues, morpholino oligonucleotides (*dlx3* MO and *dlx7* MO) were generated. Morpholinos are antisense, chemically modified oligonucleotides that have been shown to bind and block translation of mRNA in vitro, in tissue cell culture (Summerton et al., 1997; Summerton et al., 1999), and in some in vivo applications (Arora et al., 2000; Qin et al., 2000; Heasman et al., 2000). Recently, it has been demonstrated that morpholinos function as highly effective and specific translational inhibitors in zebrafish (Nasevicius and Ekker, 2000). We injected these morpholinos both individually and together (*dlx3+7* MO) into one-cell stage wild-type embryos and then examined these embryos for morphological defects. To address the issue of morpholino specificity for the target genes, we generated two morpholinos complementary to non-overlapping sequence for each gene, and we injected these in all possible combinations in the co-injection analyses. In addition, we also injected a control morpholino.

Embryos injected with *dlx7* MO do not exhibit any morphologically detectable defects and are indistinguishable from wild type (Fig. 2A,B). By contrast, otic vesicles are smaller than wild-type in ~98% (79/81) of *dlx3* MO-injected embryos. By 24 h, the otic vesicles of these embryos exhibit smaller lumens and typically only one otolith (Fig. 2C, Fig. 3). This effect is very consistent over a wide range of concentrations of injected MO (see Materials and Methods) and is observed for both *dlx3* MOs. We did not detect any other visible defects in the injected embryos up to 7 days of development, and no defects were observed in embryos injected with the control morpholino (not shown). We conclude from these results that *dlx3* is necessary for proper otic development, whereas *dlx7* is dispensable in this process.

Embryos co-injected with *dlx3+7* MO displayed even more severe otic defects than observed in embryos injected with *dlx3* MO alone (Fig. 2D). Whereas 89% (72/81) of embryos injected with *dlx3* MO developed at least one otolith, 100% (57/57) of embryos injected with *dlx3+7* MO completely lacked otoliths and formed only very small lumens (Fig. 3). Because the *dlx7* MO does not produce any defects when injected singly, it appears that *dlx3* and *dlx7* are partially functionally redundant in the process of otic development. This also suggests that the otic phenotype of *b380* mutants is mediated by the loss of *dlx3* and *dlx7*.

To address the redundancy of *dlx3* and *dlx7* further, and to test the specificity of the *dlx3* MO and *dlx7* MO, we attempted to rescue the otic defects of MO-injected embryos by co-injection of either *dlx3* or *dlx7* in vitro synthesized mRNA. Forty percent (21/53) of embryos co-injected with *dlx3* MO and *dlx3* mRNA had larger lumens and two otoliths in at least one otic vesicle, compared with only 2% (2/81) of embryos injected with *dlx3* MO alone (Fig. 2G,H; Fig. 3). In addition, we find that 53% (21/40) of embryos co-injected with *dlx7* mRNA and *dlx3+7* MO had larger lumens than those injected with *dlx3+7* MO and at least one otolith (Fig. 2I,J; Fig. 3). Increasing amounts of either *dlx3* or *dlx7* mRNA led to toxicity and developmental disruption of the injected embryos. We have not been able to elicit otolith formation in *b380* mutant embryos by injecting *dlx3* and/or *dlx7* mRNA, although we can occasionally observe formation of a small otic vesicle (data not

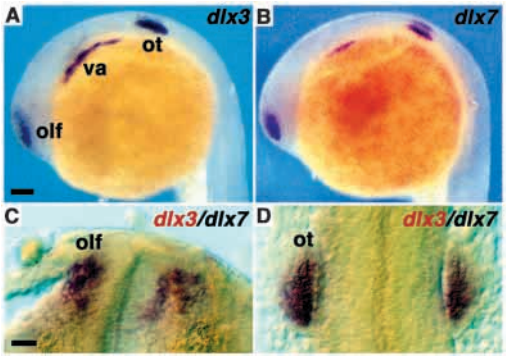

Fig. 1. *dlx3* and *dlx7* are co-expressed by the same otic and olfactory cells. (A,B) Whole-mount RNA in situ hybridization with probes for (A) *dlx3* and (B) *dlx7* at 18 somites. Lateral views, anterior towards the left. (C,D) Flat-mount double in situ hybridization showing (C) olfactory and (D) otic expression of *dlx3* and *dlx7* at 18 somites. *dlx3* expression is shown in red in both panels with *dlx7* expression shown in purple. Anterior is towards the top. ot, otic vesicle; olf, olfactory placode; va, visceral arches. Scale bars: in A, 100 μm for A,B; in C, 25 μm for C,D.

shown). This could be due to the limiting amounts of mRNA that can be injected into embryos.

Otic and olfactory development is blocked prior to differentiation

To determine at what stage placode development fails when *dlx3* and/or *dlx7* function is lost, we examined the expression of otic and olfactory markers that are known to be transcribed during different stages of placode formation and differentiation. *eya1* is a member of the *eyes absent*-like gene family, which encodes a group of transcriptional coactivators, and it is transcribed in both the otic and olfactory structures as they undergo differentiation (Sahly et al., 1999). At 18 somites, *eya1* transcripts are detectable in the otic vesicles, olfactory placodes, anterior pituitary, anterior and posterior lateral line placodes, and in the developing somites in wild-type embryos (Fig. 4A,G), embryos injected with a control MO (Fig. 4B,H), and in embryos injected with *dlx7* MO (Fig. 4C,I). However, the number of *eya1*-expressing otic and olfactory cells is reduced in embryos injected with *dlx3* MO (Fig. 4D,J). *eya1* expression is reduced even further in otic domains of embryos injected with *dlx3+7* MO (Fig. 4E) and is reduced to a level comparable with that observed for *dlx3* MO in the olfactory domain (Fig. 4K). Expression is undetectable in the otic domain of *b380* mutants (Fig. 4F), and olfactory expression is reduced to almost a single cell (Fig. 4L). Similar results were obtained for expression of *anxV* (Fig. 4M-R), a later marker of olfactory placodes (S. Farber and M. E. Halpern, personal communication). These results show that otic and olfactory placode development are already blocked at the time when patterning and differentiation normally take place, suggesting an earlier role for *dlx3* and *dlx7* function in the development of these structures.

Otic placode formation is blocked prior to *pax2.1* otic expression but after initiation of *pax8* expression

To determine whether *dlx3* and *dlx7* are required earlier for placode formation, we examined the expression of two early markers of sensory placodes, *pax8* and *pax2.1*. *pax8* is the earliest known marker for otic development in both fish and mammals (Pfeffer et al., 1998). In zebrafish, *pax8* expression is detectable in the presumptive otic primordia as early as 80% epiboly, around the same time that *dlx3* expression becomes detectable and several hours before otic placodes become morphologically visible. We find that otic expression of *pax8* in *b380* and *dlx3* MO-, *dlx7* MO- and *dlx3+7* MO-injected embryos is indistinguishable from wild type at 3 somites (Fig. 5A-E). All of these embryos show strong *pax8* expression in the presumptive otic primordia and the pronephros at this stage.

pax2.1 expression begins in the otic primordia later than *pax8*, at ~3 somites, and expression in this domain lasts through the otic vesicle stage (Krauss et al., 1991; Pfeffer et al., 1998). In addition to the otic expression domain, *pax2.1* is also expressed strongly in the optic stalk, midbrain-hindbrain boundary (mhb) and pronephros at this stage (Fig. 5F). Expression of this gene appears normal at 3 somites in embryos injected with *dlx7* MO (Fig. 5G). By contrast, embryos injected with *dlx3* MO show reduced expression of *pax2.1* in the presumptive otic primordia (Fig. 5H). This reduction in expression is specific; expression levels in the optic stalk, mhb

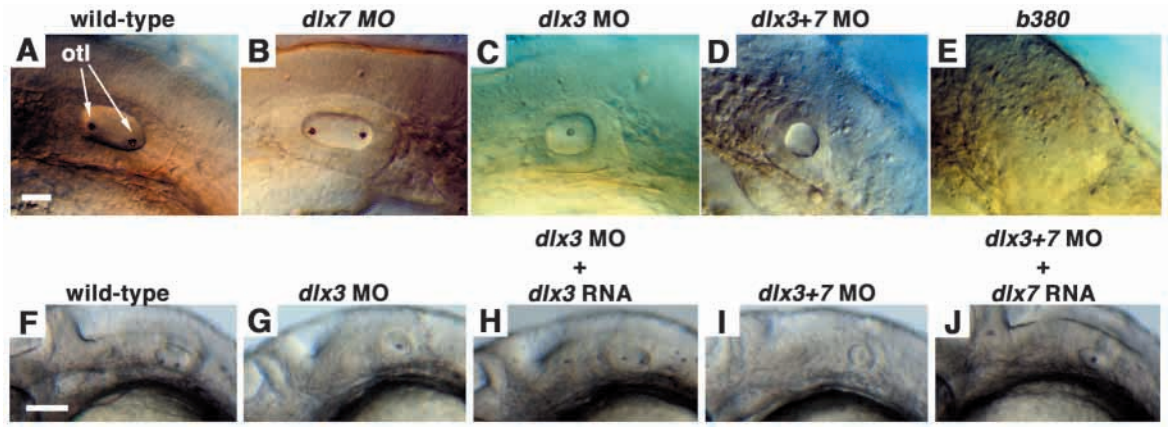

Fig. 2. Loss of *dlx3* and *dlx7* function leads to a reduction in otic vesicle size. Lateral views, anterior towards the left, of otic vesicles in (A-E) 24 hour and (F-J) 27 hour embryos. (A,F) Wild-type, (B) *dlx7* MO, (C,G) *dlx3* MO, (D,I) *dlx3+7* MO, (E) *b380* mutant, (H) *dlx3* MO + *dlx3* RNA and (J) *dlx3+7* MO + *dlx7* RNA embryos. otl, otolith. Scale bars: in A, 25 μm for A-G; in F, 75 μm for F-J.

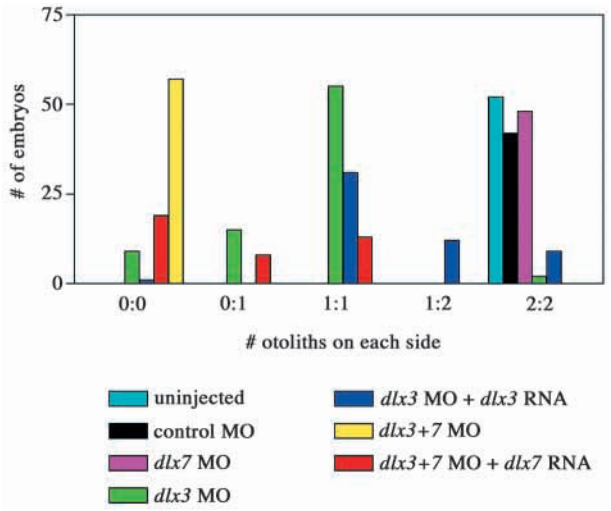

Fig. 3. Morphological data for morpholino injection and RNA rescue. Embryos were injected with morpholinos alone or with RNA, as indicated, and otic vesicles were examined in individual embryos. Severity of otic defect was scored by counting the number of otoliths visible in each otic vesicle: 0:0, no otoliths detectable in either otic vesicle of embryo; 0:1, one otolith detectable in one otic vesicle, etc.

and pronephros are comparable with those seen in wild-type and *dlx7* MO-injected embryos. We are unable to detect any *pax2.1* expression in the otic domain of embryos co-injected with *dlx3+7* MO or in *b380* mutants (Fig. 5I,J). This loss of expression is also specific to the otic domain, similar to the pattern seen in *dlx3* MO-injected embryos. Thus, in the absence of dlx3 and dlx7 function, otic placode formation appears to be blocked after initiation of *pax8* transcription in the otic domain but before *pax2.1* otic expression. It is during this period from late gastrulation to early somitogenesis that *dlx3* and *dlx7* are expressed in a stripe surrounding the neural plate.

Evidence for cross-regulation of *dlx3* and *dlx7* in morpholino-injected embryos

An additional level of complexity in understanding the function of *dlx3* and *dlx7* comes from potential cross-regulatory interactions between these two genes, which have been shown to exist among some Dlx genes (Zerucha et al., 1997; Zerucha et al., 2000). Therefore, we wanted to determine whether any similar cross-regulation exists between *dlx3* and *dlx7*. Because morpholinos target gene expression at the level of translation, we might expect *dlx3* and *dlx7* mRNA expression patterns to be unaffected in injected embryos in the absence of cross-regulation. However, while *dlx3* expression in embryos injected with *dlx7* MO is comparable with wild type at 6 somites (Fig. 6A,B), expression is noticeably upregulated in embryos injected with either *dlx3* MO alone (Fig. 6C) or co-injected with *dlx7* MO (Fig. 6D). At this same stage, *dlx7* expression is also upregulated in embryos injected with *dlx3* MO (Fig. 6G). By contrast, *dlx7* expression is downregulated in embryos injected with *dlx7* MO alone (Fig. 6F), and appears similar to wild-type in co-injected embryos (Fig. 6E,H) (see Table 1 for summary of data). Except for the noted up- and downregulation, the patterns of *dlx3* and *dlx7* expression appear to otherwise be normal in all of these MO-injected embryos. We also examined expression of *dlx3* and *dlx7* in *b380* homozygotes and, as expected, no transcripts for these genes were detected (not shown).

To exclude artifacts of injection or in situ hybridization, we performed these experiments three separate times with the same results. In addition, *dlx3* expression in *dlx7* MO embryos serves as in internal control for this experiment; we never observed an upregulation of expression in these embryos, making it unlikely that the injection process itself increases the permeability of the embryos during the hybridization procedure, leading to a perceived upregulation. In addition, we never observed an up- or downregulation of other markers we examined in embryos injected with the control morpholino (see Fig. 4B,H,N, for example). We independently confirmed aspects of this cross-regulation in a collaboration with K.

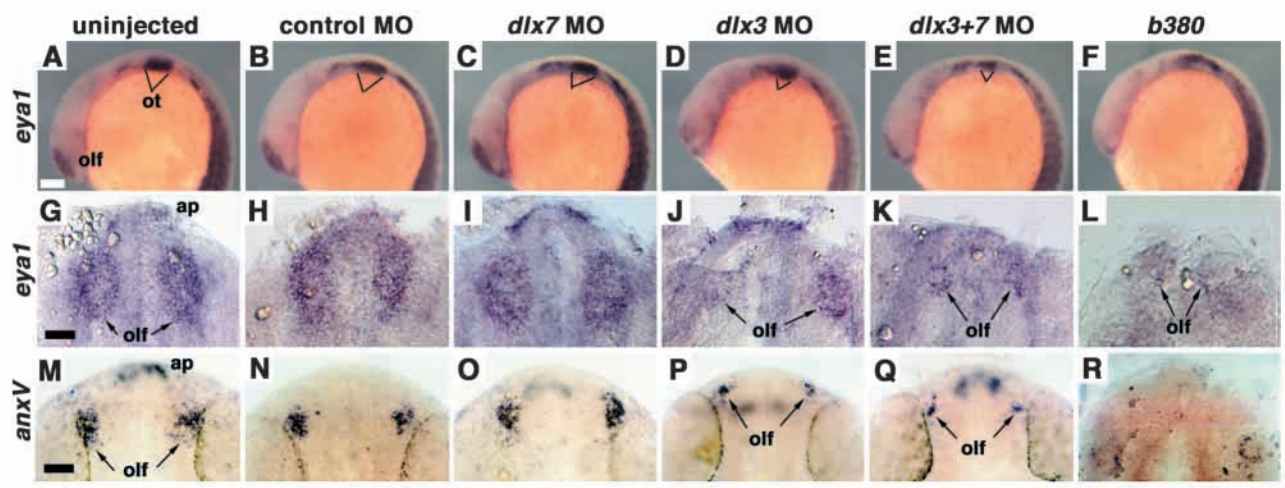

Fig. 4. Expression of otic and olfactory differentiation markers is reduced by loss of *dlx3* and *dlx7* function. (A,G,M) Wild-type, (B,H,N) control MO, (C,I,O) *dlx7* MO, (D,J,P) *dlx3*MO, (E,K,Q) *dlx3+7* MO and (F,L,R) *b380* mutant embryos. (A-L) Expression of *eya1* at 18 somites in (A-F) whole-mount and (G-L) flat-mount embryos. (M-R) Flat-mount in situ hybridization with *anxV* at 24 hours. (A-F) Lateral views, anterior towards the left. (G-R) Dorsal view, anterior towards the top of the panels. ot, otic vesicle; olf, olfactory placode; ap, anterior pituitary. Scale bars: in A, 100 μm in A-F; in G, 25 μm in G-L; in M, 25 μm in M-R.

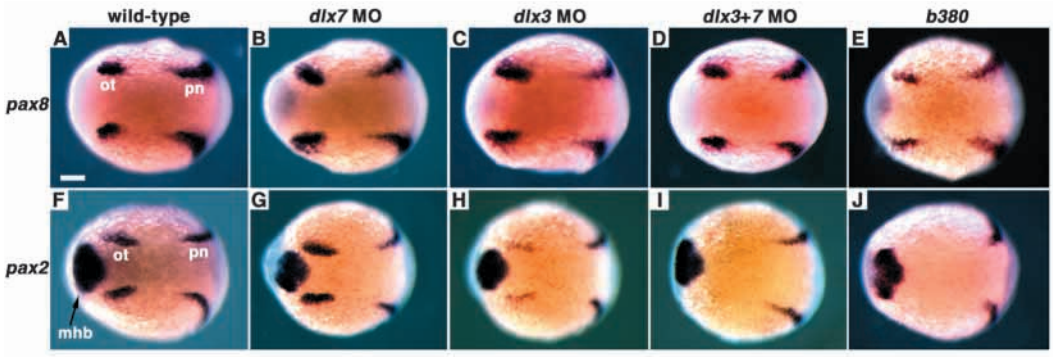

Fig. 5. *dlx3* and *dlx7* affect otic placode formation before the onset of *pax2.1* otic expression but after that of *pax8*. Whole-mount RNA in situ hybridization at 3 somites with (A,F) wild-type, (B,G) *dlx7* MO, (C,H) *dlx3*MO, (D,I) *dlx3+7* MO and (E,J) *b380* mutant embryos. (A-E) *pax8* is expressed in the presumptive otic primordia (ot) and the pronephros (pn) at this stage. Expression of this gene is unaffected by a loss of *dlx3* or *dlx7* function. (F-J) *pax2.1* is expressed in the presumptive otic primordia (ot), pronephros (pn), midbrain-hindbrain boundary (mhb) and optic stalk (not shown) at this stage. Expression is reduced specifically in the otic primordia of (H) *dlx3* MO-injected embryos, and is undetectable in (I) embryos injected with *dlx3+7* MO and in (J) *b380* mutants. All panels show dorsal views with anterior towards the left. Scale bar: 100 μm.

Wuennenberg-Stapleton in J. Ngai's laboratory (UC Berkeley, personal communication), who performed a microarray gene chip analysis comparing expression levels between RNA isolated from 16 h wild-type, *b380* mutant and *dlx3+7* MO injected embryos. In this analysis, a *dlx3* clone was spotted twice to serve as a control in the *b380* to wild-type RNA comparison. As expected, *dlx3* was strongly downregulated in RNA from *b380* embryos (see Table 2). These experiments also showed a statistically significant and reproducible upregulation of *dlx3* (approximately twofold) in the RNA from *dlx3+7* MO injected embryos.

We find that this overexpression of *dlx3* begins to dissipate by mid-to-late somitogenesis. At 21 somites, *dlx3* expression in *dlx7* MO injected embryos (Fig. 6J,N) is still comparable with wild type (Fig. 6I,M). Expression still appears to be upregulated in *dlx3* MO embryos, most notably in the visceral arches (Fig. 6K,O). However, the total number of cells expressing *dlx3* within the otic region is reduced, similar to the trend observed with other otic markers expressed during differentiation, such as *eya1* (Fig. 4A-E). Upregulation is not nearly as notable in *dlx3+7* embryos at the 21 somite stage (Fig. 6L,P). Expression may still be upregulated in otic cells, but the total number of expressing cells in this region is reduced even more than in *dlx3* MO embryos. Both intensity and size of the regions expressing *dlx3* in the olfactory organ and the visceral arches appear to be reduced in these embryos.

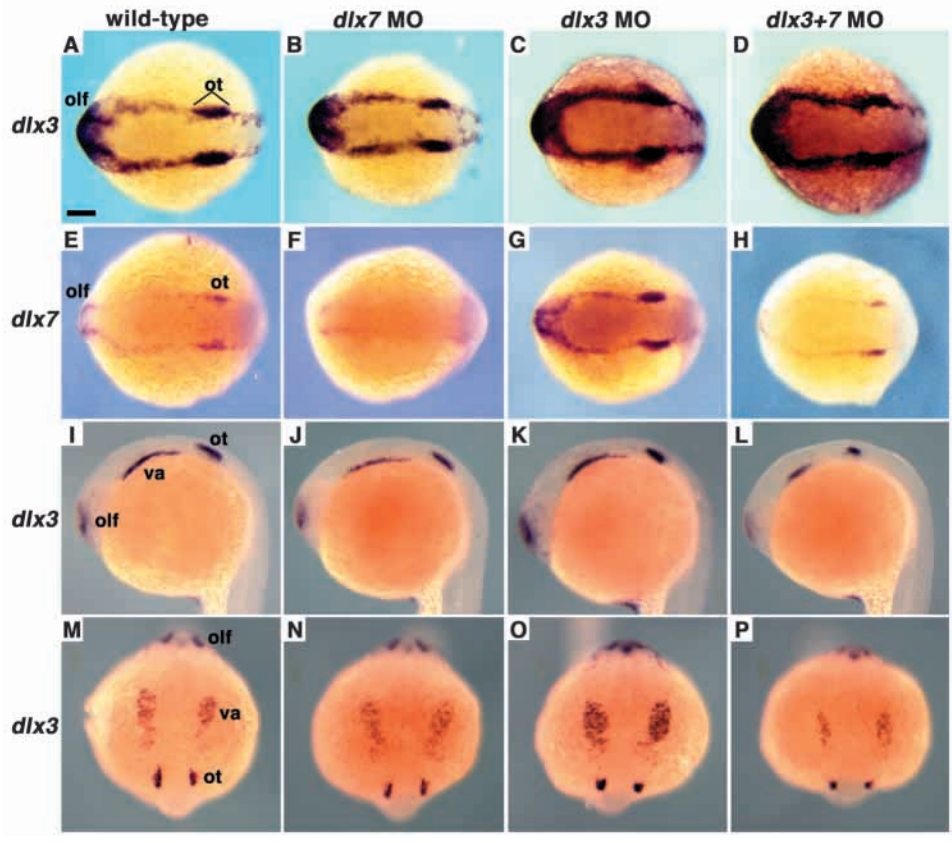

Fig. 6. Cross- and autoregulatory mechanisms monitor expression levels of *dlx3* and *dlx7*. Whole-mount in situ hybridization with (A,E,I,M) wild-type, (B,F,J,N) *dlx7* MO, (C,G,K,O) *dlx3* MO and (D,H,L,P) *dlx3+7* MO embryos. (A-D) *dlx3* and (E-F) *dlx7* expression at 6 somites, dorsal views, anterior towards the left. (I-P) *dlx3* expression at 21 somites. (I-L) Lateral views with anterior towards the left, (M-P) dorsoanterior views with anterior towards the top. ot, otic region; olf, olfactory region; va, visceral arches. Scale bar: 100 μm.

DISCUSSION

dlx3, *dlx7* and sensory placode formation

The zebrafish *dlx3* and *dlx7* genes have been identified as genes expressed during the initial and formative stages of sensory placode development (Akimenko et al., 1994; Ekker et al., 1992; Ellies et al., 1997; Torres and Giraldez, 1998). We

Table 1. Summary of cross- and autoregulation data

	Wild type	*dlx7* MO	*dlx3* MO	*dlx3+7* MO
dlx3 expression, 6 somites	0	0	+1	+1
dlx7 expression, 6 somites	0	–1	+1	0
dlx3 expression, 21 somites	0	0	+1	–1

0, expression levels similar to wild type; +1, increased expression levels compared with wild type; –1, decreased expression levels compared with wild type.

Table 2. Microarray data for *dlx3* expression

	Wild type	*b380*	*dlx3+7* MO
dlx3-1	0	3.11	–0.94
dlx3-2	0	2.63	–0.93

dlx3-1 and *dlx3-2* refer to two independent spots.
The numbers compare expression levels with wild type and are based on a log2 scale. Positive numbers indicate downregulation with respect to wild type. Values higher than 0.8 or lower than –0.8 are considered significant (K. Wuennenberg-Stapleton, personal communication).

provide functional evidence that *dlx3* and *dlx7* act in concert to promote otic and olfactory placode formation. Our data demonstrate that the expression domains of *dlx3* and *dlx7* are completely overlapping through the first 24 hours of development, creating the potential for redundant functions. The removal of both *dlx3* and *dlx7* activity by the *b380* deletion mutation leads to a failure of otic and olfactory placode development, both by morphological and gene expression criteria. Furthermore, the morpholino mediated knockdown experiments strongly support the view that the sensory placode phenotype elicited by *b380* mutant embryos is due to the lack of *dlx3* and *dlx7* gene activity. Specifically, loss of *dlx3* and *dlx7* function results in a severe reduction of the otic and olfactory placodes, mimicking the phenotype of *b380* mutant embryos. Interestingly, our findings suggest that while the functions of *dlx3* and *dlx7* are redundant, they are not equivalent. The loss of *dlx7* activity appears not to result in any detectable phenotype. However, *dlx3* MO injections lead to a clearly visible reduction in the size of the otic placode and inner ear. This suggests that *dlx3* alone is fully capable of supporting otic placode and inner ear formation, while *dlx7* can only partially compensate for the loss of *dlx3*.

Transplantation and extirpation experiments in a number of organisms have shown that competence to respond to placode-inducing signals is initially broadly distributed throughout ectoderm (Baker and Bronner-Fraser, 2001; Torres and Giraldez, 1998). As development proceeds, competent ectoderm becomes progressively restricted to a region lateral to the neural plate and subsequently commits to the appropriate placodal fates. Expression of *dlx3* and *dlx7* recapitulates these steps, beginning by late gastrulation (90% epiboly), and appears to coincide with the region competent to respond to placode inducing signals. Shortly after onset of somitogenesis, *dlx3* and *dlx7* expression is downregulated along the margin of the neural plate but upregulated in the prospective otic and olfactory placodes, possibly in concert with commitment to those fates.

Given our observations for *dlx3* and *dlx7* inactivation, we can envision two possible models for how these genes may function in the process of otic and olfactory placode development. One possibility is that *dlx3* and *dlx7* function prior to somitogenesis to maintain competence in ectodermal cells to respond to signals required for placode induction. Alternatively, these genes could be required later, immediately after their upregulation during early somitogenesis, for the proper development and differentiation of otic and olfactory placodal cells that have already been induced and committed to placodal fates. In support of the latter model, we find that at least some induction of otic characteristics occurs in the absence of *dlx3/7*. Expression of *pax8* is the earliest known manifestation of otic induction (80% epiboly) (Pfeffer et al., 1998), and *pax8* appears to be induced and expressed normally in *dlx3/dlx7*-deficient embryos. However, our *pax2.1* expression data better support the first model. *pax2.1* otic expression is initiated shortly after the onset of somitogenesis, and coincides with the time when *dlx3* and *dlx7* expression is upregulated in the otic anlagen (Krauss et al., 1991; Pfeffer et al., 1998). If *dlx3* and *dlx7* function is required only during this later stage for placodal differentiation after all inductive events have occurred, we would expect that at least initiation of *pax2.1* expression would occur normally. However, *pax2.1* is not expressed in *b380* mutant embryos or *dlx3/7* MO embryos, implying an earlier function for *dlx3* and *dlx7* in placodal development.

It has recently been shown that induction of the otic placode begins earlier than previously thought. Phillips et al. have demonstrated that zebrafish *fgf3* and *fgf8* cooperate during mid-gastrulation to induce the otic placode and *pax8* expression (Phillips et al., 2001). However, it has also been shown that induction of the otic placode is required and proceeds over a period of time in zebrafish (Mendonsa and Riley, 1999) and in other species [see Groves and Bronner-Fraser (Groves and Bronner-Fraser, 2000), for recent molecular data on chick]. The importance of continued inductive influences is further supported by mutations in genes that presumably disrupt hindbrain-derived signals and lead to inner ear defects (Chisaka et al., 1992; Cordes and Barsh, 1994; Epstein et al., 1991; Lufkin et al., 1991; Moens et al., 1998). For example, mutant alleles of zebrafish *valentino* gene result in inner ear abnormalities, and hindbrain expression of this gene does not begin until early somitogenesis (Moens et al., 1998; Moens et al., 1996). Thus, even after induction of *pax8*, the placodal ectoderm needs to respond to further signals, and it is possible that *dlx3* and *dlx7* are necessary to maintain this competence.

It should be noted that these two models are not mutually exclusive. For example, *dlx3* and *dlx7* could be required early for competence in preplacodal ectoderm, and later for proper patterning or differentiation of the inner ear and olfactory organ. We will be better able to pinpoint the time of action of these genes as more early markers for otic and olfactory primordia become available. However, we might have to await the availability of conditional mutations in zebrafish to fully determine the role of these genes.

Our results indicate that *pax8* expression is not sufficient to promote otic placode formation, or even *pax2.1* expression, which is highly overlapping with *pax8* during early somite stages. Targeted inactivation of the mouse *Pax8* gene also suggests that it is not required for otic placode development (Mansouri et al., 1998). Disruption of mouse *Pax2* (Favor et

al., 1996; Torres et al., 1996) or zebrafish *pax2.1* (Brand et al., 1996) results in later perturbations in otic vesicle development but does not prevent placode formation. Of other genes expressed in the otic placode, targeted disruption of mouse *Eya1* leads to the most severe inner ear defect described so far (Xu et al., 1999). However, initial otic placode induction and *Pax2/8* expression occur normally in these *Eya1*-deficient embryos. Inner ear development arrests later at the otic vesicle stage, and this is followed by apoptotic regression of the otic primordia. Our observations provide a possible explanation for the resilience of sensory placodes to disruption by mutations. First, the signals required for placode induction are redundant [*fgf3/8* for otic placode (Phillips et al., 2001)]. Second, the responding ectoderm itself expresses functionally redundant molecules that support the initial steps of sensory placode formation.

Cross-regulatory interactions between Dlx genes

Possible crossregulatory interactions between Dlx genes have previously been proposed based on the overlapping expression domains observed for both paralogous and orthologous Dlx genes (Zerucha et al., 1997). In zebrafish, *dlx3* has been shown to act as a transcriptional activator of *dlx4* through a 1.7 kb fragment flanking the 5′ region of *dlx4* (Zerucha et al., 1997). More recently, Zerucha et al. (Zerucha et al., 2000) have shown that a highly conserved DNA element found in the intergenic region between mouse *Dlx5*/*Dlx6* and zebrafish *dlx4*/*dlx6* is sufficient to direct the forebrain specific expression seen with the endogenous genes. Furthermore, Dlx proteins bind to this intergenic enhancer in vitro and can upregulate transcription of a reporter gene in a co-transfection assay.

Our results suggest that both cross- and autoregulatory interactions exist among *dlx3* and *dlx7* that modulate the levels of their activities. These interactions are evident in early somitogenesis and, at least in the case of *dlx3*, are still functioning by mid-to-late somitogenesis stages (summarized in Table 1). In particular, morpholino-mediated knockdown of Dlx3 protein consistently leads to an upregulation of both *dlx3* and *dlx7* message, suggesting that Dlx3 might act through a shared enhancer element. This upregulation is observed in both RNA in situ hybridization as well as with microarray analysis. However, further experiments will be required to address whether the observed feedback mechanisms involve direct interactions between Dlx3/Dlx7 proteins and the regulatory regions of their genes.

The evolution of Dlx gene function

Similar to the Hox genes, the Dlx gene family has arisen through several rounds of duplication, which is thought to have facilitated the development of more complex and novel morphological structures in vertebrates (Ohno, 1970). In support of this, we have shown that two Dlx genes act in concert to promote the development of sensory structures that are absent in primitive chordates that only possess one Dlx gene, such as amphioxus (Holland et al., 1996). However, it remains unclear whether the roles of Dlx3 and Dlx7 are conserved during vertebrate evolution. In lamprey, Dlx genes are expressed in the otic and olfactory placodes, suggesting an ancient role for these genes in sensory organ development (Neidert et al., 2001). Support for a conserved role of the *dlx3* gene in sensory placode development comes from expression analysis in chick and axolotl. Axolotl *Dlx3-3* is expressed in the lateral line placodes and the otic vesicle (Metscher, 1997), and chick *DLX3* is expressed in the otic and olfactory placodes (Pera and Kessel, 1999). Although in chick, *DLX5* transcription surrounding the anterior neural plate, including the prospective olfactory placode, starts earlier than *DLX3* transcription (Pera et al., 1999), and is more reminiscent of the zebrafish *dlx3* expression pattern.

Functional studies of *DLX3* in *Xenopus* (Beanan and Sargent, 2000; Dirksen et al., 1994), mouse (Morasso et al., 1999) and humans (Price et al., 1998) do not provide evidence for a conserved role in sensory placode development, and expression of these orthologs in mouse and *Xenopus* differs from zebrafish. Mouse embryos homozygous for a *Dlx3* targeted gene disruption die around day 10 because of placental patterning defects, and no otic or olfactory defect has been reported for these embryos (Morasso et al., 1999). A four base pair deletion in the human *DLX3* gene, resulting in a truncation of the protein close to the C terminus, leads to Tricho-Dento-Osseus (TDO) syndrome, which is characterized by craniofacial abnormalities, kinky hair and tooth defects (Price et al., 1998). However, the dominant nature of this mutation complicates the interpretation of the role of DLX3 in human development. The *Xenopus dlx3* homolog, *X-dll2*, appears to promote the development of epidermis and may suppress the development of mesoderm and neural tissue (Beanan and Sargent, 2000; Dirksen et al., 1994). Developmental function of the *dlx7* ortholog remains to be addressed in other vertebrates. Whether the discrepancies of *dlx3* expression and function observed among different vertebrate species reflect an actual functional difference or rather a problem in the assignment of orthology is unclear.

However, at least in mouse, the linked *Dlx5/Dlx6* genes are expressed in a very similar pattern to zebrafish *dlx3/dlx7* during gastrulation, and therefore it has been suggested that these paralogous genes are more likely to be functionally equivalent (Quint et al., 2000). Notably, mouse embryos homozygous for a targeted *Dlx5* gene disruption display inner ear patterning defects and olfactory epithelium abnormalities (Acampora et al., 1999; Depew et al., 1999). This may correspond to the phenotype that we observe in *dlx3* MO-injected embryos, although that remains to be addressed. No *Dlx5/Dlx6* double mutant has been reported in mouse, although it would be interesting to examine whether the phenotype of such a mutant would reveal redundant functions for these genes. Functional redundancy for *Dlx1* and *Dlx2* has been observed in mice with targeted disruptions of these genes. Mice that lack only Dlx1 or Dlx2 display craniofacial malformations (Qiu et al., 1997; Qiu et al., 1995). In addition to craniofacial abnormalities, mice that lack both proteins have severe subcortical telencephalon abnormalities and lack maxillary molars, phenotypes not observed in either of the single mutants (Qiu et al., 1997). This suggests that mouse *Dlx1* and *Dlx2* posses both redundant and nonredundant functions. Although we observe partially redundant functions for zebrafish *dlx3* and *dlx7*, our results indicate that *dlx3* function alone is sufficient for sensory placode formation. Functional redundancy of *dlx3* and *dlx7* is further supported by the observation that both *dlx3* mRNA and *dlx7* mRNA are able to partially rescue the *dlx3+7* MO phenotype. Thus, it remains unclear why both of these seemingly redundant genes have been preserved through

evolutionary time. Several theoretical models predict that one copy of a redundant gene pair will eventually become silenced by drift if the two genes are entirely equivalent (Kimura, 1983; Li, 1980; Watterson, 1983). However, one model proposes that selection will maintain both copies of a redundant pair if one member is slightly less efficacious and also mutates at a slightly lower rate than the other gene (Krakauer and Nowak, 1999). Our data fits this model in that *dlx7* appears to be a slightly less efficacious member of a redundant gene pair. It will be interesting to determine whether *dlx7* also possesses a lower mutation rate than *dlx3*, providing further support for this evolutionary model.

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