Pax8 and Pax2a function synergistically in otic specification, downstream of the Foxi1 and Dlx3b transcription factors

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Summary

The vertebrate inner ear arises from an ectodermal thickening, the otic placode, that forms adjacent to the presumptive hindbrain. Previous studies have suggested that competent ectodermal cells respond to Fgf signals from adjacent tissues and express two highly related paired box transcription factors Pax2a and Pax8 in the developing placode. We show that compromising the functions of both Pax2a and Pax8 together blocks zebrafish ear development, leaving only a few residual otic cells. This suggests that Pax2a and Pax8 are the main effectors downstream of Fgf signals. Our results further provide evidence that *pax8* expression and *pax2a* expression are regulated by two independent factors, Foxi1 and Dlx3b, respectively. Combined loss of both factors eliminates all indications of otic specification. We suggest that the Foxi1-Pax8 pathway provides an early 'jumpstart' of otic specification that is maintained by the Dlx3b-Pax2a pathway.

Key words: *dlx3b*, *fgf3*, *fgf8*, *foxi1*, Inner ear, Morpholino, Otic placode, *pax8*, *pax2a*, *sox9a*, *sox9b*, Zebrafish

Introduction

The vertebrate inner ear is the sensory organ that provides the auditory and vestibular functions responsible for hearing and balance. It develops from a transient embryonic structure, the otic placode, a thickening of head ectoderm adjacent to the developing hindbrain. Through interactions with adjacent tissues, the otic placode develops into the otic vesicle that subsequently forms epithelial and neuronal cells of the inner ear (Noden and van de Water, 1986; Couly et al., 1993; Fritzsch et al., 1997; Barald and Kelley, 2004).

Studies in various species suggest that signals from the underlying mesoderm and adjacent hindbrain induce ectodermal cells to form the otic placode (reviewed by Fritzsch et al., 1997; Torres and Giráldez, 1998; Baker and Bronner-Fraser, 2001; Whitfield et al., 2002). In zebrafish, Fgf3 and Fgf8 appear to have overlapping functions in otic placode induction. The genes are expressed in the future hindbrain by late gastrula stages, and *fgf3* is also expressed at this stage in the underlying mesendoderm. Loss of either *fgf3* or *fgf8* leads to a reduction in ear size and loss of both *fgf3* and *fgf8* together results in near or total ablation of otic tissue (Phillips et al., 2001; Maroon et al., 2002; Leger and Brand, 2002). Furthermore, Fgf signaling is sufficient and necessary for otic induction, indicating a direct role for Fgf3 and Fgf8 (Phillips et al., 2004). In the mouse, Fgf3 and Fgf10 act as redundant signals during otic induction (Wright and Mansour, 2003; Alvarez et al., 2003). We have previously shown that Fgf signals are required for the preotic expression of some, but not all, of the transcription factors involved in otic induction (Liu et al., 2003). For example, the four transcription factors Dlx3b (Ekker et al., 1992), Dlx4b (Stock et al., 1996; Ellies et al., 1997), Sox9a (Chiang et al., 2001; Yan et al., 2002) and Sox9b (Chiang et al., 2001; Li et al., 2002) are all required for otic placode specification. *sox9a* and to some extent *sox9b* require Fgf signaling for their proper expression in the preotic region, whereas *dlx3b* and *dlx4b* do not. When Fgf3 and Fgf8 functions are removed, *dlx3b* and *dlx4b* gene expression is unaffected during induction and early patterning stages. Later expression of *dlx3b* and *dlx4b* in the otic anlagen is reduced when Fgf signals are blocked, but this is probably an indirect effect caused by the loss of *sox9a* function (Liu et al., 2003).

Fate-mapping experiments at mid-gastrula stages indicate that precursors of cranial placodes are arranged in an anteroposterior order at the lateral border of the prospective anterior neural plate (Kozlowski et al., 1997). We have previously shown that *dlx3b* and *dlx4b* are both expressed in late gastrula stage embryos in this same region, a stripe corresponding to cells of the future neural plate border; expression of both genes becomes restricted to cells of the future olfactory and otic placodes by the beginning of somitogenesis (Akimenko et al., 1994; Ekker et al., 1992; Ellies et al., 1997). We also showed by fate mapping that a subset of cells in the anterior part of the *dlx3b* stripe later contribute to the olfactory placodes (Whitlock and Westerfield, 2000). Knockdown of *dlx3b* and *dlx4b* causes a severe loss of otic tissue even in the presence of functional Fgf signaling (Solomon and Fritz, 2002; Liu et al., 2003), indicating that these genes are required to specify the competence of cells to form the ear. Expression of the forkhead class winged helix transcription factor, *foxi1*, is progressively restricted at late gastrula stages to bilateral domains, including the presumptive otic placode, and, subsequently, *foxi1* expression is downregulated prior to placode formation. Disruption of *foxi1* leads to severe defects in otic placode formation and highly

variable ear phenotypes (Solomon et al., 2003; Nissen et al., 2003), suggesting that *foxi1* also influences otic competence.

In addition to these genes implicated in otic development, some Pax genes that encode paired box transcription factors are expressed at the right time and place to be involved in otic specification. The Pax gene family is subdivided into four distinct classes based on sequence similarities (Noll, 1993; Mansouri et al., 1996). The *Pax2, Pax5* and *Pax8* genes constitute one such class and encode highly related transcription factors with similar biochemical activities (Pfeffer et al., 1998). *Pax2-Pax5-Pax8* genes have important roles in embryonic development and organogenesis of the eye, ear, kidney and thyroid (Dressler et al., 1990; Nornes et al., 1990; Plachov et al., 1990). The best-studied *Pax2-Pax5-Pax8* gene function is in development of the midbrain-hindbrain boundary (isthmus). In the mouse, *Pax2* or *Pax2* and *Pax5*, depending on the genetic strain, tops a hierarchy of genes that function together to form the isthmus. Pax2 and Pax5 act at multiple stages in this process and are also required for maintenance of *Pax2* (Urbanek et al., 1994; Torres et al., 1996; Mansouri et al., 1998). Implantation of Fgf8-soaked beads into chick embryos showed further that Fgf8 acts in this positive feedback loop maintaining *Pax2* expression in the isthmus (Martinez et al., 1999). In zebrafish, *pax2a* has been shown to function in this process; loss of *pax2a* leads to failed formation of the isthmus (Brand et al., 1996; Lun and Brand, 1998). Similar to amniote embryos, maintenance but not induction of zebrafish *pax2a* depends on both *pax2a* and *fgf8* (Lun and Brand, 1998; Reifers et al., 1998). Gene replacement in the mouse has shown that *Pax5* can functionally substitute for *Pax2*, indicating that the Pax2-Pax5-Pax8 proteins are interchangeable (Bouchard et al., 2000). Combined, redundant gene function has also been shown for *Pax2* and *Pax8* during development of the mouse urogenital system (Bouchard et al., 2002).

The relative roles of Pax2 and Pax8 in otic specification are still somewhat unclear. *Pax8* is one of the earliest known markers of otic cells in vertebrates, showing onset of otic expression before *Pax2* (Pfeffer et al., 1998; Heller and Brandli, 1999; Hutson et al., 1999; Groves and Bronner-Frasier, 2000). In mouse, loss of *Pax8* does not prevent expression of *Pax2* or proper inner ear formation (Mansouri et al., 1998) and loss of *Pax2* has no effect on otic induction but variably affects formation of the cochlea (Torres et al., 1996). Zebrafish have two Pax2 genes, *pax2a* and *pax2b*, both expressed in the preotic region with *pax2a* expressed at higher levels several hours earlier than *pax2b* (Pfeffer et al., 1998). Loss of *pax2a*, *pax2b* or both alters hair cell development but does not hinder otic placode induction (Riley et al., 1999; Whitfield et al., 2002). Cells of the zebrafish otic placode also express *pax8* (Pfeffer et al., 1998); functional studies have not previously been described.

We show that Pax8-depleted zebrafish embryos, like *pax2a*$^{-}$ mutants, have only mild ear defects. By contrast, Pax8 depleted *pax2a*$^{-}$ mutants fail to form a differentiated otic vesicle or inner ear, although a few residual cells express genes characteristic of otic fate. These data demonstrate that Pax2a and Pax8 have similar functions required for the correct expression of *sox9a*, *sox9b* and *pax2a*, and to a much lesser extent, *dlx3b*. However, *pax8* and *pax2a* are regulated by two independent factors, Foxi1 and Dlx3b, respectively; removal of both factors is required to block the establishment of otic fate. Our results integrate *pax2a* and *pax8* gene functions into the previously known genetic pathways that regulate otic placode induction; Foxi1 and Pax8 mediate early Fgf dependent otic specification, whereas Dlx3b and Pax2a mediate later Fgf signaling required for maintained development.

Materials and methods

Animals

Embryos were obtained from the University of Oregon zebrafish facility, produced using standard procedures (Westerfield, 2000) and staged according to standard criteria (Kimmel et al., 1995) or by hours post fertilization at 28°C (h). The wild-type line used was AB. The lines, *no isthmus*^{tu29a}, a null allele of *pax2a*, and *acerebellar*^{ti282a}, a strong hypomorphic allele of *fgf8*, have been described previously (Brand et al., 1996) and we refer to the homozygous mutants as *pax2a*$^{-}$ and *fgf8*$^{-}$, respectively. The mutation in *foxi1* was described by Solomon et al. (Solomon et al., 2003), and we refer to the homozygous mutants as *foxi1*$^{-}$. Homozygous *pax2a*$^{-}$ mutants were either scored by the loss of the midbrain-hindbrain boundary or by the use of Pax2 antibody (α-Pax2; Covance); homozygous *foxi1*$^{-}$ mutants were scored by absence of *pax8* expression or by PCR (Solomon et al., 2003). Homozygous *pax2a*$^{-}$;*foxi1*$^{-}$ double mutants were scored for both phenotypes.

Genes and markers

Approved gene and protein names that follow the zebrafish nomenclature conventions (http://zfin.org/zf_info/nomen.html) are used.

Immunocytochemistry

Antibody staining was carried out as described previously (Westerfield, 2000) with some modifications. Primary antibodies were used in the following concentrations: α-Pax2 (Covance), 1:100; α-Dlx3b (Liu et al., 2003), 1:50; rabbit polyclonal IgG α-Myc (Santa Cruz Biotechnology), 1:500. The following secondary antibodies were used: goat α-mouse Alexa Fluor 488 (Molecular Probes), goat α-mouse Alexa Fluor 568 (Molecular Probes), 1:100; goat α-rabbit Alexa Fluor 488 (Molecular Probes), 1:100. Embryos were analyzed using a Zeiss Axiophot 2 microscope.

In situ hybridization and mRNA synthesis

cDNA probes that detect the following genes were used: *dlx3b* (Ekker et al., 1992); *sox9a* (Chiang et al., 2001); *sox9b* (Chiang et al., 2001; Li et al., 2002); *egr2b* (previously *krox20*) (Oxtoby and Jowett, 1993); *cldna* (Kollmar et al., 2001); and *pax2a* (Krauss et al., 1991). For detection of *pax2a* in dn*pax2a-myc*-injected embryos, the 5′ and 3′ UTRs of *pax2a* were amplified by PCR, subcloned into pBluescript, linearized with *Not*I and *Eco*RI, respectively, and transcribed with T7 RNA polymerase. Probe synthesis and single or double-color in situ hybridization was performed essentially as previously described (Thisse et al., 1993; Jowett and Yan, 1996; Whitlock and Westerfield, 2000). We purified the in vitro synthesized mRNA and probes using an RNeasy mini column (Qiagen GmbH). In vitro mRNA synthesis was performed using an SP6 RNA synthesis kit (Ambion). The construct encoding dnPax2a-myc was generated by PCR amplification of the *pax2a* gene coding for the first 295 amino acids, which were fused in-frame with six Myc-epitopes and cloned into the CS2+ vector (Turner and Weintraub, 1994). For RNA injections, 1-3 nl of a 300 ng/μl solution was delivered into the cytoplasm of one cell at the two-cell stage.

Morpholinos (MOs)

We have described the *dlx3b*-MO, *fgf3*-MOs and *fgf8*-MOs previously (Liu et al., 2003; Maves et al., 2002). Splice-blocking *pax8*-MOs

were: E2/I2, 5′-GTGTGTGTTCACCTGCCCAGGATCT; E3/I3, 5′-GTGTGTACCGGTTGATGGAGCTGAC; E4/I4, 5′-CACAGCACTTACTCAGTGTGTGTCC; E5/I5, 5′-TTTCTGCACTCACTGTCATCGTGTC; and E9/I9, 5′-ACCGGCGGCAGCTCACCTGATACCA. About 1-3 nl of MO-solution was injected into the cytoplasm of one-cell stage embryos. The concentration of the *pax8* splice-blocking MOs was 1 μg/μl each for *pax8*-E5/I5 and *pax8*-E9/I9.

Results

pax8 expression precedes and diverges from *pax2a* expression in the otic placode

In zebrafish, the otic anlagen express *pax2a*, *pax2b* and *pax8* prior to formation of the placode. Expression of *pax8* is initiated at 85-90% epiboly and upregulated at bud stage [for a detailed description, see Phillips et al. (Phillips et al., 2001)]. Strong expression persists until the 9- to 10-somite stage (Fig. 1A,B), when the otic placode becomes morphologically visible. Subsequently, *pax8* is rapidly downregulated (Fig. 1C) and we are no longer able to detect *pax8* transcripts in the otic placode (Fig. 1D), in contrast to previous reports describing a low level expression of *pax8* until formation of the otic vesicle at 18-somite stage (Pfeffer et al., 1998; Phillips et al., 2001). *pax2a* expression begins in presumptive otic cells at the three-somite stage. High levels of *pax2a* can be detected as the placode develops and expression is maintained throughout the placode (Fig. 1A-D). After formation of the otic vesicle, *pax2a* transcripts become localized to the ventromedial region of the otic vesicle and are eventually retained only in sensory hair cells (Riley et al., 1999). To determine whether the same population of preotic cells expresses *pax2a* and *pax8*, we double labeled for both *pax2a* and *pax8* from preplacodal to placodal stages. The preotic *pax8* expression domain overlaps with *pax2a* from the onset of *pax2a* expression until *pax8* is downregulated. The two domains share the same medial border that abuts the hindbrain, but the *pax8* domain extends farther lateral than does the *pax2a* domain (Fig. 1E-H). *pax2b* is also expressed in the preotic region overlapping with *pax2a* (not shown). However, *pax2b* is expressed later, just prior to formation of the otic placode, and at low levels. It was therefore not included in this report.

Residual otic cells express *pax2a* and *pax8* in the absence of Fgf3 and Fgf8 signaling

Previous studies have suggested that Fgf3 and Fgf8 play overlapping roles in otic induction and that both *pax2a* and *pax8* require Fgf3 and Fgf8 signaling for their proper expression (Phillips et al., 2001; Maroon et al., 2002; Leger and Brand, 2002). However, we have shown that even in the absence of Fgf3 and Fgf8 signaling, some residual cells express *pax2a* (Liu et al., 2003). We, thus, examined whether this is also true for *pax8*.

We find that knockdown of Fgf3 and Fgf8 in wild-type embryos or knockdown of Fgf3 in *fgf8*$^{-}$ mutants significantly reduces *pax8* expression in the preotic region, although weak residual expression can still be detected (Fig. 1I,J). This finding is similar to the observations of Maroon et al. (Maroon et al., 2002), but contrasts with the results of Phillips et al. (Phillips et al., 2001) and Leger and Brand (Leger and Brand, 2002) who reported complete loss of *pax8* when Fgf3 and Fgf8 are knocked-down by antisense morpholino oligonucleotide (MO) injection. Although the discrepancies between these studies might be explained by incomplete effectiveness of the MOs, the Fgf receptor blocking drug SU5402 also led to differing results: Leger and Brand (Leger and Brand, 2002) reported a complete loss of *pax8* expression whereas Maroon et al. (Maroon et al., 2002) found that *pax8* was unaffected. Thus, taken together, these results could indicate either that *pax8* is highly sensitive to Fgf signaling and only complete loss of the Fgf signal leads to loss of *pax8* expression or that *pax8* expression is regulated partly by some factor in addition to Fgf. *foxi1*$^{-}$ mutants fail to initiate *pax8* expression (Solomon et al., 2003; Nissen et al., 2003), suggesting that this forkhead-related transcription factor may be the other regulator of *pax8* expression. To test this interpretation, we examined whether *foxi1* acts independently of Fgf signaling. We found that knockdown of Fgf3 and Fgf8 in wild-type embryos or knockdown of Fgf3 in *fgf8*$^{-}$ mutants has no significant effect

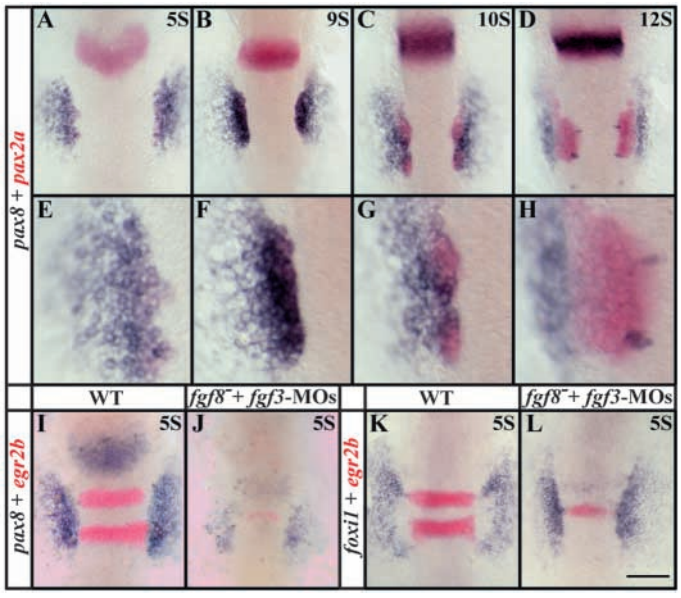

Fig. 1. *pax8* expression partially overlaps with *pax2a* in the preotic region and, like *pax2a*, depends upon Fgf signaling. Expression domains of *pax8* (blue) and *pax2a* (red) coincide in the preotic region (A-C) but diverge after formation of the placode (D). At five-somite (A,E) to nine-somite (B,F) stages, the *pax8* domain encompasses *pax2a* but also extends further laterally. By the 10-somite stage, when the otic placode is forming, *pax8* expression diminishes in the placode (C,G) and is completely absent from the placode by the 12-somite stage (D,H) but is obvious just lateral to the placode. The bilateral pairs of medial blue spots in D,H are hindbrain neurons that express *pax8*. (E-H) High-magnification views of embryos shown in A-D. (I,J) Fgf 3 and Fgf8 depletion leads to a severe reduction of *pax8* expression at the five-somite stage but not to complete loss. In Fgf3- and Fgf8-depleted embryos, only weak expression can be detected (J) compared with uninjected wild-type embryos (I). Expression of *egr2b* (red) in the hindbrain (I) is also reduced when Fgf signals are lost (J) (Maves et al., 2002). (K,L) *foxi1* is not dependent on the Fgf3 and Fgf8 signal. In wild-type embryos at the five-somite stage, *foxi1* is expressed in two patches lateral to the neural plate (K) and depletion of Fgf3 and Fgf8 has no obvious effect on *foxi1* expression (L). Dorsal views, anterior towards the top. Scale bar: 120 μm for A-D,I-L; 40 μm for E-H.

on *foxi1* expression (Fig.1K,L), supporting the hypothesis that *foxi1* expression is independent of Fgf signaling, but required for cells to respond to Fgf signaling (Nissen et al., 2003).

Pax2a and Pax8 function synergistically in otic specification

Because *pax8*$^{-}$ mutants are not available, we used MOs to knock down gene function in a gene-specific manner (Nasevicius and Ekker, 2000). In addition to their ability to block the translation of mRNAs in the cytoplasm, MOs can inhibit pre-mRNA splicing (Draper et al., 2001), thus interfering with the transport of transcript from the nucleus to the cytoplasm (Yan et al., 2002). This inappropriate retention of transcripts in the nucleus can be used as an assay for MO efficacy in the absence of an antibody to test for the production of a translated product (Yan et al., 2002). We used this splice-blocking strategy for *pax8* because neither a Pax8 antibody nor the sequence of the 5′ terminus of the *pax8* mRNA (Pfeffer et al., 1998) was available. We determined the sequence of several introns, designed specific splice-blocking MOs (Fig. 2A), and injected the MOs alone and in various combinations. As a control for the efficacy of the MOs, we visualized the nuclear localization of *pax8* messenger by in situ hybridization; the most efficacious combination of MOs was used in this study (Fig. 2A-C).

Injection of *pax8*-MOs into wild-type embryos has only a subtle effect: the morphological development of the otic placode is delayed (Fig. 2F,J) compared with wild-type embryos injected with control MOs or to un-injected wild-type embryos (Fig. 2D,H). Consistent with their eventual otic fates, cells in the embryos injected with *pax8*-MOs express otic markers such as Dlx3b, *claudin a* (*cldna*) and *fibronectin 1* (*fn1*) (Fig. 2N,R; not shown), again showing only a slight developmental delay compared with control embryos (Fig. 2L,P). The lack of a stronger phenotype could be due to compensation for *pax8* by *pax2a*. *pax2a*$^{-}$ mutants exhibit only a weak neurogenic phenotype in the ear that probably results from reduced Delta signaling (Riley et al., 1999) but are

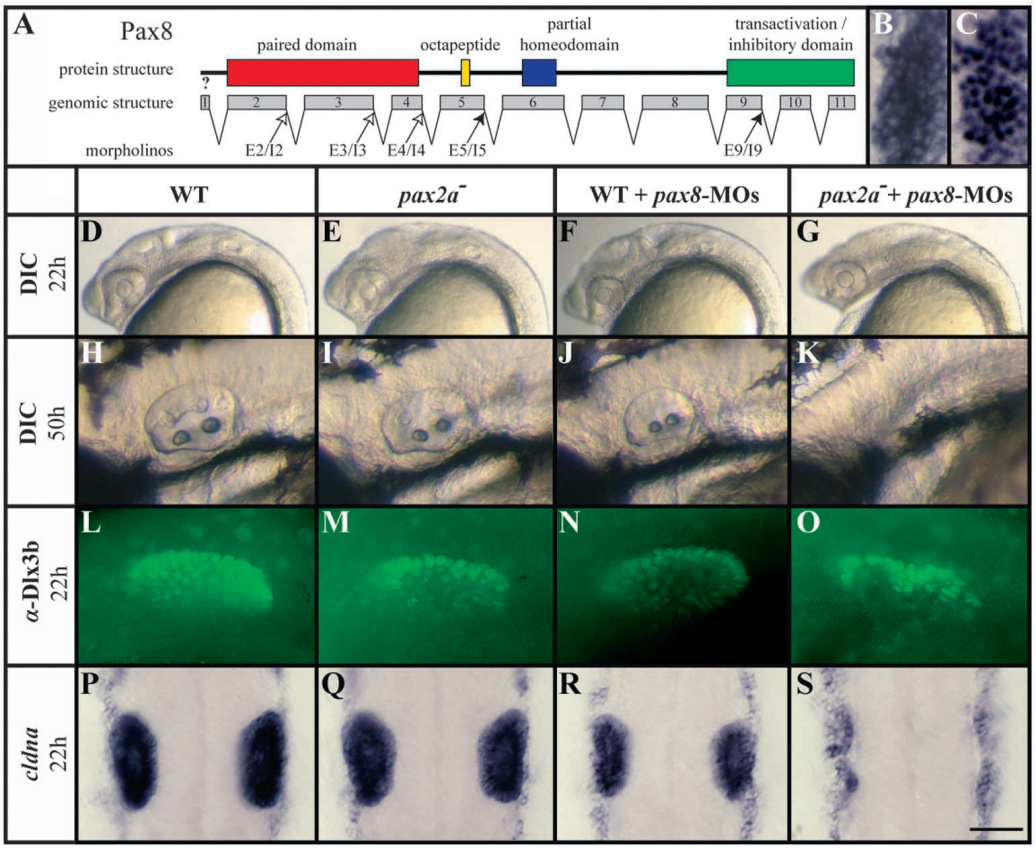

Fig. 2. Pax2a and Pax8 have overlapping functions in ear development. (A) Schematic map showing the genomic structure of *pax8* (after Pfeffer et al., 1998), the protein domain structure and the positions of the splice-blocking morpholinos. Black arrows indicate MO E5/I5 and E9/I9, the most efficacious combination used in this study. The white arrows E2/I2, E3/I3 and E4/I4 show positions of the other three splice-blocking MOs that yielded only weak or no phenotype. The sequence of the first exon (?) is possibly incomplete. The efficacy of E5/I5 and E9/I9 *pax8*-MOs combination is visualized by in situ hybridization (B,C). (B) In wild-type embryos at the one-somite stage, *pax8* transcripts in the preotic region are localized primarily in the cytoplasm leaving the nuclei relatively clear. (C) In E5/I5 and E9/I9 *pax8*-MOs injected embryos, *pax8* transcripts are localized mostly in nuclei leaving the cytoplasm free of signal. (D-S) Knockdown of Pax8 in *pax2a*$^{-}$ mutants has a severe otic phenotype. Wild-type embryos (D,H,L,P), *pax2a*$^{-}$ mutants (E,I,M,Q) and *pax8*-MO-injected wild-type embryos (F,J,N,R) are similar, but, in contrast to *pax2a*$^{-}$ mutants, are depleted of Pax8 (G,K,O,S). The otic vesicle is absent in *pax8*-MO-injected *pax2a*$^{-}$ mutant embryos and cannot be detected at 22 h (G) or 50 h (K). However, Dlx3b (L-O) and *cldna* (P-S) are present in *pax2a*$^{-}$ mutants depleted of Pax8. (D-O) Side views, anterior towards the left, dorsal towards the top; (B,C,P-S) dorsal views, anterior towards the top. Scale bar: 30 μm for B,C; 200 μm for D-G; 75 μm for H-K; 60 μm for L-O; 100 μm for P-S.

otherwise relatively unaffected (Fig. 2E,I,M,Q). Injection of *pax8*-MOs into *pax2a*$^{-}$ mutants produces a different and highly penetrant phenotype, including severe disruption of otic development. Most of the injected *pax2a*$^{-}$ embryos (79%, 44/56) do not form an otic vesicle (Fig. 2G,K), although a few (12/56) form an extremely small vesicle that lacks otoliths (not shown). Nevertheless, analysis of the otic markers, Dlx3b, *cldna* and *fn1* reveals the presence of residual otic cells, even in embryos with no visible otic vesicles (Fig. 2O,S; not shown). Thus, *pax2a* and *pax8* have overlapping and, apparently, synergistic functions required for formation of the ear, although removal of both gene functions is insufficient to eliminate all otic cells.

Pax2a and Pax8 are required for maintenance of otic *sox9a*, *sox9b* and *pax2a* expression, but neither induction nor maintenance of *dlx3b* expression

To study the placement of *pax2a* and *pax8* in the genetic pathway regulating otic development, we examined the expression of several otic markers at preplacodal, placodal and vesicle stages. We concentrated on the transcription factor genes, *sox9a*, *sox9b* and *dlx3b* (Yan et al., 2002; Chiang et al., 2001; Akimenko et al., 1994), that are essential for formation of the ear (Liu et al., 2003). We also examined *pax2a* expression that is reduced in *pax2a*$^{-}$ mutants at later stages (Brand et al., 1996).

Loss of Pax2a, together with Pax8 knockdown, affects *sox9a* and *sox9b* expression. *sox9a* is broadly expressed in the preotic region at the three-somite stage (Fig. 3A) and expression is maintained in the placode (Fig. 3C) and in the vesicle (not shown). In *pax2a*$^{-}$ mutants injected with *pax8*-MOs, the expression of *sox9a* is reduced in extent and level (Fig. 3B). At the 12-somite stage, when the placode is morphologically visible in wild-type embryos, we detect no *sox9a* expression in *pax2a*$^{-}$ mutants injected with *pax8*-MOs (Fig. 3D). The *sox9a* duplicate, *sox9b*, is also expressed from preplacodal to vesicle stages, although it is initiated later in development (Fig. 3E,G). Like *sox9a*, *sox9b* expression is compromised at preplacodal stages in *pax2a*$^{-}$ mutants depleted of *pax8* (Fig. 3E) and absent at the 12-somite stage (Fig. 3H).

dlx3b expression is less affected by loss of Pax2a and Pax8 knockdown. By the end of gastrulation, a band of cells expresses *dlx3b* surrounding the neural plate, particularly in the region that corresponds to the future otic placode (Fig. 3I). *dlx3b* expression persists throughout the placode until formation of the otic vesicle (Fig. 3K). In *pax2a*$^{-}$ mutants injected with *pax8*-MOs, the *dlx3b* expression domain is smaller, but the concentration of cells in the region where the placode would normally form can still be recognized (Fig. 3J). In contrast to *sox9a* and *sox9b*, *dlx3b* expression can still be detected in some cells in *pax2a*$^{-}$ mutants depleted of *pax8* at the 12-somite stage (Fig. 3L) and even at the stage when the vesicle would form in normal embryos (Fig. 2O). Previously, we have shown that loss of Sox9a leads to strong reduction of Dlx3b in the preotic domain (Liu et al., 2003) and because the phenotype of these embryos is essentially the same as *pax2a*$^{-}$ mutants depleted of Pax8, we conclude that reduction of *dlx3b* gene expression in the preotic domain in the absence of Pax2a and Pax8 is presumably due to concomitant reduction of Sox9a.

Maintenance of *pax2a* expression depends strongly on Pax2a or Pax8. In *pax2a*$^{-}$ mutants, *pax2a* transcription initiates normally and we cannot distinguish between wild-type, *pax2a*$^{-}$ mutants and *pax2a*$^{-}$ mutants injected with *pax8*-MOs at

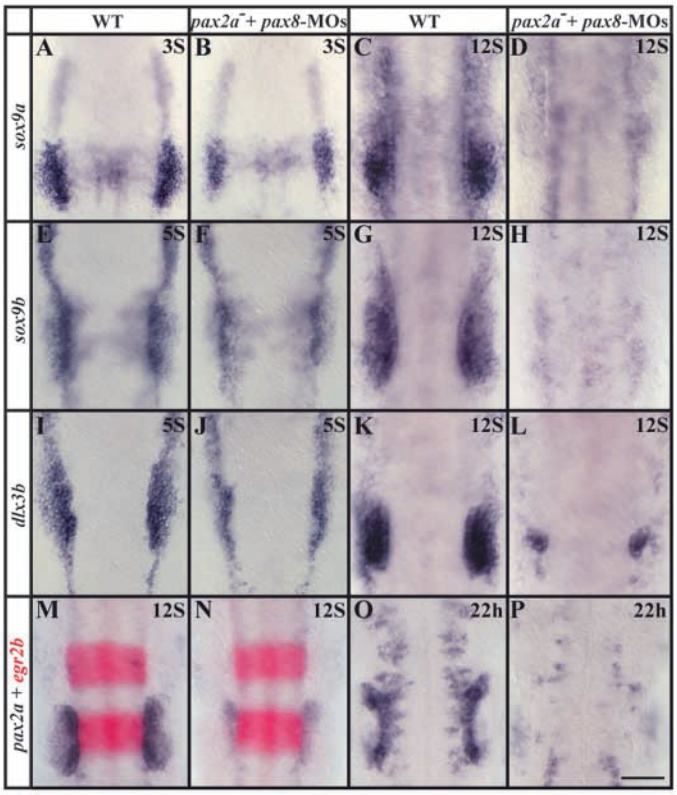

Fig. 3. Pax2a and Pax8 are required for maintenance of otic cell fates. Pax2a and Pax8 are required together for preotic expression of *sox9a* (A-D), *sox9b* (E-H) and *pax2a* (M-P) but not of *dlx3b* (I-L). Cells of the presumptive otic placode express *sox9a* in wild-type embryos at the three-somite stage (A) at higher levels than in *pax2a*$^{-}$ mutants after *pax8*-MOs injection (B). At the 12-somite stage, *sox9a* is expressed throughout the otic placode in wild-type embryos (C) but no otic *sox9a* expression can be detected in *pax2a*$^{-}$ mutants depleted of Pax8 (D). (E-H) The *sox9a* duplicate, *sox9b*, shows similar behavior. In wild type at the five-somite stage, *sox9b* is expressed in the preotic region and neural crest (E). The neural crest expression is unaffected in *pax2a*$^{-}$ mutants injected with *pax8*-MOs, but expression in the preotic domain is reduced (F). At the 12-somite stage, *sox9b* is expressed strongly in the otic placode in wild-type embryos (G) but is absent in *pax2a*$^{-}$ mutants injected with *pax8*-MOs (H). *dlx3b* expression is strong in cells of the future otic placode in wild-type embryos (I) at the five-somite stage and this domain is smaller but still recognizable in *pax2a*$^{-}$ mutants after *pax8*-MOs injection (J). At the 12-somite stage, *dlx3b* is expressed throughout the otic placode in wild-type embryos (K) but in *pax2a*$^{-}$ mutants with a knockdown of Pax8, only a few residual cells express *dlx3b* (L). *pax2a* expression is strong in the otic placode of wild-type embryos at the 12-somite stage (M) but expression is severely reduced in *pax2a*$^{-}$ mutants after *pax8*-MOs injection (N). At 22 h, when the otic vesicle has formed in wild-type embryos, *pax2a* expression is restricted to the ventromedial region (O) but is completely absent in *pax2a*$^{-}$ mutants depleted of Pax8 (P). Expression of *egr2b* (red) in rhombomeres 3 and 5 is unchanged in *pax2a*$^{-}$ mutants after *pax8*-MOs injection (N) in comparison with uninjected wild-type embryos (M). Dorsal views, anterior towards the top. Scale bar: 120 μm.

preplacodal stages (not shown). However, there is severe downregulation of *pax2a* in *pax2a*$^{-}$ mutants injected with *pax8*-MOs by the 12-somite stage (Fig. 3N) and *pax2a* is completely lost by vesicle stages (Fig. 3P). Labeling for *fgf3* or *fgf8* expression and *egr2b* or *mafb* (previously *valentino*) expression, both downstream targets of Fgf signaling (Maves et al., 2002), reveals that expression of *fgf3* or *fgf8* and patterning of the hindbrain occurs normally in *pax2a*$^{-}$ mutants depleted of Pax8 (Fig. 3N, and not shown). These results indicate that Pax2a and Pax8 act synergistically downstream of Fgf3 and Fgf8; when Pax2a and Pax8 functions are both compromised, otic induction is weaker and otic fate is not maintained, even in the presence of normal Fgf signaling.

sox9a, *sox9b* and *pax2a*, but not *dlx3b*, are transcriptional targets of Pax8

Because we see some residual specification of otic cells in Pax2a and Pax8 knockdown embryos, we were concerned that some Pax protein function remained. Structure-function analyses have shown that proteins of the Pax2-Pax5-Pax8 family have overlapping biochemical activities; their DNA-binding specificities are highly similar and they can substitute for each other (Bouchard et al., 2000). Thus, to block all Pax2-Pax5-Pax8 function, we generated a dominant-negative form of Pax2a (dn*pax2a-myc*) by replacing the C-terminal transactivation-inhibitory domain with six Myc epitope tags (Fig. 4A). We injected mRNA from this *pax2a* variant into wild-type embryos and analyzed subsequent otic development. To assess the effectiveness of dn*pax2a-myc*, we examined *eng3* expression in injected embryos. Expression of *eng3*, a downstream target of Pax2a in the midbrain-hindbrain boundary region, is initiated at the one-somite stage in wild-type embryos (Fig. 4B) but is never activated in strong *pax2a*$^{-}$ mutants (Lun and Brand, 1998). In dn*pax2a-myc* mRNA-injected embryos, we identify regions expressing the variant protein by the presence of the Myc-epitopes. In these regions, *eng3* transcription is severely reduced or completely absent (Fig. 4C). The nuclear localization of the Myc-epitopes and the severe downregulation of *eng3* show that this Pax2a variant enters the nucleus and competes with the endogenous Pax2-Pax5-Pax8 proteins for binding sites. We expect that, owing to its abundance, this construct is able to out compete the endogenous proteins and act in a dominant-negative fashion.

Injection of dn*pax2a-myc* strongly affects initial otic development. Expression of both *sox9a* and *sox9b* is severely reduced or absent in the presence of the Pax2a variant, interfering with expression not only in the preotic domain but also in the neural crest and neural tube (Fig. 4D′,D″,E′,E″). Because *pax8* is the only member of the Pax2-Pax5-Pax8 family known to be expressed in the preotic region at this early developmental stage, our result suggests that the dominant-negative Pax2a construct interferes with Pax8 function and that Pax8 is required for correct early otic expression of *sox9a* and *sox9b*. In addition, *pax2a* transcription is affected in the preotic domain of these injected embryos but not in the isthmus (Fig. 4F′,F″) indicating that otic *pax2a*, but not isthmic *pax2a*, expression depends on Pax2-Pax5-Pax8 proteins at this stage. By contrast, *dlx3b* expression is less affected in the presence of the dominant-negative form of Pax2a; the preotic *dlx3b* domain is slightly reduced, presumably owing to loss of *sox9a*, but can still be easily recognized (Fig. 4G′,G″). Thus, injection of the dominant-negative form of Pax2a affects expression of *sox9a*, *sox9b* and *dlx3b*, similar to loss of *pax2a* together with Pax8 knockdown. By contrast, *pax8*-MO injection into *pax2a*$^{-}$ mutants has only a mild effect on initiation of *pax2a* expression, whereas injection of dn*pax2a-myc* blocks *pax2a* induction. This discrepancy could indicate that the *pax8*-MOs are only partially effective in blocking Pax8 function, or alternatively that a Pax protein other than Pax8 is required for

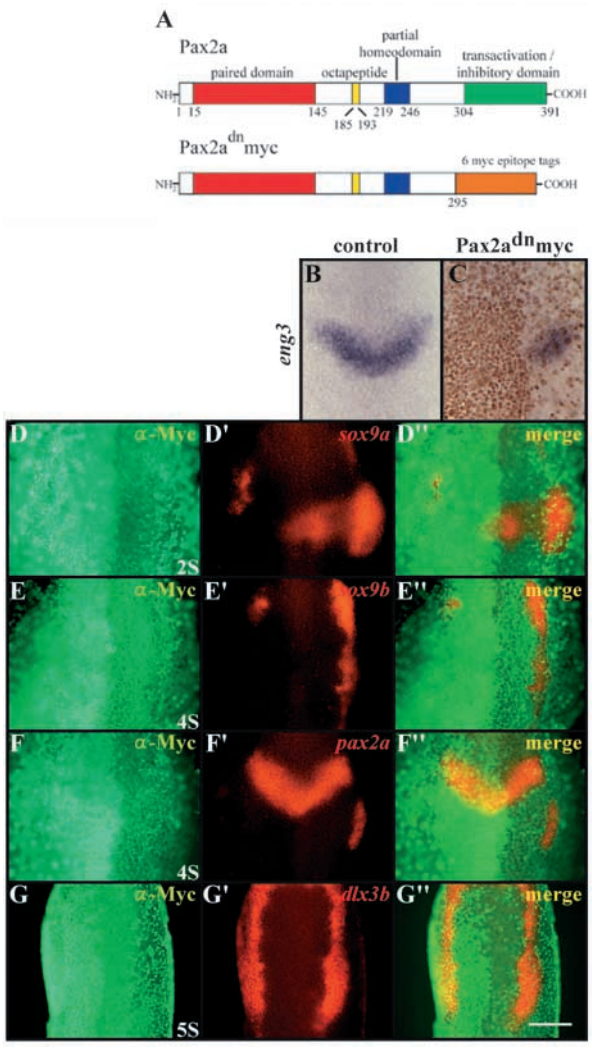

Fig. 4. Pax2a and Pax8 are required for induction of otic cell fates. (A) Schematic map of protein domain structure of Pax2a and the dominant-negative variant, dnPax2a-myc. In dnPax2a-myc, the 295 N-terminal amino acids are fused in frame with six Myc-epitopes. The numbers indicate amino acids of the domains (Lun and Brand, 1998). (B,C) dnPax2a-myc acts in a dominant-negative fashion. At the three-somite stage, *eng3* (blue) is expressed in the isthmic region (B), whereas in wild-type embryos injected with dn*pax2a-myc*, the side expressing the transgene shows reduced or no *eng3* (blue) expression (C). The distribution of the Myc-epitope (brown) indicates the localization of the Pax2a variant in nuclei. Expression of dominant-negative Pax2a leads to downregulation of *sox9a* (D,D′,D″), *sox9b* (E,E′,E″) and *pax2a* (F,F′,F″) but has only slight effects on *dlx3b* (G-G′). (D-G) Fluorescence images of α-Myc antibody labeling; (D′-G′) fluorescence images of mRNA in situ hybridization probes; (D″-G″) merged fluorescence images. (B-G″) Dorsal views, anterior towards the top. Scale bar: 120 μm.

initiation of *pax2a* expression. Analysis of the transcript structure of *pax8* (B. Riley, personal communication) shows that *pax8* is subject to alternative splicing, producing some transcripts that are not targeted by these *pax8*-MOs; thus, incomplete depletion of Pax8 is likely. Nevertheless, these results together demonstrate that Pax8 function is required for correct otic expression of *sox9a*, *sox9b*, and *pax2a* and, to a much lesser extent, *dlx3b*.

Pax8 and Fgf control otic expression of *pax2a* synergistically

Recent studies implicate combined functions of Fgf3 and Fgf8 in otic *pax2a* induction (Phillips et al., 2001; Maroon et al., 2002; Leger and Brand, 2002). In addition, Pax2a and Fgf8 are both required to maintain *pax2a* expression in the isthmus (Lun and Brand, 1998; Reifers et al., 1998). To test whether Pax8 also acts together with Fgf signals to promote *pax2a* expression, we injected *pax8*-MOs into *fgf8*$^{-}$ mutants. *pax2a* expression in *fgf8*$^{-}$ mutants alone shows normal timing, but the size of the otic expression domain is significantly reduced (Fig. 5A,B) (Phillips et al., 2001), and the otic vesicle is subsequently smaller (Fig. 5D,E) (Phillips et al., 2001). *fgf8*$^{-}$ mutants depleted of *pax8* show an even stronger reduction of *pax2a* expression in the preotic region (Fig. 5C) and form an even smaller ear (Fig. 5F). This observation suggests that Fgf8 and probably Fgf3 act through and in parallel with Pax8 to promote proper *pax2a* expression.

Foxi1 is required for patterning of otic placode precursors

The forkhead domain transcription factor, Foxi1, has been implicated as a regulator of *pax2a* and *pax8* (Solomon et al., 2003). *foxi1*$^{-}$ mutants display highly variable otic morphologies, initiation of otic *pax8* fails and otic *pax2a* expression is severely delayed. Early *dlx3b* expression is unaffected in *foxi1*$^{-}$ mutants, but concentration of *dlx3b*-expressing cells in the preotic region is delayed and patchy (Solomon et al., 2003; Nissen et al., 2003). Previously, we have shown that Dlx3b is required for the correct temporal onset of *pax2a* expression in the preotic region (Liu et al., 2003). Thus, reduced expression of Dlx3b could explain the delayed and patchy expression of *pax2a* in *foxi1*$^{-}$ mutants. To determine whether the residual patchy Pax2a is co-expressed with Dlx3b, we used double fluorescent antibody labeling (Fig. 6I-J′). In wild-type embryos at the 12-somite stage, all cells of the otic placode express Pax2 and Dlx3b (Fig. 6I-I″). In *foxi1*$^{-}$ mutants, all otic cells that express Dlx3b are also Pax2 positive and when Dlx3b expression appears in two or more smaller patches, Pax2 expression is affected in an identical manner (Fig. 6J-J′).

To place Foxi1 in the genetic pathway regulating otic specification, we examined whether *foxi1* is required for correct expression of the two Sox9 genes. In *foxi1*$^{-}$ mutants, both the size of the expression domain and the level of expression of *sox9a* in the preotic region are significantly reduced at the three-somite stage (Fig. 6B) in comparison with

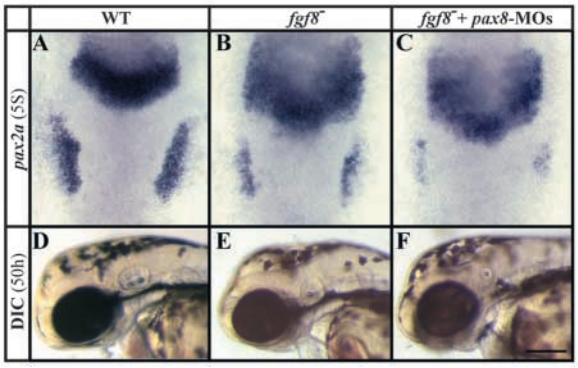

Fig. 5. Pax8 mediates the early Fgf-dependent induction of *pax2a* expression. In *fgf8*$^{-}$ mutant embryos at the five-somite stage, the preotic *pax2a* expression is reduced in size (B) compared with wild-type embryos of the same age (A). *fgf8*$^{-}$ mutant embryos after Pax8 depletion show an even further reduction of preotic *pax2a* expression (C). The defects in Pax2a expression are later manifested in a slightly reduced ear in *fgf8*$^{-}$ mutant embryos (E) or more severely reduced ear in *fgf8*$^{-}$ embryos depleted of Pax8 (F) in comparison with wild-type embryos at 50 h (D). (A-C) Dorsal views, anterior towards the top; (D-F) side views, anterior towards the left, dorsal towards the top. Scale bar: 120 μm for A-C; 180 μm for D-F.

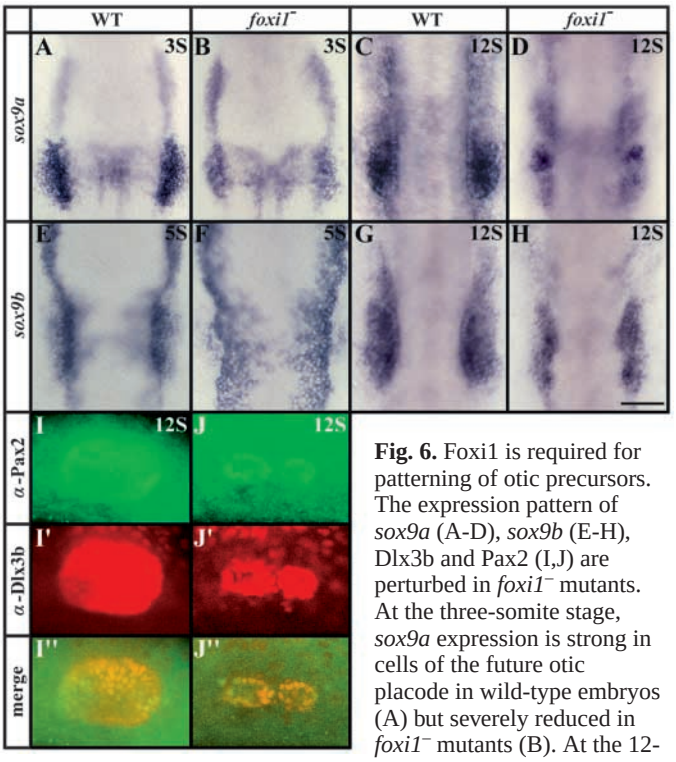

Fig. 6. Foxi1 is required for patterning of otic precursors. The expression pattern of *sox9a* (A-D), *sox9b* (E-H), Dlx3b and Pax2 (I,J) are perturbed in *foxi1*$^{-}$ mutants. At the three-somite stage, *sox9a* expression is strong in cells of the future otic placode in wild-type embryos (A) but severely reduced in *foxi1*$^{-}$ mutants (B). At the 12-somite stage, *sox9a* is expressed throughout the otic placode in wild-type embryos (C) in contrast to *foxi1*$^{-}$ mutants of the same age that have only a few cells expressing *sox9a* (D). (E-H) Loss of *foxi1* affects *sox9b* differently. At the five-somite stage, *sox9b* expression is reduced in the preotic region in *foxi1*$^{-}$ mutants (F) compared with wild-type embryos (E) but by the 12-somite stage, *sox9b* expression in *foxi1*$^{-}$ mutants has recovered (H), although not to wild-type levels (G). (I-J′) Dlx3b and Pax2 proteins coincide in the otic region. In wild-type embryos at the 12-somite stage, Dlx3b and Pax2 are co-expressed in cells throughout the otic placode (I-I″). The overlapping pattern is also present in *foxi1*$^{-}$ mutants at this stage (J-J″) showing that expression of one correlates with expression of the other. (A-H) Dorsal views, anterior towards the top; (I-J′) side views, anterior towards the left, dorsal towards the top. Scale bar: 120 μm for A-H; 50 μm for I,J.

wild-type embryos (Fig. 6A). These reductions are even more significant than in *pax2a*$^{-}$ mutants injected with *pax8*-MOs (compare with Fig. 3B). In *foxi1*$^{-}$ mutants at the 12-somite stage, *sox9a* is variably expressed in the otic region; tightly aggregated patches of cells express high levels of sox9a (Fig. 6D), but the total number of *sox9a*-expressing cells is reduced in comparison with wild-type embryos (Fig. 6C). The *sox9a* duplicate, *sox9b*, has a similar expression pattern in *foxi1*$^{-}$ mutants. At the five-somite stage, we detect very low levels of *sox9b* expression in the preotic region (Fig. 6F), even lower than in *pax2a*$^{-}$ mutants depleted of Pax8 (compare with Fig. 3F). By the 12-somite stage, *sox9b* expression somewhat recovers in *foxi1*$^{-}$ mutants (Fig. 6H), although not to wild-type levels (Fig. 6G).

Together, these results show that in the absence of Foxi1 function, and hence also in the absence of *pax8* expression, only weak induction of otic *sox9a* and *sox9b* occurs, and otic *pax2a* expression is restricted to Dlx3b-positive cells.

Pax2a acts partially independently of Foxi1

Our analysis of *foxi1*$^{-}$ mutants, dominant-negative Pax2a and *pax2a*$^{-}$ mutants depleted of Pax8 suggest that Pax2a may provide a Foxi1-independent pathway for otic specification. To test this hypothesis, we generated *foxi1*$^{-}$;*pax2a*$^{-}$ double mutants and analyzed them for otic specification. The ears of *pax2a*$^{-}$ single mutants are virtually indistinguishable from wild-type embryos by morphology (Fig. 2D,E,H,I) or by gene expression (Fig. 2L,M,P,Q). *foxi1*$^{-}$ single mutants are highly variable; some mutants develop a small lumen with only one or no otolith, whereas others have small split lumens, each with a single otolith (Solomon et al., 2003; Nissen et al., 2003). The *foxi1* mutant allele we used typically forms a small lumen with one otolith (Fig. 7B,G) and consistently retains some expression of the otic markers Dlx3b, *cldna* and *fn1* (Fig. 7L,O; not shown).

foxi1^{-};*pax2a*$^{-}$ double mutants never show any morphological sign of otic specification (Fig. 7C,H) and the otic markers Dlx3b and *fn1* are lost (Fig. 7M; not shown). Labeling for *cldna* expression, however, reveals the presence of some residual 'otic' cells that can be distinguished from *cldna*-expressing cells of the anterior and posterior lateral line placodes by expression levels and position (Fig. 7P). At the 12-somite stage, *foxi1*$^{-}$;*pax2a*$^{-}$ double mutants are similar to *pax2a*$^{-}$ mutants injected with *pax8*-MOs: they lack *sox9a* and *sox9b* expression and have reduced *dlx3b* expression (not shown). *pax2a* expression, however, is more severely reduced or completely lost in the *foxi1*$^{-}$;*pax2a*$^{-}$ embryos compared with *pax2a*$^{-}$ mutants injected with *pax8*-MOs (compare Fig. 7U with Fig. 3N). Thus, embryos with compromised Foxi1 and Pax2a show an even stronger loss of otic specification than *pax2a*$^{-}$ mutants depleted of Pax8, although a few residual cells still assume an 'otic' fate. These results further

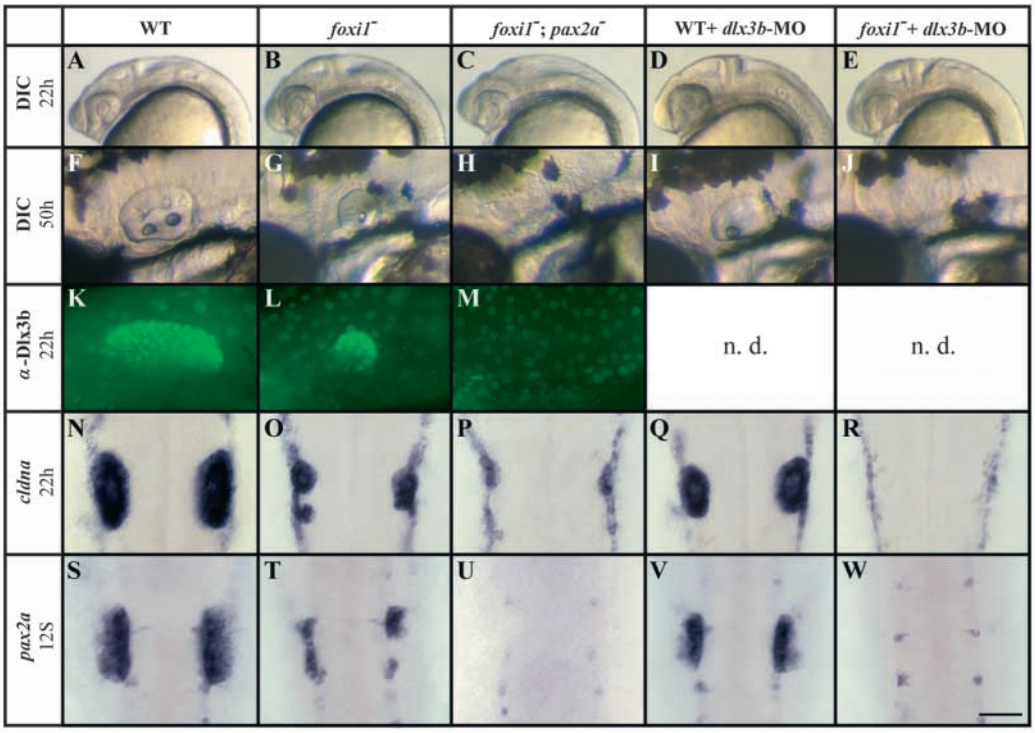

Fig. 7. Foxi1 and Dlx3b mediate convergent pathways of otic development. In *foxi1*$^{-}$ mutants, otic tissue is reduced (B,G,L,O,T) compared with wild-type embryos (A,F,K,N,S) assessed both by morphology (A,B,F,G) and markers, including Dlx3b (K,L), *cldna* (N,O) and *pax2a* (S,T). *foxi1*$^{-}$;*pax2a*$^{-}$ double mutants show no morphological sign of an otic vesicle (C,H) and no expression of Dlx3b (M) or *pax2a* (U), although some residual 'otic' cells can be detected with *cldna* (P). Injection of *dlx3b*-MO into wild-type embryos leads to reduction of overall ear size (D,I) that is preceded by reduced expression of *cldna* (Q) and *pax2a* (V). In *foxi1*$^{-}$ mutant embryos depleted of Dlx3b, all otic specification is absent as indicated by morphology (E,J) and transcription of *cldna* (R) and *pax2a* (W). (A-M) Side views, anterior towards the left, dorsal towards the top; (N-W) dorsal views, anterior towards the top. Scale bar: 200 μm for A-E; 75 μm for F-J; 60 μm for K-M; 100 μm for N-W. n.d., not done.

support our interpretation that Pax2a acts synergistically with Pax8 in otic specification and demonstrate that the majority of otic cells specified in the absence of Foxi1 are Pax2a dependent.

Foxi1 and Dlx3b mediate convergent pathways required for otic development

Our observation that a few residual cells express otic markers even in *foxi1*$^{-}$;*pax2a*$^{-}$ double mutants suggests the possibility that an additional factor participates in otic specification; Dlx3b is a likely candidate. To test this possibility, we compromised Dlx3b function using morpholino injection. Reduction of Dlx3b in wild-type embryos impairs complete maturation of the otic vesicle and most embryos form a smaller vesicle with only one otolith (Fig. 7D,I) (Solomon and Fritz, 2002; Liu et al., 2003) and reduced *cldna* expression (Fig. 7Q). By contrast, *foxi1*$^{-}$ mutants injected with *dlx3b*-MO show no morphological signs of otic specification (Fig. 7E,J) and no otic expression of *cldna* (Fig. 7R). In wild-type embryos depleted of Dlx3b, otic specification is delayed as indicated by delayed onset and reduced expression of *pax2a* (Fig. 7V). However, in *foxi1*$^{-}$ mutants injected with *dlx3b*-MO, otic *pax2a* expression is undetectable (Fig. 7W). Taken together, these results show that removal of the two factors, Foxi1 and Dlx3b, leads to a complete absence of otic specification.

Discussion

Pax2a and Pax8 synergistically mediate Fgf induction of the otic placode

Previous studies have suggested that Pax2, Pax5 and Pax8 have overlapping functions (Urbanek et al., 1994; Torres et al., 1996; Mansouri et al., 1998; Bouchard et al., 2002). To define the roles of *pax2a* and *pax8* in otic induction, we analyzed the knockdown of *pax8* by morpholino injection into wild-type embryos and into *pax2a*$^{-}$ mutants. Our results show that removal of both Pax2a and Pax8 together prevents the formation of the otic vesicle and leads to a substantial loss of otic tissue (Fig. 2), whereas neither the single null mutation of *pax2a* nor the knockdown of *pax8* alone is sufficient to block otic placode induction. This result demonstrates that Pax2a and Pax8 have overlapping functions in otic development.

Otic vesicle formation in *pax2a*$^{-}$ mutants is virtually the same as in wild-type embryos; *pax2a*$^{-}$ mutants show a weak neurogenic phenotype, probably owing to reduced Delta signaling (Riley et al., 1999). This result indicates that in the absence of Pax2a, Pax8 is sufficient for most aspects of otic development, even though there are differences in the expression patterns of these two Pax genes. Specifically, *pax8* is expressed prior to *pax2*a, and in contrast to *pax8*, *pax2a* transcription continues after the otic placode becomes morphologically visible (Fig. 1) until it is subsequently restricted to sensory hair cells (Riley et al., 1999). The presence of the *pax2a* duplicate, *pax2b*, cannot account for the absence of a more dramatic phenotype because depletion of Pax2a and Pax2b together leads to no loss of otic structures (Whitfield et al., 2002). These observations suggest either that Pax8 protein is stable and can provide sufficient function at later stages after transcription has ended or that Pax2-Pax5-Pax8 function is not required after placode formation. We are unable to distinguish between these two interpretations because no antibody against Pax8 is currently available.

The otic phenotype of *pax2a*$^{-}$ mutants depleted of Pax8 is similar to embryos depleted of both Fgf3 and Fgf8. Previous studies have shown that knockdown of Fgf3 and Fgf8 in wild-type embryos or knockdown of Fgf3 in *fgf8*$^{-}$ mutants causes a synergistic loss of otic tissue, indicating that *fgf3* and *fgf8* encode overlapping functions required for otic specification (Phillips et al., 2001; Maroon et al., 2002; Leger and Brand, 2002; Liu et al., 2003). The failure of otic tissue formation in the absence of Fgf function is preceded by a strong reduction of *pax2a* and *pax8* expression (Phillips et al., 2001; Leger and Brand, 2002). Furthermore, compromising Fgf signals leads to an absence of *sox9a* expression and significant reduction of *sox9b* expression in the preotic region (Liu et al., 2003), similar to the Fgf dependence of Sox9 in *Xenopus* (Saint-Germain, 2004). By contrast, early *dlx3b* (Leger and Brand, 2002; Liu et al., 2003) and *foxi1* (Fig. 1) expression is less affected by loss of Fgf signaling and effects on *dlx3b* expression are caused at least in part by reduced levels of *sox9a* at early stages and by reduced levels of sox9a and *sox9b* at later stages (Liu et al., 2003). Together, these observations lead to the conclusion that *pax2a* and *pax8* act downstream of Fgf3 and Fgf8, but upstream of *sox9a* and *sox9b*, and that Pax2a and Pax8 are mediators of Fgf signals during otic placode induction (Fig. 8). Recent experiments in *Xenopus* have led to the suggestion that Sox9 may act upstream of Pax8, although the results reported do not rule out the possibility that Sox9 and Pax8 interact to maintain each other's expression (Saint-Germain et al., 2004).

Pax8 helps initiate and Pax2a maintains Fgf dependent *pax2a* expression

The persistence and probably initial induction of *pax2a* expression depend upon Fgf signaling and Pax function. Normal activation of *pax2a* requires Pax8 in cooperation with Fgf8 (Fig. 5) and, because *pax8* expression is not maintained after the placode forms (Fig. 1), long-term maintenance of *pax2a* expression is probably supported by Pax2a activity. This interpretation is consistent with studies in mouse, chick and zebrafish that indicate a positive feedback loop in the isthmic region that requires Pax2 and Fgf8 activity for maintenance of *Pax2* expression (Urbanek et al., 1994; Torres et al., 1996; Mansouri et al., 1998; Martinez et al., 1999; Brand et al., 1996; Lun and Brand, 1998; Reifers et al., 1998). The presence of a high-affinity Pax2-Pax5-Pax8-binding site conserved among human, mouse and *fugu* in the upstream promoter sequence of the *Pax2* gene is consistent with the auto-regulatory function of Pax2 (Pfeffer et al., 2002). This element is also conserved in the zebrafish *pax2a* promoter (data not shown). Despite the importance of Pax protein for *pax2a* expression, induction may occur in the absence of Pax activity. In *foxi1*$^{-}$ mutants (Solomon et al., 2003; Nissen et al., 2003) (Fig. 7), where *pax8* expression is undetectable in the preotic region, *pax2a* expression nevertheless appears, although delayed and variable. These observations may suggest that when Pax8 is reduced, longer exposure to Fgf is required to induce *pax2a* expression. Consistent with this interpretation, we find that embryos injected with the dominant-negative Pax2a construct completely lack otic *pax2a* expression (Fig. 4), presumably because Pax function is more effectively blocked.

Foxi1-Pax8 and Dlx3b-Pax2a mediate two phases of otic specification

Our results are consistent with those of Nissen et al. (Nissen et al., 2003) and Solomon et al. (Solomon et al., 2004) and suggest that Foxi1 is required for the initial Fgf-dependent induction of *pax8*. In *foxi1*$^{-}$ mutants, early expression of *sox9a* and *sox9b* is also severely affected (Fig. 6), similar to the effects of Pax8 depletion in *pax2a*$^{-}$ mutants. However, unlike embryos lacking both Pax2a and Pax8, *foxi1*$^{-}$ mutants later recover *sox9a* and *sox9b* expression (Fig. 6). This recovery is due to Pax2a (Fig. 7); once expression begins, Pax2a protein maintains its own expression and activates downstream *sox9* target genes. Thus, it is likely that variability in the onset of *pax2a* expression, in the absence of Foxi1 and, hence, Pax8 (Fig. 8), produces the highly variable phenotype of *foxi1*$^{-}$ mutants. Supporting this interpretation, *foxi1*$^{-}$;*pax2a*$^{-}$ double mutants exhibit consistent, more severe reduction of otic tissue (Fig. 7).

Our data indicate that Pax2a and Pax8 participate in the same otic developmental pathway: Foxi1 and Pax8 mediate the initial Fgf dependent induction that includes initiation of Dlx3b-dependent *pax2a* expression. Pax2a subsequently maintains its own expression. This model contrasts somewhat from previous suggestions (Riley and Phillips, 2003) primarily based on studies in mouse where loss of *Foxi1* (Hulander et al., 1998; Hulander et al., 2003) or Pax8 (Mansouri et al., 1998) does not prevent otic *Pax2* expression or early patterning and morphogenesis of the otic vesicle. This apparent discrepancy in Foxi1 function between zebrafish and mouse may be due to temporal differences in development. Otic induction in response to Fgf signals occurs over a much longer time period in mice than in zebrafish, which provides more time for cells in mammalian embryos to respond to Fgf signals, even in the absence of Pax8. Analysis of *Pax2*;*Pax8* double mutant mice will be necessary to test this interpretation definitively.

Foxi1 and Dlx3b provide competence to respond to Fgf signals

Our results also provide further insight into Fgf-dependent and -independent processes and the mechanisms underlying competence in otic development. Previously, we have demonstrated that loss of either Fgf3 and Fgf8 or loss of Dlx3b, Dlx4b and Sox9a results in nearly complete loss of otic tissue, although a few residual cells express otic markers including *pax2a*, *fn1* and *cldna* (Liu et al., 2003). Loss of both Fgf signals, and all three of these transcription factors completely blocks all indications of otic induction, suggesting that Fgf-dependent and Fgf-independent processes of otic induction act synergistically. We propose that induction of otic fate by Fgf signals takes place only when cells are competent to respond, and that this competence is provided by Foxi1 and Dlx3b (Fig. 8). A direct role for Foxi1 and Dlx3b in competence needs to be demonstrated, for example by ectopic expression and transplantation experiments. Foxi1 and Dlx3b function by regulating *pax8* and *pax2a* expression, respectively, in an Fgf-dependent fashion. In Dlx3b-deficient embryos, expression of *pax8* is indistinguishable from that in wild-type embryos, presumably owing to normal Foxi1 and Fgf signaling. However, otic *pax2a* expression is initiated only very late and weakly (Solomon and Fritz, 2002; Liu et al., 2003). By contrast, otic *pax8* expression fails and *pax2a* expression is present although delayed in *foxi1*$^{-}$ mutants (Solomon et al., 2003; Nissen et al., 2003). Inhibition of both factors, Foxi1 and Dlx3b, completely blocks otic specification even in the presence of functional Fgf signaling (Fig. 7). By activating Pax8, Foxi1 thus provides competence to otic precursor cells to respond to early Fgf signaling; Dlx3b and Pax2a subsequently maintain this competence (Fig. 8).

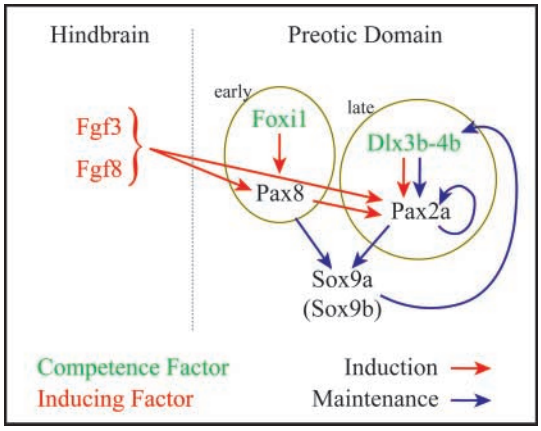

Fig. 8. Pax8 and Pax2a function synergistically in otic specification, downstream of the Foxi1 and Dlx3b transcription factors. Two-phase model summarizing genetic interactions during otic placode induction. Cells of the future otic placode are able to respond to Fgf signals, emanating from the hindbrain, owing to the competence factor Foxi1 (green) and induce Pax8 (red arrows) during an early phase of development (85% epiboly). Pax8 is among the first factors expressed and its activity is required for the expression of Sox9a that maintains (blue arrows) expression of a second pair of competence factors, Dlx3b and Dlx4b, in the preotic domain. Together with Fgf signals and Pax8, Dlx3b-4b is required for the proper initiation of Pax2a in a second, later phase of development (three-somite stage). Once expressed, Pax2a functions with Pax8 in an overlapping manner. Pax2a maintains its own expression and establishes a positive feedback loop through Sox9a and Dlx3b that maintains expression of these factors even after the placode has formed and otic Pax8 expression has stopped. Later in development (as indicated by parentheses), expression of Sox9b probably also helps maintain this pathway.

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