Zebrafish pax8 is required for otic placode induction and plays a redundant role with Pax2 genes in the maintenance of the otic placode

Melinda D. Mackereth1,*, Su-Jin Kwak2,*, Andreas Fritz¹ and Bruce B. Riley2,†

1Department of Biology, Emory University, Atlanta, GA 30322, USA
2Biology Department, Texas A&M University, College Station, TX 77843-3258, USA
*These authors contributed equally to this work
†Author for correspondence (e-mail: briley@mail.bio.tamu.edu)

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Summary
Vertebrate Pax2 and Pax8 proteins are closely related transcription factors hypothesized to regulate early aspects of inner ear development. In zebrafish and mouse, Pax8 expression is the earliest known marker of otic induction, and Pax2 homologs are expressed at slightly later stages of placodal development. Analysis of compound mutants has not been reported. To facilitate analysis of zebrafish pax8, we completed sequencing of the entire gene, including the 5′ and 3′ UTRs. pax8 transcripts undergo complex alternative splicing to generate at least ten distinct isoforms. Two different subclasses of pax8 splice isoforms encode different translation initiation sites. Antisense morpholinos (MOs) were designed to block translation from both start sites, and four additional MOs were designed to target different exon-intron boundaries to block splicing. Injection of MOs, individually and in various combinations, generated similar phenotypes. Otic induction was impaired, and otic vesicles were small. Regional ear markers were expressed correctly, but hair cell production was significantly reduced. This phenotype was strongly enhanced by simultaneously disrupting either of the co-inducers fgf3 or fgf8, or another early regulator, dlx3b, which is thought to act in a parallel pathway. In contrast, the phenotype caused by disrupting foxi1, which is required for pax8 expression, was not enhanced by simultaneously disrupting pax8. Disrupting pax8, pax2a and pax2b did not further impair otic induction relative to loss of pax8 alone. However, the amount of otic tissue gradually decreased in pax8-pax2a-pax2b-deficient embryos such that no otic tissue was detectable by 24 hours post-fertilization. Loss of otic tissue did not correlate with increased cell death, suggesting that otic cells dedifferentiate or redifferentiate as other cell type(s). These data show that pax8 is initially required for normal otic induction, and subsequently pax8, pax2a and pax2b act redundantly to maintain otic fate.

Key words: Preplacodal domain, Otic placode, Genetic network, Paired, Fibroblast growth factor, forkhead, distal-less, mssC

Introduction
Pax proteins are key regulators of developmental processes including specification, differentiation, growth, survival, migration and morphogenesis (Chi and Epstein, 2002; Dahl et al., 1997; Mansouri et al., 1996; Stuart et al., 1994). Pax genes are present in organisms ranging from worms to mammals (Czerny et al., 1997; Dahl et al., 1997; Walther et al., 1991). Pax proteins are named for and defined by the presence of a C-terminal regulatory region containing both activating and inhibitory domains (Dorfler and Busslinger, 1996).

Extensive alternative splicing has been reported for many vertebrate Pax genes, including mammalian Pax2, Pax3, Pax5, Pax6, Pax7 and Pax8, and zebrafish pax2a, pax3, pax7, and pax9 (Barber et al., 1999; Barr et al., 1999; Epstein et al., 1994; Kozmik et al., 1997; Kozmik et al., 1993; Nornes et al., 1996; Puschel et al., 1992; Seo et al., 1998; Tavassoli et al., 1997; Vogan et al., 1996; Zwollo et al., 1997). Similarly, Pax2/5/8 transcripts of the invertebrate chordate amphioxus (Branchiostoma floridae) are alternatively spliced (Krelova et al., 2002). In most cases, alternative splicing has been shown to produce protein isoforms with drastically different DNA binding specificities and transactivation potentials (Barber et al., 1999; Barr et al., 1999; Epstein et al., 1994; Kozmik et al., 1997; Kozmik et al., 1993; Nornes et al., 1996; Puschel et al., 1992; Seo et al., 1998; Tavassoli et al., 1997; Vogan et al., 1996; Zwollo et al., 1997). Thus, alternative splicing is a highly conserved means of increasing the functional repertoire of Pax genes.

The nine vertebrate Pax genes are grouped into four categories, with Pax2/5/8 constituting one of these classes.
Development present in echinoderms, nematodes, and flies (Czerny et al., 1998). Pax2 and Pax8 homologs are also expressed in the otic placode and pronephros in these species (Pfeffer et al., 1998; Plachov et al., 1990). Recent evidence has shown that Pax2 and Pax8 perform redundant functions during mammalian kidney development and are required for the earliest steps of this process (Bouchard et al., 2002; Mansouri et al., 1998). However, otic development in Pax2/Pax8-deficient mouse embryos has not yet been described. In both zebrafish and mouse, Pax8 is strongly expressed in the primordium of the otic placode during late gastrulation, making it the earliest known marker of otic induction (Pfeffer et al., 1998). Pax8 expression is maintained throughout placode development and is lost soon after formation of the otic vesicle (Pfeffer et al., 1998). Pax2 homologs are expressed in the otic anlagen during early somitogenesis stages and are maintained in portions of the otic vesicle (Pfeffer et al., 1998). The Pax8 knockout mouse does not show an otic phenotype (Bouchard et al., 2002; Mansouri et al., 1998), and the Pax2 knockout mouse shows variable defects in derivatives of the medial otic vesicle where Pax2 is expressed after the vesicle forms (Bouchard et al., 2000; Burton et al., 2004; Favor et al., 1996; Torres et al., 1996). The absence of an earlier or more severe phenotype may reflect redundancy between these genes. There are two Pax2 homologs in zebrafish, pax2a and pax2b, and functional disruption of both genes reduces hair cell production but does not impair formation of the placode or vesicle (Whitfield et al., 2002). The extent to which pax8 compensates for loss of pax2a and pax2b is not known.

Several upstream regulators of otic induction have been identified. The forkhead class transcription factor gene foxi1 is expressed in the ventral ectoderm beginning at 50% epiboly. By mid-gastrulation foxi1 expression is upregulated in the future otic placode prior to induction of pax8. Loss of foxi1 prevents expression of pax8 in the otic domain and severely compromises otic induction. Furthermore, misexpression of foxi1 is sufficient to induce ectopic pax8 (Nissen et al., 2003; Solomon et al., 2003). At least two other genes, fgf3 and fgf8, are also necessary for pax8 expression. These genes encode Fgf ligands that are expressed in the developing hindbrain between the prospective otic placodes. Loss of both fgf genes blocks otic induction, whereas misexpression of either gene is sufficient to induce ectopic otic tissue (Leger and Brand, 2002; Maroon et al., 2002; Phillips et al., 2001; Phillips et al., 2004). Thus, Fgf signaling and foxi1 function converge to induce pax8, suggesting that pax8 could be an important mediator of otic induction. In addition, zebrafish dlx3b and dlx4b, transcription factors with homeo-domains similar to Drosophila distal-less (Ekker et al., 1992a; Ellies et al., 1997), are required for otic placode formation. Combined loss of function of dlx3b/dlx4b leads to a reduction or absence of otic placodes and pax2a expression in otic cells, but pax8 expression initiates normally (Liu et al., 2003; Solomon and Fritz, 2002).

In this paper, we describe a role for pax8 during otic development. We have cloned full-length transcripts of zebrafish pax8 and show that there are three main splice variants that encode proteins with different N-terminal sequences. Depletion of Pax8 function leads to compromised otic vesicle and inner ear morphology, and our data suggest that different isoforms have both overlapping and unique functions. We show a strong genetic interaction between pax8 and pax2a, and to a lesser extent pax2b, implicating these genes in the maintenance of otic cell fate. Depletion of pax8 enhances otic placode and vesicle defects in embryos with reduced Fgf signaling or in embryos that have been depleted for dlx3b function. In contrast, depletion of pax8 does not enhance defects in embryos depleted for foxi1. These and other data support the hypothesis that pax8 helps mediate otic induction downstream of foxi1 and fgf3 and 8 and but in parallel with dlx3b. At later stages, pax8 acts redundantly with Pax2 genes to maintain otic fate.

Materials and methods

Fish strains

Embryos were developed at 28.5°C and staged according to standard criteria (Kimmel et al., 1995). Wild-type fish were derived from the AB line. noip226 and ace7282 were derived from the Tu line (Brand et al., 1996) and used to assess functions of pax2a and fgf8, respectively. noip226 is thought to be a null allele (Lun and Brand, 1998) whereas ace7282 is a strong hypomorph (Draper et al., 2001). Because pax2a and fgf8 are closely linked, producing ace-noi double homozygotes that we first produce a recombinant line in which both mutant loci reside on the same chromosome. The rate of recombination between fgf8 and pax2a is roughly 1.5%, so nearly 25% of intercross progeny produced in this line are double homozygotes.

pax8 5’ and 3’ RACE cloning and sequencing

RNA was isolated from 3-5 somite and 24-hour embryos using TRIPURE reagent (Roche). For the 5’ RACE reaction, 3-5 somite stage RNA was processed using the First Choice RLM-RACE kit from Ambion. cDNA was synthesized using a pax8-specific primer (CAGCGCGCAGCCGAAAAGT) and C. therm polymerase (Roche) at 68°C for reverse transcription. Subsequently, PCR was performed using a second, nested pax8-specific primer (GCCGGCGGTCGATTGGCAAAACTGTA) and the 5’ RACE adaptor outer primer (Ambion). A fraction of this reaction was used as template in a second PCR amplification with a third, nested pax8-specific primer (AAGGGCCCGAGATGACGGAGGACGAA) and the 5’ RACE adaptor inner primer. All PCRs were performed using the Clontech Advantage-GC2 protocol with a final concentration of 1 M GC-melt. The resulting amplification products were cloned into pCRTI Topo vector (Invitrogen) and sequenced. The 24-hour RNA was reverse transcribed using the CDS primer from the SMART II kit (Clontech) and C. therm polymerase. 3’ RACE was performed using a pax8-specific primer (CATCAATGGGCTGCTGGGAATCA) and the CDS primer (Clontech) in an initial PCR. A fraction of this reaction was used as template in a second PCR amplification with a nested pax8-specific primer (TCCGGGCGAGATGACGGAGGACGAA) and the 5’ RACE adaptor inner primer. All PCRs were performed using the Clontech Advantage-GC2 protocol with a final concentration of 1 M GC-melt. The resulting amplification products were cloned into pCRTI Topo vector and sequenced.

For the splice variant analysis, pax8-specific primers located in the 5' UTR [exon 1a (Fig. 1A); GACAGACAACGGGAACACCCAA-CAC] and the 3' UTR [exon 13 (Fig. 1A); ACCCGCGCTCAC-
Fig. 1. Sequence and splice variants of pax8. (A) The complete sequence of the pax8 variant 3 transcript. Exons predicted by comparison to genomic sequence are indicated above the sequence. The transcription start site, preceded by a TATAA box, begins with exon 1a. Exon 1 is contiguous with genomic DNA sequence, but has been subdivided into exons 1a, 1b, and 1c to indicate that the splice variants shown in Fig. 1B contain different parts of exon 1. The arrow indicates the putative start codon used in variant 1 transcripts. The star indicates that some transcripts use an alternate splice donor site that extends exon 9 by 33 bp (sequence not shown). The canonical Paired domain and predicted transactivation and inhibitory domains are indicated below the sequence. Polyadenylation (polyA) sites are underlined in purple; the majority of transcripts (52/54) use the first polyA site. (B) A schematic representation (not to scale) of the splice variants observed in pax8 transcripts. The variant name (e.g. 1.1, 1.2), total number of representative transcripts out of 54 total sequenced, and percentage abundance are indicated after each schematic transcript. The unlabeled box between exon 9 and exon 10 in variants 1.4 and 1.5 indicates an additional 11 amino acid insert (see also Fig. 1A) resulting from the use of an alternate splice donor site. The predicted coding sequence for variants 2 and 3 begins with the start codon in exon 1c; all variant 1 transcripts use the start codon in exon 2 indicated by the arrow in Fig. 1A.
CTAACACATCAATAG] were used to amplify pax8 transcripts from 24-hour cDNA (described above), using the Advantage-GC2 PCR protocol with a 1 M concentration of GC-melt. The resulting products were cloned into the pCRII Topo vector and sequenced.

**Morpholino injections**

Morpholino oligonucleotides obtained from Gene Tools Inc. were diluted and injected as previously described (Nasevicius and Ekker, 2000; Phillips et al., 2001). A total of 1-5 ng MO was injected per embryo. At least 25 specimens were examined for each experiment.

To knockdown pax8, translation-blocking morpholinos and splice-blocking morpholinos were generated as follows: translation blocker for splice variant 1 (variant 1 MO): 5′ GGTACCAAAACATGCTCTCTCTTAGTG 3′; translation blocker for splice variants 2 and 3 (variant 2/3 MO): 5′ GACCTGCCCAGTGCTGGTGAAT 3′; splice blocker exonnt/l (splice donor site): 5′ CTCGACTCAGTGTACAGCTCCTC 3′; splice blocker exonnt/l (splice acceptor site): 5′ CAGCGTCAGTTGCTCAGCACAAC 3′; splice blocker exonnt/l (paired domain): 5′ GGAGGGTGCACACCCCTCCGCCC 3′; splice blocker exonnt/l (homeo domain): 5′ TGGCTGTTGCTGCACCTCGTCTGTGCT 3′. Unless stated otherwise, pax8 morphants were injected with 2.5 ng each of variant 1 MO and variant 2/3 MO to disrupt pax8 function.

To knockdown fgf3, two translation-blocking sequences were co-injected: fgf3-MO #1, 5′ CATGTTGCATCGGCGATGCGGC 3′; fgf3-MO #2, 5′ GTGCCCTCATCAGAAGATCATTTG 3′. Other morpholino sequences were as follows: dlx3b-MO translation blocker, 5′ ATATGTCGGTCCACTCATCCTTTAAT 3′; foxl-MO translation blocker, 5′ TAATCGCCTCTCCCTCCAGAAA 3′; pax2b-MO translation blocker: 5′ GGTCTGCTCTACAGTGAATATCCAT 3′.

**Immunofluorescent staining**

Embryos were raised in 0.3% PTU solution to inhibit the formation of melanocytes. Embryos were fixed and stained as previously described (Riley et al., 1999) using polyclonal anti-mouse Pax2 antibody (Berkeley Antibody company, 1:100 dilution) and monoclonal anti-acetylated tubulin antibody (Sigma T-6793, 1:100). The sequence analysis shows that pax8 transcripts are subject to extensive alternative splicing. To address the potential functional significance of different Pax8 isoforms, artificial mRNA for variants 1.1 or 2.1 (full length) and 1.3 or 2.3 (nonfunctional transactivation domain) were injected into one-cell embryos. Ectopic overexpression of either full length variant leads to severe gastrulation defects, precluding a meaningful interpretation of pax8 function in otic placode formation. Conversely, injection of the isoforms lacking the transactivation domain did not lead to any detectable phenotypes in otic placode or vesicle morphology (data not shown).

**Functional analysis of pax8**

We designed morpholino oligonucleotides (MO) to knock down pax8 function. Four MOs were designed to block pre-mRNA splicing at distinct splice junction sites (Draper et al., 2001), and two additional MOs were designed to target the sequence around each of the predicted start codons (Nasevicius and Ekker, 2000). Together, these two MOs are predicted to block translation of all isoforms (Fig. 1A). Co-injection of the translation-blocking MOs resulted in the most consistent and reproducible phenotypes, and this approach was used in all
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Fig. 2. Assessment of Pax8 function in otic induction. (A,D) Head region of live wild-type (A) and pax8-MO injected (D) embryos at 30 hpf. The pax8-MO-injected embryo has a narrow midbrain-hindbrain border (asterisk), and small otic vesicles (ov). (B,E) Otic vesicles at 30 hpf in live wild-type (B) and pax8-MO-injected (E) embryos. (C,F) Otic vesicles at 30 hpf in embryos injected with v1-MO to knockdown variant 1 isoforms of pax8 (C) or v2/3-MO to knockdown variant 2 and 3 isoforms of pax8 (F).

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We also examined later aspects of otic patterning in pax8 morphants. General features of anterior-posterior, dorso-ventral and medio-lateral patterning appear unaffected, as shown by normal expression of pax5 in the anterior (Fig. 3B,F), dlx3b in the dorsal (Fig. 3C,G), and pax2a in the ventromedial portions of the otic vesicle (Fig. 3A,E). However, hair cell production is reduced by an average of 42±11% (n=59; compare Fig. 3A and E), and severely affected specimens produce only a single macula per ear (not shown). Cristae typically form by 72 hpf and express msxC, although the level of expression is usually reduced in the lateral crista (Fig. 3D,H). In severely affected specimens with very small otic vesicles, one or more cristae are fused or missing (not shown). Later stages of otic development become increasingly aberrant as embryos begin to degenerate and die (not shown). Since pax8 is not expressed in the ear past 19 hpf, reduction in the vesicle and sensory epithelia presumably arise from earlier deficits in the placode or preplacode resulting from reduced levels of pax8.

Interactions between pax8 and Fgf genes

Fgf signaling appears to be the principal inducer of otic development in all vertebrates (Noramly and Grainger, 2002; Riley and Phillips, 2003). In zebrafish, fgf3 and fgf8 are expressed in periotic tissues during gastrulation and are essential for otic induction (Leger and Brand, 2002; Maroon et al., 2002; Phillips et al., 2001). Furthermore, in the experimental context of the whole embryo, misexpression of fgf3 or fgf8 can lead to the formation of ectopic otic placodes (Phillips et al., 2004). Induction of pax8 expression is the earliest response to Fgf signaling and does not occur in the absence of Fgf signaling. We hypothesized that pax8 helps mediate otic induction or early differentiation in response to Fgf signaling. In support of this hypothesis, the phenotype
observed in pax8 morphants (Figs 2 and 3) resembles that observed in embryos disrupted for either fgf3 or fgf8 (Fig. 4E,M). We tested this relationship further by examining the effects of disrupting pax8 and either fgf3 or fgf8. Because fgf3 and fgf8 are partially redundant, blocking either function alone causes only moderate reduction in the expression domains of preotic markers pax8, pax2a, and dlx3b (Fig. 4F-H,N-P), with corresponding reduction in the size of the otic vesicle (Fig. 4E,M). These tissues are further reduced in embryos depleted for both pax8 and fgf3 (Fig. 4I-L). Depleting pax8 in ace (fgf8) mutants causes even more severe deficiencies in otic development: at early stages, expression domains of pax8 and pax2a are strongly reduced, upregulation of dlx3b does not occur, and at later stages the otic vesicle is rudimentary and produces no hair cells or otoliths (Fig. 4Q-T). It is unclear whether the stronger interaction of pax8 with fgf8 compared to fgf3 reflects functional differences between these ligands or differing degrees of functional disruption. A recent report suggests that fgf8 plays a more dominant role than fgf3 in early hindbrain patterning (Wiellette and Sive, 2004), and may thus also have a more pronounced role in otic induction. In either case, the above data are consistent with the hypothesis that pax8 helps mediate the effects of both Fgfs. The fact that otic development is not totally ablated is consistent with the notion that pax8 is not the only gene involved in the early response to Fgf signaling.

Interactions between pax8 and Pax2 genes

Two other Pax genes are coexpressed with pax8 in preotic cells, albeit at slightly later stages: pax2a is expressed in the otic anlagen by 11 hpf (3 somites) and pax2b is expressed by 13.5 hpf (9 somites) (Pfeffer et al., 1998). As closely allied members of the Pax family, pax2a and pax2b are thought to play a role in the development of the otic vesicle. Similar to the effects observed in pax8 morphants, depletion of pax2a and pax2b results in a reduction of the expression domains of preotic markers pax8 and dlx3b (Fig. 4F-H,N-P). These reductions are further exacerbated by blocking the function of either fgf3 or fgf8, indicating that these Fgfs play a role in the regulation of pax2a and pax2b expression.

Fig. 3. Assessment of Pax8 function in otic vesicle patterning. (A,E) Otic vesicles at 32 hpf stained with anti-Pax2 (red) and anti-acetylated tubulin (green) antibodies to label hair cells (Riley et al., 1999) in wild-type (A) and pax8-MO-injected (E) embryos. Hair cell patches are indicated (white arrows). Injected embryos produce fewer hair cells than normal. In the experiment shown here, an average of 5.2±1.1 hair cells were seen in pax8 morphants (n=59 ears), compared to 9.2±1.4 hair cells in wild type embryos (n=10 ears); 5-10% of pax8 morphants produce only one macula per ear (not shown). (B,F) Pax5 expression at 24 hpf in wild-type (B) and pax8-MO-injected (F) embryos. (C,G) dlx3b expression at 33 hpf in wild-type (C) and pax8-MO-injected (G) embryos. (D,H) msxC expression marks developing cristae in the otic vesicle at 78 hpf in wild-type (D) and pax8-MO-injected (H) embryos. The lateral crista is present in the injected embryo but shows strongly reduced expression of msxC. Defects in development of cristae and semicircular canals are highly variable in pax8 morphants. Images show lateral views with anterior to the left and dorsal to the top. ac, anterior crista; lc, lateral crista; pc, posterior crista. Scale bar in H: 90 µm for D,H, 40 µm for C,G, and 30 µm for A,B,E,F.

Fig. 4. Interaction of pax8 with fgf3 and fgf8. Wild-type embryos (A-D), wild-type embryos injected with fgf3-MO (E-H), wild-type embryos co-injected with fgf3-MO and pax8-MO (I-L), ace (fgf8) mutants (M-P), and ace mutants injected with pax8-MO (Q-T). Images show lateral views of the otic vesicle at 30 hpf (A,E,I,M,Q), and dorsal views of pax8 expression at 10 hpf (B,F,J,N,R), pax2a expression at 12 hpf (C,G,K,O,S) and dlx3b expression at 12 hpf (D,H,L,P,T). op, otic placode. Arrowheads mark the otic region where dlx3b expression normally shows marked upregulation. Anterior is to the left in all specimens. Op, optic placode. Scale bar in T: 30 µm for A,E,I,M; 200 µm for B-D,F-H,J,L,N-P,R,T.
pax2/5/8 family, these three genes could provide multiple levels of redundancy during otic development. Notably, embryos deficient in both pax2a and pax2b produce relative normal otic vesicles, with defects being limited to reduced hair cell production (Whitfield et al., 2002). This surprisingly mild phenotype possibly reflects compensation by pax8. Similarly, later expression of pax2a and pax2b could ameliorate the effects of disrupting pax8 function. To address these possibilities, we injected pax8-MO and pax2b-MO into noi (pax2a) mutants (Fig. 5A′-D′). Early placode development in pax2a−pax2b−pax8-deficient embryos is similar to that in pax8 morphants. However, by 18 hpf, the otic domain of pax2a is severely reduced in pax2a−pax2b−pax8-deficient embryos (Fig. 5B′). By 24 hpf, the otic domain of pax2a is lost entirely in about half of pax2a−pax2b−pax8-deficient embryos (47%, n=36; Fig. 5C′), and there are no morphological signs of otic development (Fig. 5D′). Staining with acridine orange indicates that there is no noticeable increase in cell death in the periotic region (not shown), suggesting that otic cells dedifferentiate in the absence of pax2/8 function. In agreement, about half of pax2a−pax2b−pax8-deficient embryos show diffuse expression of dlx3b in the otic region or no otic expression at all (11/30 and 3/30, respectively, Fig. 5E′,F′). In addition, a scattering of dlx3b-expressing cells appears

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**Fig. 5.** Relative functions of pax8, pax2a and pax2b. (A-C) pax2a expression in wild-type embryos at 14 hpf (A), 18 hpf (B) and 24 hpf (C). (D) Head region of an un.injected noi (pax2a) mutant at 30 hpf. The otic vesicle has essentially normal morphology. A′-D′, noi (pax2a) mutants co-injected with pax8-MO and pax2b-MO showing pax2a expression at 14 hpf (A′), 18 hpf (B′) and 24 hpf (C′), and a live specimen with no otic vesicles at 30 hpf (D′). krox20 expression in the hindbrain is shown in red (B,B′), (E,E′,F′) dlx3b expression at 24 hpf in a wild-type embryo (E), a wild-type embryo injected with pax8-MO (F), and noi (pax2a) mutants co-injected with pax8-MO and pax2b-MO (E′,F′). The specimen in E′ lacks morphological otic vesicles but does show a scattering of dlx3b-expressing cells in the otic region (asterisk). The specimen in F′ lacks morphological signs of otic development and also shows no dlx3b expression in the otic region (asterisk). G,G′,H,H′, fgf3 expression in wild-type embryos at 14 hpf (G) and 19 hpf (H) and in pax2a−pax2b−pax8-deficient embryos at 14 hpf (G′) and 19 hpf (H′). Expression in the hindbrain (r4) is normal in all specimens, whereas expression in the otic vesicle (ov) is strongly reduced at 19 hpf in pax2a−pax2b−pax8-deficient embryos (compare H,H′). (I′-L′) sox9a expression at 13.5 hpf in wild-type (I) and pax2a−pax2b−pax8-deficient (I′) embryos. (J′-L′) claudinA expression in wild-type embryos at 13.5 hpf (J) and 24 hpf (K,L) and in pax2a−pax2b−pax8-deficient embryos at 13.5 hpf (J′) and 24 hpf (K′,L′). M-P, wild-type embryos co-injected with pax8-MO and pax2b-MO showing expression of pax2a at 14 hpf (M) and 18 hpf (N), the head region of a live specimen with a small otic vesicle at 30 hpf (O), and an enlargement of the otic vesicle of the same specimen (P). Images show dorsal views with anterior to the left (A,A′,G,G′,J′-J′,M), dorsal views with anterior to the top (B,B′,C,C′,K,K′,N), or lateral views with anterior to the left (D-D′,F′,L′,O,P). a, pharyngeal arches; asterisk, region where otic vesicle normally forms; mhb, midbrain-hindbrain boundary; op, otic placode; ov, otic vesicle; r4, rhombomere 4 of the hindbrain. Scale bar in P: 170 µm for A,A′,G,G′,J′-J′,M; 75 µm for B-F,B′-F′,H,H′,K,K′,L′,L′,O; 19 µm for P.
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between the otic territory and pharyngeal arches, a region normally devoid of dlx3b expression. It is possible that these ectopic dlx3b-expressing cells are the dispersed remnants of the otic vesicle. Expression of other otic markers shows similar results. For example, sox9a expression is initially induced but is barely detectable in pax2a-pax2b-pax8-deficient embryos by 13.5 hpf (Fig. 5L'). claudinA is expressed in a reduced otic domain at 13.5 hpf. By 24 hpf, about half of pax2a-pax2b-pax8-deficient embryos show either no claudinA expression (9/25, Fig. 5K') or a diffuse scattering of expressing cells, again suggesting dispersal of otic remnants (5/25, Fig. 5L'). In summary, otic induction appears no worse in pax2a-pax2b-pax8-deficient embryos than in pax8 morphants, but the otic placode is not maintained properly at later stages. Various hindbrain markers including krox20, fgf3, fgf8, wnt8, and wnt8b (Fig. 5B',G',H', and data not shown) are induced and maintained normally, suggesting that the failure to maintain otic fate is not due to loss of hindbrain signaling.

A similar phenotype to the pax2a-pax2b-pax8-deficient phenotype is seen in noi (pax2a)-pax8-deficient embryos (not shown). Because pax2b is still expressed, we infer that pax2b alone is not sufficient to maintain otic development. Nevertheless, the frequency of total ear loss in noi (pax2a)-pax8-deficient embryos (22%, n=23) is lower than in pax2a-pax2b-pax8-deficient embryos (47%, n=36), suggesting that pax2b can contribute to otic maintenance. To test this further, we injected wild-type embryos with pax2b-MO and pax8-MO. Otic development is similar to that seen in pax8-deficient embryos through 18 hpf (Fig. 5M,N, and data not shown). However, pax2b-pax8-deficient embryos produce a much smaller otic vesicle than pax8-deficient embryos and usually lacks otoliths (Fig. 5O,P), suggesting significant loss of otic tissue after the vesicle begins to form. Thus, both pax2a and pax2b play a role in otic maintenance, but the requirement for pax2a appears more critical.

Because of the strong interaction between pax8 and pax2a, we used the noi (pax2a) mutation to provide a sensitized background in which to test the relative roles of different pax8 splice variants. pax8 variant 1 MO blocks translation of variant 1 isoforms, which lack the N-terminal Paired domain, whereas pax8 variant 2/3 MO blocks translation of isoforms predicted to include the entire Paired domain. Injection of pax8 variant 1 MO into noi (pax2a) mutants usually results in production of a moderately reduced otic vesicle containing hair cells but no otoliths (83%, n=84, Fig. 6A,B). In contrast, injection of pax8 variant 2/3 MO into noi (pax2a) mutants ablates the ear entirely (21/76) or results in production of a relatively small otic vesicle (55/76). In the latter case, however, otoliths are usually produced (Fig. 6C,D). The two translation blockers also differentially affect brain development in the region of the midbrain-hindbrain border (MHB). The MHB does not form in noi (pax2a) mutants. Mutants injected with pax8 variant 2/3 MO invariably show a persistent and intense band of cell death localized to the ventral midline of the MHB region (Fig. 6C,H). This pattern of cell death is never

**Fig. 6.** Distinct functions of pax8 splice isoforms. (A-D) Lateral views of the head and otic structures at 30 hpf in noi (pax2a) mutants injected either with pax8 variant 1 MO (A,B) or noi mutants injected with pax8 variant 2/3 MO (C,D). Most noi mutants knocked down for variant 1 isoforms produce a moderate-sized otic vesicle containing hair cells but lacking otoliths (B). In contrast, noi mutants knocked down for variant 2 and 3 isoforms typically produce a small otic vesicle containing both hair cells and otoliths (D) or no otic vesicle at all (data not shown). In addition, all noi mutants knocked down for variant 2 and 3 isoforms show persistent cell death (cd) in the midbrain-hindbrain region. (E-H) Dorsal views of the midbrain-hindbrain border region at 30 hpf in an uninjected wild-type embryo (E), an uninjected noi mutant injected with pax8 variant 1 MO (G) and a noi mutant injected with pax8 variant 2/3 MO (H). Increased cell death is not evident in the majority of noi mutants knocked down for variant 1 isoforms (A) and, if present (G), cell death is diffuse and limited to dorsolateral tissue. In noi mutants knocked down for variant 2 and 3 isoforms, cell death is invariably present, intense, and localized to the midline of the midbrain-hindbrain border region (H). Anterior is to the left (A-D) or to the top (E-F). cd, cell death; mhb, midbrain-hindbrain border; ot, optic tectum. Scale bar in H: 75 µm for A,C; 50 µm for E-H; 19 µm for B,D.

**Fig. 7.** pax2a interacts with fgf8. Wild-type embryos (A-C), ace (fgf8) single mutants (D-F) and ace-noi (fgf8-pax2a) double mutants (G-I). Images show dorsal views of pax2a expression at 12 hpf (A,D,G) and 14 hpf (B,E,H), and lateral views of otic vesicles at 30 hpf (C,F,I). The specimen in B is the same as in Fig. 5A, and the specimen in C is the same as in Fig. 2B. Anterior is to the left in all specimens. mhb, midbrain-hindbrain border; op, optic placode. Scale bar in I: 170 µm for A,B,D,E,G,H; 35 µm for C,F,I.
observed in uninjected noi (pax2a) mutants nor in mutants injected with pax8 variant 1 MO (Fig. 6A,F). Instead, 20-30% of noi (pax2a) mutants injected with pax8 variant 1 MO show a moderate increase in cell death in the dorsolateral MHB region (Fig. 6G). The significance of these differences is unclear at present, but the data strongly suggest that Pax8 isoforms containing a complete vs. partial Paired domain have at least some distinct developmental functions.

Interactions between pax2a and Fgf genes
A role for Pax2 genes in placode maintenance has not been previously reported, so this function was further investigated by analyzing the interaction between fgf genes and pax2a. In ace-noi (fgf8-pax2a) double mutants, expression of preotic markers is initially comparable to that seen in ace single mutants. However, the otic domain of pax2a shrinks dramatically in ace-noi double mutants such that, by 14 hpf, it is much smaller than in either ace or noi single mutants (Fig. 7D,E,G,H). ace-noi double mutants produce only very small otic vesicles containing few hair cells and no otoliths (Fig. 7I; and data not shown). In severely affected specimens, otic vesicles are so small that they can only be detected at high magnification using DIC optics. Similar results are obtained when fgf3 is knocked down in noi (pax2a) mutants (not shown). Presumably, Fgf depletion impairs early preotic development such that pax2a function becomes critical for this process. When pax2a is disrupted in addition to fgf3 or fgf8, the majority of placodal cells are unable to maintain otic fate.

Pax8 interaction with other transcription factors, Foxi1 and Dlx3b
foxi1 is one of the earliest regulators of preotic development identified (Nissen et al., 2003; Solomon et al., 2003). Loss of foxi1 causes a severe phenotype characterized by production of very small otic vesicles or, in severe cases, complete loss of otic tissue. Expression of pax8 in the otic domain is not detectable in foxi1 mutants, presumably contributing to the mutant phenotype. Conversely, misexpression of foxi1 is sufficient to induce ectopic expression of pax8, suggesting that Foxi1 serves as an upstream activator of pax8 expression. To test this predicted epistatic relationship, we co-injected foxi1-MO and pax8-MO into wild-type embryos. Knockdown of foxi1 and pax8 causes defects in otic development that are indistinguishable from the effects of injecting foxi1-MO alone (Fig. 8A-F), supporting the notion that foxi1 and pax8 function in a simple linear pathway.

dlx3b is another early regulator of preotic development, and mutants homozygous for a deletion that removes dlx3b (as well as dlx4b and sox9a) show severe deficiency of otic tissue. However, they show nearly normal expression of pax8 (Solomon and Fritz, 2002). Furthermore, early expression of dlx3b along the edges of the neural plate is independent of Fgf signaling and pax8 function (Fig. 4). These and other data strongly suggest that pax8 and dlx3b are at least initially in independent pathways. To investigate the epistatic relationship between these genes, embryos were co-injected with dlx3b-MO and pax8-MO. In dlx3b-pax8 double morphant embryos, preotic domains of dlx3b and pax2a are reduced relative to those seen in embryos depleted for dlx3b or pax8 alone (Fig. 8H,I,K,L). Otic vesicles are dramatically reduced in size and typically produce no hair cells or otoliths (Fig. 8G,J; and data not shown), indicating severe deficiencies in otic differentiation. These findings show that pax8 and dlx3b do not operate in a simple linear pathway.

Discussion
Exonic structure of pax8
We have completed the pax8 cDNA sequence and identified three main variants of transcripts with several subvariants present. While most features of the exon-intron structure are conserved with mammalian Pax8, the zebrafish sequence shows several unique features. Most significantly, we identified three main categories of splice variants that vary in their N-terminal sequences, leading to the use of two alternative start codons. The predicted ORF for variant 1 begins ten amino acids within the canonical Paired domain and would presumably disrupt the DNA-binding ability of the N-terminal portion of this domain (Xu et al., 1995). Variants 2 and 3 encode proteins that contain the entire canonical Paired domain. The Paired domain comprises N-terminal and C-
terminal subdomains, which mediate binding to distinct parts of a conserved DNA consensus sequence. Although no isoforms using alternate start codons have been identified in the mouse or human, one mammalian isoform, Pax8(S), contains an additional serine residue in the N-terminal subdomain. This insertion functionally inactivates the N-terminal subdomain, causing Pax8(S) to bind a different DNA consensus sequence through its C-terminal subdomain, perhaps analogous to the zebrafish variant 1 vs. variant 2-3 isoforms. Pax8(S) accounts for roughly one-third of Pax8 produced in all tissues, extending the range of target genes potentially regulated by the Pax8 locus (Kozmik et al., 1997). It is possible that the zebrafish isoform with an incomplete N-terminal subdomain has altered binding properties similar to mammalian Pax8(S). Interestingly, Fugu pax8 also encodes a methionine at position 10 of the Paired domain (Pfeffer et al., 1998), and thus may encode a set of Pax8 proteins similar to zebrafish. Although the exact functional differences between these isoforms remain to be elucidated, our data suggest that variant 1 and variant 2/3 isoforms appear to have both overlapping and unique functions as revealed by knockdown in the noi (pax2) mutant background.

At least six splice variants found in the mouse and human show changes in C-terminal sequences (Kozmik et al., 1993), and even more C-terminal variants are found in zebrafish. Other Pax8 functional domains, including the transactivation domain and the inhibitory domain, are disrupted in these isoforms. The functional significance of C-terminal variation is presently unknown; however, altering the structure of the functional domains may create proteins with altered DNA sequence specificity or varying transactivation potentials, as has been previously reported for other members of the zebrafish and mammalian Pax gene families (Barber et al., 1999; Barr et al., 1999; Epstein et al., 1994; Kozmik et al., 1997; Kozmik et al., 1993; Nornes et al., 1996; Puschel et al., 1992; Seo et al., 1998; Tavassoli et al., 1997; Vogan et al., 1996; Zwollo et al., 1997). It should be noted that these alternatively spliced isoforms appear to be rare in zebrafish.

Redundancy among Pax2 and pax8 genes

Knockdown of pax8 causes significant reduction in the amount of otic tissue produced during induction, and the deficit persists through subsequent stages of otic development. The small vesicle that is eventually produced expresses regional markers normally but shows deficiencies in sensory epithelia. In severe cases, various maculae or cristae are missing or fused, possibly as a nonspecific consequence of the presence of too little otic tissue. The closely related genes pax2a and pax2b are expressed at slightly later stages of preotic development and appear to partially overlap in function with pax8. Disruption of both pax2a and pax2b function causes only subtle defects in otic development, suggesting that pax8 can substantially compensate for their loss. When the function of all three Pax genes is disrupted, otic tissue shows progressive diminution during placodal development and is lost entirely by 24 hpf. Staining with acridine orange does not reveal an obvious increase in otic cell death, suggesting that otic these cells eventually dedifferentiate in the absence of otic maintenance mediated by pax2a, pax2b, and pax8. This notion is further supported by the observation that the otic domain of dlx3b expression appears to be progressively lost beginning around 24 hpf. These data strongly support the hypothesis that pax8 and pax2 functions are partially redundant. A similar relationship among murine Pax2/5/8 family members seems likely as well. Pax8 and Pax2 are expressed in the developing murine ear at the same relative stages as in zebrafish (Pfeffer et al., 1998). No ear defects have been reported in Pax8 knockout mice (Bouchard et al., 2002; Mansouri et al., 1998), and defects in Pax2 knockout mice are limited to disturbances in medial otic vesicle development (Bouchard et al., 2000; Burton et al., 2004; Favor et al., 1996; Torres et al., 1996). Otic development has not yet been described in Pax8-Pax2 double knockout mice, but it seems likely that much more severe otic defects will result in such embryos. Indeed, such has been observed with respect to kidney development (Bouchard et al., 2002). The developing kidney undergoes apoptotic cell death at an early stage in Pax8-Pax2 double mutants, a phenotype not observed in either of the single mutants (Bouchard et al., 2002).

pax8 as part of a genetic network.

Induction of pax8 expression requires at least two distinct pathways, one mediated by foxi1 and the other by Fgf signaling (Leger and Brand, 2002; Maroon et al., 2002; Nissen et al., 2003; Phillips et al., 2001; Solomon et al., 2003; Solomon et al., 2004). These inductive pathways are partially independent, but some aspects of foxi1 expression appear to be regulated by Fgf signaling. foxi1 is initially expressed in ventral ectoderm but then shows upregulation in periotic ectoderm roughly 30-60 minutes before induction of pax8. The spatial pattern of foxi1 expression is unaltered in embryos depleted for Fgf3 and Fgf8 (Solomon et al., 2004), but misexpression of Fgf3 or Fgf8 is sufficient to induce foxi1 expression in ectopic locations (Phillips et al., 2004). It is possible that foxi1 is sensitive to residual Fgf signaling in Fgf morphants or, alternatively, Fgf3 and Fgf8 may act in concert with other factors to regulate foxi1. In any case, expression of pax8 occurs in the region where foxi1 and Fgf signaling overlap, and serves as an important nexus linking these pathways.

Our data also indicate that pax8 positively regulates its own expression since the level of pax8 expression is reduced in pax8 morphants. We speculate that pax8 helps mediate otic induction and that this feedback loop magnifies the efficacy of Fgf signaling, extending the range of Fgf action to cells farther from the source. Thus, loss of pax8 would be expected to limit otic induction to cells in close proximity to the signaling source, a prediction borne out by our studies. Subsequent expression of pax2a and pax2b, which require Fgf signaling but not pax8, presumably stabilizes otic fate within the diminished population of preotic cells. Such a model could explain why eliminating Pax8 in the mouse has such mild consequences; in the mouse, Fgf3 is expressed directly within preotic cells, making the need for signal amplification less critical during initial stages of otic induction. Later expression of Pax2 might then be sufficient to stabilize otic development initiated by prior Fgf signaling.

A number of other transcription factors have been implicated in early otic development, the best characterized of which are dlx3b and dlx4b. dlx3b/4b are initially expressed in ventral ectoderm but become restricted by 9 hpf to a contiguous line of cells surrounding the neural plate (Akimenko et al., 1994). By 11 hpf, dlx3b/4b show strong upregulation in preotic cells. The early phases of dlx3b/4b expression are independent of Fgf
signaling, but later upregulation in the otic anlagen fails to occur in embryos depleted for Fgf3 and Fgf8 (Liu et al., 2003; Solomon, 2004); (this report). As such, dlx3b and dlx4b could serve as another mediator of Fgf signaling (Solomon, 2004). Knockdown of either dlx gene causes mild to moderate deficiencies in otic development, with much more severe deficiencies seen in embryos knocked down for both (Liu et al., 2003; Solomon and Fritz, 2002). Embryos homozygous for a deletion that removes dlx3b, dlx4b and sox9a (a third preotic marker under control of Fgf signaling) fail to produce an ear, although roughly one-third of mutant embryos produce a few disorganized otic cells that belatedly express pax8. This severe disruption occurs despite the fact that pax8 is initially expressed normally (Solomon and Fritz, 2002). Thus, pax8 is clearly not sufficient to sustain early otic development. Other transcription factors also play crucial roles during otic induction.

In this paper, we have shown that knockdown of both pax2a and pax8 causes much more severe loss of ear tissue than knocking down either alone. We have previously shown that foxi1, which is required for pax8 expression in the otic domain, and dlx3b act in parallel pathways in early otic placode formation and show a strong synergistic genetic interaction (Solomon, 2004). The pax8-dlx3b morphant analysis confirms these previous results and furthermore suggests that a significant aspect but not all of foxi1 function is mediated by pax8. Thus, there appear to be multiple regulatory genes that respond to Fgf signaling and help mediate its effects. Each is likely to control both redundant and specific functions; hence there is neither a single ‘master regulator’, analogous to the likely to control both redundant and specific functions; hence there is neither a single ‘master regulator’, analogous to the

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