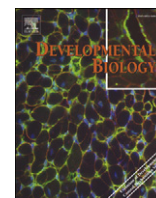


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Pax2/8 proteins coordinate sequential induction of otic and epibranchial placodes through differential regulation of *foxi1*, *sox3* and *fgf24*

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ABSTRACT

Vertebrate cranial placodes contribute vitally to development of sensory structures of the head. Amongst posterior placodes, the otic placode forms the inner ear whereas nearby epibranchial placodes produce sensory ganglia within branchial clefts. Though diverse in fate, these placodes show striking similarities in their early regulation. In zebrafish, both are initiated by localized Fgf signaling plus the ubiquitous competence factor Foxi1, and both express *pax8* and *sox3* in response. It has been suggested that Fgf initially induces a common otic/epibranchial field, which later subdivides in response to other signals. However, we find that otic and epibranchial placodes form at different times and by distinct mechanisms. Initially, Fgf from surrounding tissues induces otic expression of *pax8* and *sox3*, which cooperate synergistically to establish otic fate. Subsequently, *pax8* works with related genes *pax2a/pax2b* to downregulate otic expression of *foxi1*, a necessary step for further otic development. Additionally, *pax2/8* activate otic expression of *fgf24*, which induces epibranchial expression of *sox3*. Knockdown of *fgf24* or *sox3* causes severe epibranchial deficiencies but has little effect on otic development. These findings clarify the roles of *pax8* and *sox3* and support a model whereby the otic placode forms first and induces epibranchial placodes through an Fgf-relay.

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Introduction

In vertebrate embryos, cranial placodes form as a series of epithelial thickenings around the anterior neural plate and contribute to sensory structures of the head (Baker and Bronner-Fraser, 2001; Brugmann and Moody, 2005; Schlosser, 2006). All placodes are derived from a contiguous zone of preplacodal ectoderm, which forms in the head along the neural–nonneural interface during gastrulation (Streit, 2007). The preplacodal ectoderm then generates the diverse array of placodal fates in response to different regional signals. The otic placode, which gives rise to the inner ear, has been the most extensively characterized of all cranial placodes. Otic development is initiated by Fgf ligands secreted by the hindbrain and subjacent mesendoderm (Alvarez et al., 2003; Ladher et al., 2000, 2005; Léger and Brand, 2002; Liu et al., 2003; Maroon et al., 2002; Park and Saint-Jeannet, 2008; Phillips et al., 2001; Riley and Phillips, 2003; Wright and Mansour, 2003). Some of the earliest markers of otic development are members of the Pax2/8 family of transcription factors (Pfeffer et al., 1998). The functions of Pax2 and Pax8 in regulating early otic development have been most extensively studied in zebrafish (Hans et al., 2004; Mackereth et al., 2005). Otic expression of *pax8* begins during late gastrulation and requires both Fgf signaling and the otic-competence factor Foxi1 (Hans et al., 2004, 2007; Phillips et al., 2001; Solomon et al., 2003, 2004;). By early somitogenesis stages, expression of

related genes *pax2a* and *pax2b* is also detected in the preotic placode (Pfeffer et al., 1998). Otic expression of *pax2a/b* requires Fgf, but not *foxi1* (Hans et al., 2004; Léger and Brand, 2002; Solomon et al., 2003, 2004). Despite these slight differences in regulation, *pax8* and *pax2a/b* function together and provide substantial redundancy during otic development. Impairment of both *pax2a* and *pax2b* has little effect on otic induction, whereas impairment of *pax8* leads to production of a reduced otic placode (Hans et al., 2004; Mackereth et al., 2005). In *pax2a/pax2b/pax8*-depleted embryos, a small otic placode initially forms but eventually disperses as cells lose otic identity (Mackereth et al., 2005). Thus, *pax2/8* genes are together necessary for normal induction and maintenance of the otic placode. How *pax2/8* genes mediate these functions is still unknown. Moreover, because some otic tissue initially forms in the absence of *pax2/8* function, there must be additional genes that help mediate the effects of Fgf during otic induction.

Another gene coexpressed with *pax2/8* in the otic primordium is *sox3* (Nikaido et al., 2007; Sun et al., 2007). Like *pax8*, otic expression of *sox3* also requires Fgf and *foxi1* (Lee et al., 2003; Nechiporuk et al., 2007; Sun et al., 2007). In mouse and zebrafish, disruption of *Sox3* causes mild-to-moderate reduction in the size of the otic vesicle (Dee et al., 2008; Okuda et al., 2010; Rizzoti and Lovell-Badge, 2007). However, otic patterning has not been examined in detail in these backgrounds. Additionally, genetic interactions between *sox3* and *pax8* have not been investigated, leaving open the question of whether these genes cooperate to mediate otic induction.

Epibranchial placodes constitute a distinct set of placodes with fates quite different from the otic placode, yet there are striking

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parallels between early development of these placode-types (Ladher et al., 2010). Epibranchial placodes give rise to a series of sensory ganglia associated with the mouth and throat, including the facial, glossopharyngeal and vagal ganglia. Like the otic placode, epibranchial placodes require the same upstream regulators, Fgf and Foxi1, and both express *pax8* and *sox3* as early response factors. Moreover, fate-mapping studies show that otic and epibranchial precursors lie close together during early development, with epibranchial placodes emerging from an arc of ectoderm wrapping around the lateral edge of the otic territory (Streit, 2002; Sun et al., 2007). These similarities have led to the hypothesis that Fgf initially specifies a common otic/epibranchial field, which later splits into adjacent compartments with distinct fates (Freter et al., 2008; Ladher et al., 2010; Ohyama et al., 2006; Schlosser and Ahrens, 2004; Sun et al., 2007). However, close examination of early markers suggests that otic and epibranchial placodes are induced at different times, possibly by distinct mechanisms. Initially, *pax8* and *sox3* are coexpressed within a relatively small domain adjacent to rhombomere 4 of the hindbrain. This appears to correspond to the otic domain in zebrafish because at least two otic-specific markers, *atoh1b* and *fgf24*, are soon induced within the same domain (Draper et al., 2003; Millimaki et al., 2007). A dramatic transition occurs between 3 and 6 somites stage (11 and 12 hpf) as *sox3* downregulates within the otic domain and spreads outward into the prospective epibranchial domain (Nikaido et al., 2007; Sun et al., 2007). Similarly, expression of *foxi1* is abruptly lost from otic cells but is maintained at high levels in epibranchial cells. In contrast, *pax8* and *pax2a* remain highly expressed in the otic domain but shows only weak expression in the epibranchial domain (Pfeffer et al., 1998; Phillips et al., 2001). The regulation and functional significance of these dynamic changes have not been established.

Here we have reexamined early regulation of otic and epibranchial development. Our data confirm that the otic placode forms first and that *pax8* and *sox3* interact synergistically to promote otic induction. Subsequently, *pax8* works redundantly with *pax2a* and *pax2b* to promote two distinct functions in the otic placode. First, *pax2/8* repress otic expression of *foxi1*. This is necessary to maintain otic fate, as artificially maintaining *foxi1* expression blocks further otic development. Second, *pax2/8* activate otic expression of *fgf24*. Fgf24 in turn downregulates *sox3* in the otic domain and induces *sox3* in the epibranchial domain. Knockdown of *fgf24* has little effect on otic development but causes a severe deficiency of epibranchial ganglia, similar to knocking down *sox3* directly. These data support a new model wherein the otic placode forms first and subsequently induces formation of epibranchial placodes through *pax2/8*-dependent Fgf24 signaling. The data also support a key role for *pax8* in orchestrating the dynamic changes in early gene expression that distinguish otic from epibranchial fates.

Materials and methods

Strains and developmental conditions

The wild type strain was derived from AB line (Eugene, OR). The *noit^{tu29a}* mutation is a null allele (Lun and Brand, 1998) and was used to assess function of *pax2a*. Transgenic lines used in this study include *Tg(hsp70:fgf8a)^{x17}* (Millimaki et al., 2010), *Tg(hsp70:foxi1)^{x19}* (Kwon et al., 2010) and *Tg(brn3c:gap43-GFP)^{s356t}* (Xiao et al., 2005). For convenience, these transgenes are referred to in the remainder of the text as *hs:fgf8*, *hs:foxi1* and *brn3c:GFP*, respectively. Embryos were developed at standard conditions of 28.5 °C in fish water containing methylene blue and were staged based on standard protocols (Kimmel et al., 1995).

In situ hybridization

In situ hybridization was carried out at 67 °C as described previously (Jowett and Yan, 1996; Phillips et al., 2001).

Morpholinos

Translation-blocking morpholino oligomers (MOs) obtained from Gene Tools Inc. were used to block gene function. MOs were injected into embryos at one-cell. All MO sequences used in this study have been previously described and tested for efficacy and specificity. To knockdown *pax8*, wild-type embryos were injected with 2.5 ng each of variant 1 MO (5'-GTTCAAAACATGCCTCC-TAGTTGA-3') and variant 2/3 MO (5'-GACCTCGCCAGTGCTGTG-GACAT-3') as previously described (Mackereth et al., 2005). To knock down *fgf24*, embryos were coinjected with 5 ng *fgf24*-MO, 5'-GACGGCAGAACAGACATCTTGCTCA-3' (Fischer et al., 2003) and, to inhibit non-specific cell death, 7.5 ng of *p53*-MO (Robu et al., 2007). Other morpholinos used in this study include *pax2b*-MO, 5'-GGTCTGCCTTACAGTGAATATCCAT-3' (5 ng/embryo, Mackereth et al., 2005); and *sox3*-MO1 5'-TACATTCTAAAAGTGGTGTGCCAAGC-3' (5 ng/embryo, Okuda et al., 2010).

Gene misexpression

To misexpress *foxi1* or *fgf8* from heat shock-inducible transgenes, heterozygous transgenic embryos were heat shocked at 39 °C for 30 min at the indicated times. After heat shock, embryos were incubated at 33 °C until fixation.

Cell transplantation

Donor embryos were injected with lineage tracer (lysine-fixable biotinylated dextran, 10000 MW, in 0.2 M KCl) at the one-cell stage. Labeled cells were transplanted from blastula stage donors into non-labeled hosts of the same stage. Transplanted cells were identified in the hosts by streptavidin-FITC antibody staining.

Results

Previous studies have shown the importance of Fgf signaling in otic and epibranchial induction, but there is still much to learn about the factors that mediate Fgf signaling. Fgf initially induces expression of *pax8* and *sox3* in the otic primordium by 9.5 hpf (late gastrulation). Another otic marker, *pax2a*, is coexpressed in the otic domain by 11 hpf (1–3 somites stage). By 12 hpf, expression of *sox3* begins to downregulate in the otic placode as it expands outward into prospective epibranchial ectoderm (Nikaido et al., 2007; Sun et al., 2007, Fig. 1D). Weaker expression of *pax8* and *pax2a* is also detected in the epibranchial anlagen by 12 hpf, whereas higher levels persist in otic domain (Fig. 1A, B). By comparison, expression of *fgf24* remains restricted to the otic domain throughout placodal development (Draper et al., 2003; Fig. 1C).

The roles of *pax8* and *pax2a* in otic induction have been partially characterized, but their roles in epibranchial development have not been determined, nor have the roles of *sox3* and *fgf24* been determined. To address these questions, we injected morpholino oligomers (MOs) to knock down these genes and assessed the effects on otic and epibranchial development.

sox3 and *pax8* cooperate to regulate otic and epibranchial induction

We first examined the effects of knocking down the earliest otic markers, *pax8* and/or *sox3*, on otic development. Knockdown of *sox3* alone caused a 9 ± 2% reduction in the area of the otic/epibranchial domain of *pax2a* at 12 hpf, but subsequent formation of the otic vesicle was nearly normal (Fig. 2B, F). Consistent with previous findings (Mackereth et al., 2005), knockdown of *pax8* reduced the area of *pax2a* expression to 63 ± 4% of normal, with a similar reduction in the size of the otic vesicle (Fig. 2C, G). The expression domains of *pax8* and *sox3* were similarly reduced at 10 hpf (not

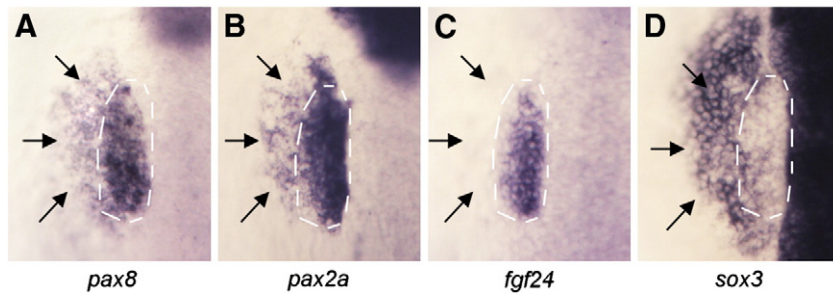


Fig. 1. Spatial domains of otic and epibranchial markers at 12 hpf. Dorsal views showing expression of *pax8* (A), *pax2a* (B), *fgf24* (C) and *sox3* (D) in wild-type embryos at 12 hpf. Otic domains (white dashed lines) and epibranchial domains (black arrows) are indicated. Unlike the other genes, *fgf24* expression is limited to the otic domain.

shown). Knockdown of both genes caused further reduction in otic development, such that the area of the *pax2a* domain was only $38 \pm 2\%$ of normal and the otic vesicle was similarly reduced (Fig. 2D, H). Patterning in the otic vesicle was relatively normal in embryos knocked down for *pax8* and/or *sox3*, though expression domains of all markers were reduced in proportion to the overall size of the otic vesicle (Fig. S1 A–L). Thus *sox3* and *pax8* both regulate otic placode induction. *pax8* function appears more critical than *sox3*, but the strong enhancement of otic deficiency in *pax8-sox3* double morphants shows that each gene provides unique functions required for early otic development.

Because *Sox3* has been implicated in regulation of sensory-neural regions of the otic vesicle in chick (Abelló et al., 2010), we also examined formation of sensory epithelia and neurons of the stato-acoustic ganglion (SAG) in embryos knocked down for *sox3* and/or *pax8*. *sox3* morphants produced sensory epithelia with roughly normal numbers of hair cells, as marked by *brn3c:GFP* expression. SAG development was also normal based on expression of proneural gene *neuroD*, as well as accumulation of mature *Islet1*-positive SAG neurons (Fig. S1 M–T, Table 1). In *pax8* morphants and *pax8-sox3* double morphants, sensory epithelia and SAG neurons formed but were reduced in size as expected from the diminished size of the otic vesicle. These data suggest that *pax8* and *sox3* are not directly required for development of sensory epithelia or SAG neurons in zebrafish.

Instead these genes interact to control the amount of otic tissue induced, which indirectly affects the amount of sensory-neural tissue produced.

To monitor epibranchial development following gene knockdown, we examined expression of *phox2a*, which marks all epibranchial ganglia by 30 hpf (Begbie et al., 1999; Lee et al., 2003; Nechiporuk et al., 2005). Previous studies have shown that *sox3* is required for normal development of epibranchial ganglia (Dee et al., 2008; Rizzoti and Lovell-Badge, 2007). We confirmed that *sox3* morphants develop with a substantial deficiency of *phox2a*-expressing epibranchial ganglia, with almost total loss of the glossopharyngeal and anterior vagal ganglia (Fig. 2J). *Pax8* has not previously been shown to regulate epibranchial placode development, but we tested this possibility because *pax8* is expressed at a low level in at least part of the epibranchial primordium by 12 hpf (Hans et al., 2004; Phillips et al., 2001; Fig. 1A). Although *pax8* morphants developed with only a slight reduction in epibranchial ganglia (Fig. 2K), *pax8-sox3* double morphants showed complete loss of all epibranchial ganglia (Fig. 2L). Similar results were obtained by visualizing expression of the general neurogenic marker, *ngn1*, though a few small disorganized clusters of *ngn1*-expressing cells were still produced in *pax8-sox3* double morphants (not shown). However, these clusters appear to be derived from neural crest as simultaneous ablation of neural crest eliminated all residual neurogenesis in the epibranchial region (our

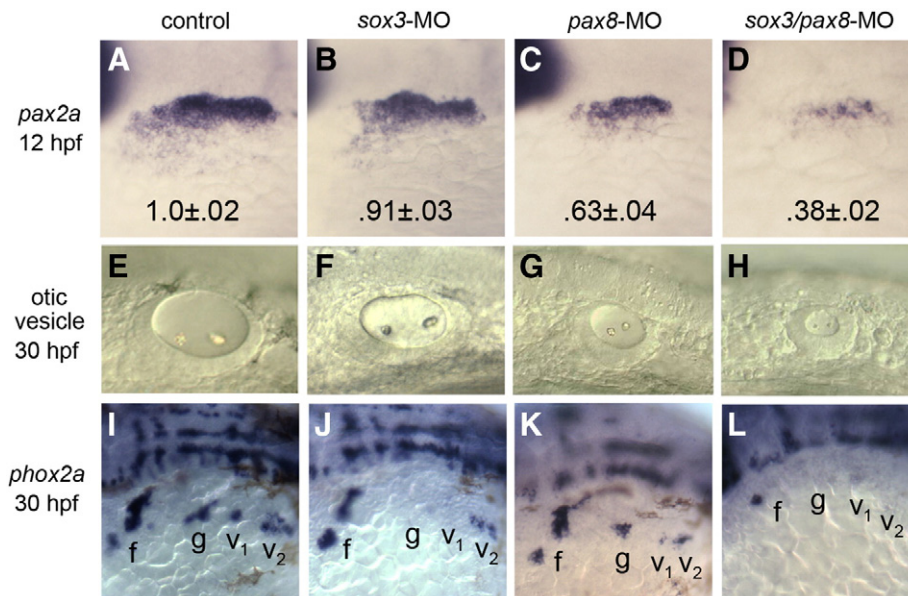


Fig. 2. *pax8* and *sox3* interact in otic and epibranchial induction. (A–D) *pax2a* expression in the otic/epibranchial domain at 12 hpf in a control embryo (A), *sox3* morphant (B), *pax8* morphant (C) and *sox3-pax8* double morphant (D). Numbers indicate normalized values for the mean \pm standard deviation of the area of the *pax2a* expression domain ($n = 10$ specimens for each background). Area was calculated by outlining otic–epibranchial domains in Photoshop and measuring the number of pixels within. Differences between the morphants and the control were highly significant ($p < .0005$) as determined by *t*-tests. (E–H) Otic vesicles at 30 hpf in a live control embryo (E), *sox3* morphant (F), *pax8* morphant (G) and *sox3-pax8* double morphant (H). (I–L) Expression of *phox2a* at 30 hpf in a control embryo (I), *sox3* morphant (J), *pax8* morphant (K) and *sox3-pax8* double morphant (L). Positions of the facial ganglion (f) glossopharyngeal ganglion (g), and vagal ganglia (v_1 and v_2) are indicated. All images show lateral views with anterior to the left.

Table 1

Number of SAG neurons and hair cells in 30 hpf embryos.

	Control	<i>sox3</i> -MO	<i>pax8</i> -MO	<i>sox3/pax8</i> MO	<i>fgf24</i> -MO
No. of SAG neurons ^a	28.9 ± 2.1 n = 20	24 ± 2.2 n = 20	12.5 ± 1.8 n = 20	8.1 ± 1.4 n = 20	27.2 ± 1.9 n = 20
No. of hair cells in the utricular macula ^a	6.3 ± 0.6 n = 14	6.2 ± 0.4 n = 14	4.8 ± 0.4 n = 14	3.1 ± 0.5 n = 14	6.3 ± 0.7 n = 20
No. of hair cells in the saccular macula ^a	3.9 ± 0.8 n = 14	3.8 ± 0.7 n = 14	2.2 ± 0.4 n = 14	2.1 ± 0.3 n = 14	3.8 ± 0.4 n = 20

^a Values expressed as mean ± SD. n, sample size. MO, morphants, hpf, hours post fertilization.

unpublished observations). Thus, *pax8* and *sox3* are together indispensable for development of epibranchial ganglia.

Downregulation of *foxi1* and *sox3* in the otic placode

Although *pax8* and *sox3* are initially coinduced in the otic anlagen by Fgf, *sox3* soon downregulates in otic cells as they develop. Because *pax8* expression persists in the otic domain, we speculated that *pax8* might directly or indirectly repress otic expression of *sox3*. Indeed, downregulation of *sox3* in the otic placode was delayed by at least 3 hours in *pax8* morphants (Fig. 3E, and data not shown). Surprisingly, induction of *sox3* in the epibranchial domain was also delayed by 3 h, consistent with a non-autonomous role for *pax8* (see below).

We also tested whether *pax8* modulates *foxi1* expression during otic/epibranchial development. *Foxi1* initially serves as a competence factor for establishing the entire preplacodal ectoderm (Kwon et al., 2010), and its expression later becomes restricted to the otic and epibranchial primordia where its function is especially critical (Hans et al., 2007; Lee et al., 2003; Nissen et al., 2003; Solomon et al., 2003). As development proceeds, *foxi1* expression normally begins to downregulate in the otic domain by 11 hpf whereas it is maintained in epibranchial ganglia through at least 36 hpf (Lee et al., 2003). In *pax8* morphants, however, exclusion of *foxi1* from the otic placode was delayed by about 2 h (data not shown). Because *pax2a* and *pax2b* are later coexpressed in the otic placode and are partially redundant with *pax8*, we tested the effects of disrupting all known *pax2/8* function. In *pax2a/pax2b/pax8*-deficient embryos, strong *foxi1* expression was maintained in the otic region through at least 24 hpf (Fig. 3F, G), by which time otic identity is lost (Mackereth et al.,

2005). These data show that Pax2/8 proteins directly or indirectly repress *foxi1* transcription in the otic placode.

Although *foxi1* is required to initiate otic development, we hypothesized that failure to downregulate *foxi1* at later stages impedes further otic development. To test this idea, we made use of a stable transgenic line to misexpress *foxi1* under the control of a heat shock promoter (Kwon et al., 2010). Global activation of *hs:foxi1* expression at 11 hpf caused a dramatic reduction in the size of the otic placode by 14 hpf (Fig. 4B). Additionally, otic expression of *pax2a* was irregular and spotty. Because global misexpression of *foxi1* possibly interferes with essential signals from other tissues, we generated mosaic embryos by transplanting cells from *hs:foxi1* transgenic embryos into non-transgenic host embryos. Activation of *hs:foxi1* in mosaic embryos caused loss of expression of *pax2a* in transgenic cells within the otic region (Fig. 4C, D). These data indicate that maintaining *foxi1* expression after 11 hpf impairs completion of otic development in a cell-autonomous manner. This could explain why otic cells eventually lose otic identity in *pax2a/pax2b/pax8*-deficient embryos (Mackereth et al., 2005).

Loss of otic fate in *pax2a/pax2b/pax8*-deficient embryos does not involve death of the otic placode, as these cells persist in the otic-epibranchial area through at least 24 hpf (Mackereth et al., 2005). We hypothesized that some of these cells might switch fate and contribute to epibranchial tissue instead. However, development of epibranchial ganglia was severely impaired in *pax2a/pax2b/pax8*-deficient embryos (Fig. 3H). These data are consistent with loss of epibranchial expression of *sox3* (Fig. 3E, and data not shown), further indicating that *pax2/8* genes are required directly or indirectly for development of epibranchial placodes.

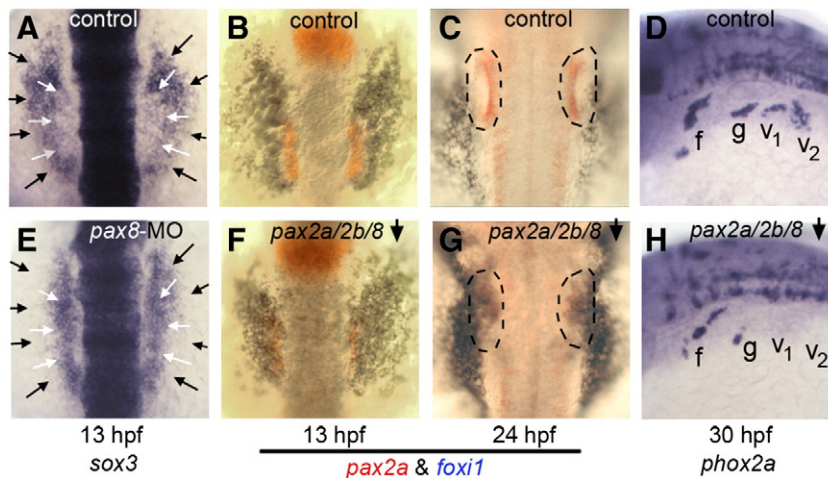


Fig. 3. Requirement for *pax2/8* in otic and epibranchial development. (A, E) Expression of *sox3* at 13 hpf in a control embryo (A) and *pax8* morphant (E). White arrows indicate the lateral edges of the otic domain and black arrows indicate the edges of the epibranchial domain. (B, C, F, G) Two color *in situ* hybridization of embryos at 13 hpf (B, F) and 24 hpf (C, G) showing expression of *pax2a* (red) and *foxi1* (blue). Outlines indicate the otic vesicle (C) or vestigial otic region (G). Expression patterns are shown in control embryos (B, C) and *pax2a/pax2b/pax8*-deficient embryos (F, G). (D, H) Expression of *phox2a* at 30 hpf in a control embryo (D) and *pax2a/2b/8*-deficient embryo (H). Images show dorsal views with anterior to the top (A–C, E–G); dorsolateral views with anterior to the left and dorsal to the top (D, H). Positions of the facial ganglion (f), glossopharyngeal ganglion (g) and vagal ganglia (v_1 and v_2) are indicated.

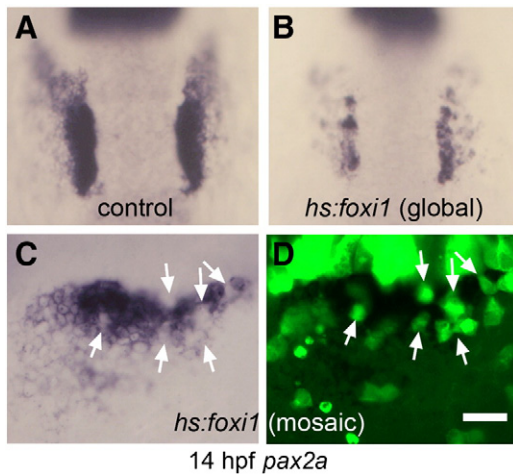


Fig. 4. Misexpression of *foxi1* inhibits otic development. (A, B) Expression of *pax2a* at 14 hpf in a control embryo (A) and a *hs:foxi1* transgenic embryo (B) heat shocked at 11 hpf. (C, D) Expression of *pax2a* at 14 hpf in a mosaic embryo as seen under bright field (C) and fluorescence imaging (D). The mosaic was produced by transplanting lineage-labeled *hs:foxi1* transgenic cells (green fluorescence) into a non-transgenic host. The embryo was heat shocked at 11 hpf to activate the transgene. Note the absence of *pax2a* expression in transgenic cells (white arrows). Images show dorsal views with anterior to the top (A–B); lateral views with anterior to the left (C–D). Scale bar, 50 μ m (A, B), 25 μ m (C, D).

Pax2/8 regulate expression of *fgf24* in the otic placode

Expression of *fgf24* is limited to the otic placode and is first expressed there by 10.5 hpf, shortly after the onset of *pax8* expression (Draper et al., 2003; Fig. 1C, and our unpublished observation). We therefore tested whether *pax8*, which is critical for controlling the size of the otic placode, is required to activate this domain of *fgf24* expression. Indeed, otic expression of *fgf24* is delayed until 13 hpf in *pax8* morphants (Fig. 5B, D). We hypothesized that

belated expression of *fgf24* reflects the activation of *pax2a* and *pax2b*. In support, *pax2a/pax2b/pax8*-deficient embryos fail to express *fgf24* in otic tissue through at least 18 hpf, although *fgf24* expression occurs normally in pharyngeal arches (Fig. 5F–H). Thus, one of the functions of Pax2/8 during otic induction is to activate expression of *fgf24*. In contrast, knockdown of *sox3* had no effect on the onset of *fgf24* expression (not shown).

Fgf24 is not required for otic development

The function of *fgf24* in otic development has not been investigated. To test this we injected wild-type embryos with morpholino to knockdown *fgf24*. *fgf24* morphants develop with a normally sized otic placode, judging by expression of *pax8* at 11 hpf (Fig. 6E). Like *pax8* morphants, *fgf24* morphants fail to downregulate expression of *sox3* in the otic placode (Fig. 6F). In contrast, expression of *foxi1* showed a normal pattern of exclusion from otic cells in *fgf24* morphants (Fig. 6C, G). Furthermore, we could detect no changes in expression of regional markers within the otic vesicle, nor in development of sensory epithelia and SAG neurons (Fig. S2 and Table 1). Thus, *fgf24* is not required for otic placode induction or subsequent development and patterning of the otic vesicle. Additionally, the data show that failure to downregulate *sox3* in the otic placode in *fgf24* morphants does not adversely affect patterning and differentiation within the otic placode and vesicle. The latter conclusion was further supported by the finding that elevating *sox3* expression by activating a heat shock-inducible transgene at 11.5 hpf does not detectably alter patterning or neurogenesis within the otic vesicle (Fig. S3).

Fgf24 regulates epibranchial development

We next examined whether the otic domain of *fgf24* acts non-autonomously to regulate epibranchial development. As in *pax8* morphants, *fgf24* morphants fail to show expansion of *sox3* into the epibranchial domain (Fig. 6F). Moreover, development of

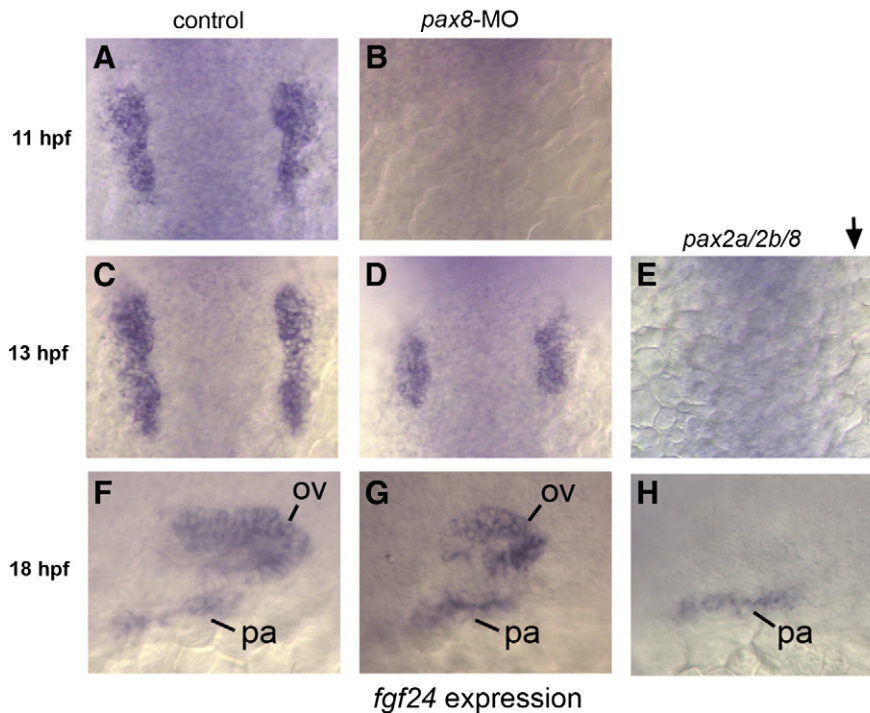


Fig. 5. *pax2/8* regulates otic expression of *fgf24*. (A, C, F) *fgf24* expression in the otic placode in control embryos at 11 hpf (A), 13 hpf (C) and 18 hpf (F). (B, D, G) *fgf24* expression in *pax8* morphants at 11 hpf (B), 13 hpf (D) and 18 hpf (G). Expression of *fgf24* is lost from preotic placodes in *pax8* morphants at 11 hpf (B) and is reduced in *pax8* morphants at 13 hpf (D) and 18 hpf (G). (E, H) *noi* (*pax2a*) mutants co-injected with *pax8*-MO and *pax2b*-MO showing loss of otic expression of *fgf24* at all time points. Expression in pharyngeal (pa) arches and the otic vesicle (ov) is indicated. Images show dorsal views with anterior to the top (A–E); lateral views with anterior to the left and dorsal to the top (F–H).

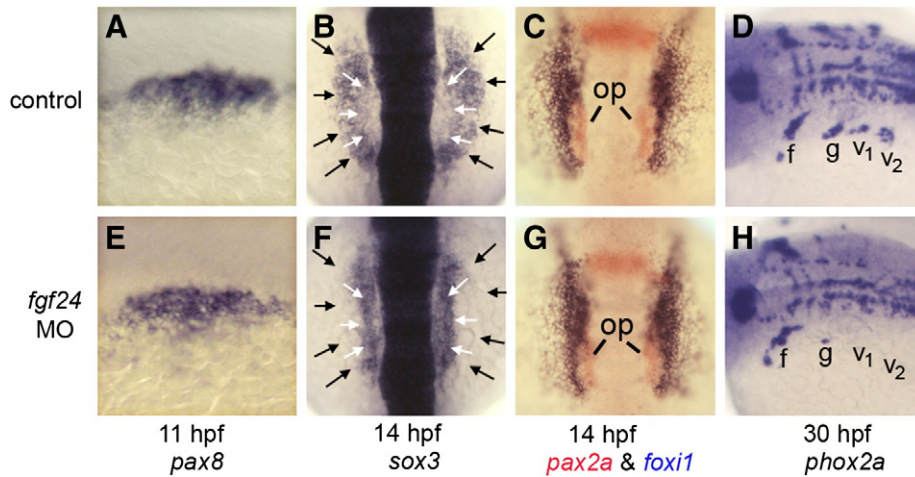


Fig. 6. *fgf24* is required for epibranchial development. (A, E) Expression of *pax8* at 11 hpf in a control embryo (A) and *fgf24* morphant (E). (B, F) Expression of *sox3* at 14 hpf in a control embryo (B) and *fgf24* morphant (F). White arrows indicate the lateral edges of the otic domain and black arrows indicate the lateral edges of the epibranchial domain. (C, G) Two color *in situ* hybridization showing *pax2a* (red) and *foxi1* (blue) in a control embryo (C) and *fgf24* morphant (G) at 14 hpf. Positions of otic placodes (op) are indicated. (D, H) Expression of *phox2a* at 30 hpf in a control embryo (D) and *fgf24* morphant (H). Positions of the facial ganglion (f), glossopharyngeal ganglion (g) and vagal ganglia (v_1 and v_2) are indicated. Images show dorsolateral views with anterior to the left (A, D, E, H); dorsal views with anterior to the left and dorsal to the top (B, C, F, G).

glossopharyngeal and vagal ganglia was almost completely blocked (Fig. 6H). These are the same ganglia adversely affected in *sox3* morphants (Fig. 2J), suggesting that the role of Fgf24 is to induce expression of *sox3* in these primordia. In contrast, development of the facial ganglion was relatively normal in *fgf24* and *sox3* morphants, indicating that other genes are able to compensate in these cells. The facial ganglion arises from the anterior-most region of the epibranchial domain, relatively far from the otic domain of *fgf24*. It is possible that some other source of Fgf regulates development of the facial ganglion, and that subsequent expression of *pax8* can partially compensate for loss of *sox3*.

Modulation of sox3 by a threshold response to Fgf

Although Fgf signaling is required to activate *sox3* expression, the observation that *fgf24* is required to downregulate *sox3* in the otic domain suggested that *sox3* is subject to repression by high levels of Fgf signaling. To test this, we used a heat shock line to misexpress *fgf8* beginning at 11 hpf. This caused *sox3* to be expressed throughout the otic and epibranchial domains, but at a significantly reduced level compared to the control embryo (Fig. 7). The low level of *sox3* expression in *hs:fgf8* embryos was comparable to the level normally seen in the otic domain of control embryos (compare Fig. 7A and C). In another control experiment, heat shock did not alter the effects of *fgf24* knockdown; *sox3* expression remained elevated in the otic domain and failed to expand into the epibranchial domain (Fig. 7B). These data support the hypothesis that *sox3* shows two distinct responses to Fgf signaling, explaining how otic expression of *fgf24* differentially regulates *sox3* in the otic and epibranchial domains.

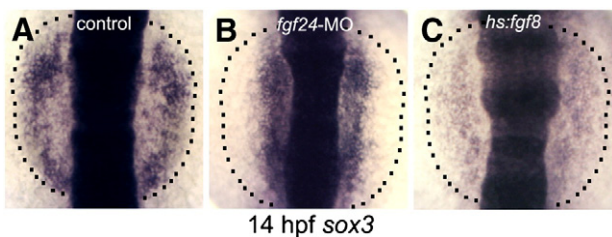


Fig. 7. Response of *sox3* to elevated Fgf signaling. Expression of *sox3* at 14 hpf in a control embryo (A), *fgf24* morphant (B) and *hs:fgf8* transgenic embryo (C). The lateral edges of the prospective epibranchial domain are indicated (dashed lines). All embryos were heat shocked at 11 hpf.

Discussion

The data provided here clarify early steps in otic placode development and support a new model for sequential induction of otic and epibranchial placodes (Fig. 8). The otic placode forms first by a previously established mechanism involving Fgf3 and Fgf8 acting locally within a broader domain of *foxi1* expression (Hans et al., 2004, 2007; Léger and Brand, 2002; Liu et al., 2003; Maroon et al., 2002; Nissen et al., 2003; Phillips et al., 2001; Solomon et al., 2003). As an initial response, *pax8* and *sox3* are coinduced in the otic domain (Nikaido et al., 2007; Sun et al., 2007). Otic expression of *pax8* stabilizes otic fate through downregulation of *foxi1*, and non-autonomously induces the majority of epibranchial placodes through

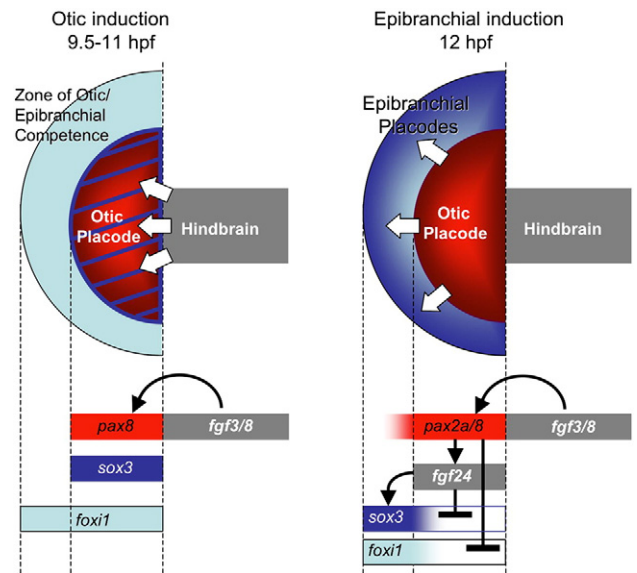


Fig. 8. Summary and model of otic and epibranchial induction. During otic induction (9.5–11 hpf), Fgf3/8 from the mesendoderm (not shown) and hindbrain (gray) induce expression of *pax8* (red) and *sox3* (blue) in preotic cells. Specific responsiveness to Fgf requires the competence factor *Foxi1*, which becomes restricted to the otic and epibranchial regions during this period. By 12 hpf, *Pax8* has induced expression of *fgf24* and repressed otic expression of *foxi1*. *Pax2a* can mediate the same functions in the absence of *Pax8*, albeit belatedly. *Fgf24* in turn downregulates otic expression of *sox3* and induces strong expression of *sox3* in adjacent epibranchial cells. Arrows represent positive regulation and cross-bars indicate negative regulation.

activation of *fgf24*. This model is compatible with previous studies showing that *Fgf3* and *Fgf8* regulate otic and epibranchial development but adds important mechanistic details, as described below. Only the facial ganglion appears to develop independently of *Fgf24*, and its regulation will be considered separately.

The roles of *pax8* and *sox3* in early otic development

Our data provide important new insights into the mechanisms by which Fgf-target genes control early otic development. Expression of *pax8* is especially critical for establishing the size of the otic placode, as shown by the production of roughly half-sized otic placodes in *pax8* morphants (Mackereth et al., 2005, and Fig. 2G). Because *pax8* is required to activate otic expression of *fgf24*, we initially hypothesized that this additional source of Fgf would serve to recruit more distant cells into the otic placode. Surprisingly, however, *fgf24* appears to provide no essential function for otic development: *fgf24* morphants and mutants show no deficit in the size of the otic placode, and there appear to be no defects in subsequent patterning in the otic vesicle (Fig. S2). Instead, the primary function of otic *fgf24* is to initiate epibranchial development through induction of *sox3* in the surrounding ectoderm (described in more detail below). How then, does *pax8* control the size of the otic domain? Because *Pax2/8* genes are autoregulatory in other developmental settings (Lun and Brand, 1998; Pfeffer et al., 1998), we speculate that Pax8 forms a feedback amplification loop in pre-otic cells, allowing cells further from the Fgf signaling source to achieve detectable expression of *pax8* and *sox3*. In *pax8* morphants, therefore, otic induction is limited to a smaller field of cells closer to the Fgf source where signal amplification is less critical. Another Fgf target gene, *sox3*, cooperates with *pax8* during otic induction. Knockdown of *sox3* alone causes only a 9% reduction in the size of the otic placode. However, knocking down both *pax8* and *sox3* causes a synergistic loss of nearly two-thirds of otic tissue. In this case, we presume that only cells immediately adjacent to the sources of Fgf are able to initiate otic development through the activation of additional as yet unknown target genes.

After helping to establish the otic placode, *pax8* later represses *foxi1* in the otic domain. This function is shared with *pax2a* and *pax2b* and appears to be essential for maintaining otic fate. In *pax2a/pax2b/pax8*-deficient embryos, *foxi1* expression persists in the otic domain (Fig. 3F, G) and expression of all otic markers is lost by 24 hpf (Mackereth et al., 2005). Furthermore, experimentally maintaining expression of *foxi1* from an inducible transgene also causes loss of otic markers. It is not clear why *foxi1* must be repressed in the otic domain since it is absolutely required for Fgf's ability to induce otic development in the first place. However, our analysis of the early role of *foxi1* in establishing preplacodal ectoderm indicates that it functions in part by repressing other regulatory genes (Kwon et al., 2010 and our unpublished observations). Thus, *pax8*-dependent downregulation of *foxi1* may alleviate repression of other genes necessary for otic differentiation.

After the onset of otic development, the later role(s) of *sox3* are still unclear. Although the otic vesicle is slightly smaller than normal in *sox3* morphants, all regional markers are expressed normally. Based on studies in chick it has been suggested that *Sox3* regulates formation of the sensory-neural domain of the otic vesicle (Abelló et al.). However, we find that knockdown of *sox3* causes no appreciable deficiency in development of sensory epithelia or SAG neurons (Fig. S1 N, R and Table 1). Otic development in *Sox3* null mice has not been studied in detail, but otic vesicles appear grossly normal and produce at least some SAG neurons (Rizzoti and Lovell-Badge, 2007). It is possible that other *SoxB1* genes compensate for loss of *Sox3* in mouse, but no other appropriately expressed genes have been identified in zebrafish. It is interesting that *fgf24* morphants fail to downregulate *sox3* in the otic placode (Fig. 6F), yet all other aspects of otic development appear normal (Fig. S2). Likewise, misexpressing *sox3* from a heat shock-

inducible transgene does not detectably alter otic development. However it must be acknowledged that failure to downregulate *sox3* could cause defects too subtle to detect using the markers at hand, even though such changes could be quite deleterious in the long-run.

The role of *Fgf24*

A novel finding central to our model is that *Fgf24* emitted by the nascent otic placode is essential for development of all epibranchial placodes posterior to the facial placode (Fig. 6H). A prominent target of *Fgf24* appears to be *sox3*. Within 1–2 h of activation of *fgf24* in the otic placode, *sox3* begins to downregulate in the otic domain while it is induced in the abutting epibranchial domain. Differential spatial regulation of *sox3* could reflect a threshold response to changing levels of *Fgf24* within a diffusion gradient. Indeed, overexpression of *Fgf8* causes *sox3* to be expressed at a low level throughout the domain of *foxi1* expression (Fig. 7). In the absence of *Fgf24*, *sox3* remains highly expressed in the otic domain and is not detected in the epibranchial domain. Disruption of *sox3* has little effect on otic development but blocks all epibranchial development posterior to the facial ganglion. This phenotype strongly resembles that of *fgf24* morphants, again supporting the notion that *sox3* is the primary mediator of *Fgf24* signaling. Otic expression of *fgf24* is in turn regulated redundantly by *pax2* and *pax8* genes. Accordingly, loss of *pax8* alone causes a 2–3-h delay in *fgf24* expression, with negligible effects on epibranchial development. In contrast, disruption of all *pax2/8* function eliminates otic expression of *fgf24* entirely and causes a deficiency in epibranchial development comparable to *fgf24*-MO. Together these data support the existence of a pathway in which otic expression of *pax8* activates expression of *fgf24*, which induces formation of epibranchial placodes in adjacent ectoderm through *sox3*.

In contrast to *sox3*, *pax2/8* genes are normally maintained at a high level in the otic placode but show only weak expression in the epibranchial domain. This pattern remains unchanged in *fgf24* morphants. Epibranchial expression of *pax2/8* appears after otic expression, possibly reflecting a delayed response to low levels of *Fgf3* and *Fgf8* from the hindbrain and subotic mesendoderm (Alvarez et al., 2003; Freter et al., 2008; Ladher et al., 2000, 2005; Léger and Brand, 2002; Liu et al., 2003; Maroon et al., 2002; Nechiporuk et al., 2007; Park and Saint-Jeannet, 2008; Phillips et al., 2001; Riley and Phillips, 2003; Wright and Mansour, 2003). It is possible that *pax2/8* provides a cell-autonomous requirement for epibranchial development, but such function(s) are evidently not sufficient to support epibranchial development in the absence of *Fgf24*.

Our model is distinct from an earlier model proposing that epibranchial placodes are induced by *Fgf3* and *Fgf8* from paraxial cephalic mesoderm (Nechiporuk et al., 2007). It is formally possible that mesodermal *Fgf3* and *Fgf8* work in parallel with otic *Fgf24* to regulate certain aspects of epibranchial development. Indeed we have confirmed that ablation of mesoderm blocks differentiation of epibranchial neurons, as shown by loss of *phox2a* and *ngn1* expression (Nechiporuk et al., 2007; and our unpublished observations). However, we find that genetic ablation of mesoderm does not block otic or epibranchial induction. For example, *pax8*, *sox3* and *fgf24* are all expressed in the otic domain by 12 hpf, after which *sox3* shows downregulation in the otic domain and upregulation in the epibranchial domain (Kwon and Riley, 2009; Mendonsa and Riley, 1999; and our unpublished observations). These data are consistent with our model and indicate that mesodermal signals are not required for epibranchial specification but are instead required for maintenance or differentiation of epibranchial ganglia.

Regulation of the facial ganglion

Epibranchial placodes and ganglia appear to follow similar regulation in general, but our data show that the facial (geniculate)

placode shows key differences from more posterior epibranchial placodes. First, development of the facial ganglion does not require *fgf24* (Fig. 6H). Similarly, there are only minor deficiencies in the facial ganglion following knockdown of *sox3* alone, *pax8* alone, or all *pax2/8* functions, whereas the other epibranchial ganglia are severely impaired or ablated under these conditions (Figs. 2J, K and 3H). However, combined knockdown of *sox3* and *pax8* ablates formation of facial ganglion (Fig. 2L). This indicates that *sox3* and *pax8* serve redundant functions in the facial placode, unlike more posterior epibranchial placodes. Such early differences in regulation could confer unique functional attributes to the facial ganglion that distinguish it from other epibranchial ganglia.

Other essential signals

In chick, frog and zebrafish, Fgfs and various Bmps secreted from pharyngeal endoderm are also required for development of epibranchial ganglia (Begbie et al., 1999; Holzschuh et al., 2005; Nechiporuk et al., 2005; Nikaïdo et al., 2007). However, these signals operate at a later stage, well after Fgf-dependent induction of *sox3*, and are required to initiate neurogenic differentiation. It is still unknown whether these endodermal signals act sequentially or are required as parallel inputs.

In mouse and chick, Wnt8a from the hindbrain is thought to distinguish otic from epibranchial fates. Accordingly, disruption of Wnt signaling blocks completion of otic development whereas elevating Wnt signaling expands otic tissue as it blocks epibranchial development (Freter et al., 2008; Ohyama et al., 2006). At first glance this appears to be an entirely different mechanism than what we describe for zebrafish, but this is not necessarily the case. Chick and mouse embryos show prominent otic expression of multiple *Fgf* genes around the time of otic induction, the functions of which have not been examined (Adamska et al., 2001; Alsina et al., 2004; Chapman et al., 2006; Pirvola et al., 2004; Wright et al., 2003). Conceivably, Wnt signaling could help modulate expression of these otic *Fgf* genes, or work in parallel with them, to affect epibranchial development. In zebrafish, Wnt signaling influences otic development indirectly through modulation of hindbrain expression of *fgf3* and *fgf8* (Phillips et al., 2004). Additional studies are needed to assess the degree to which underlying mechanisms have been conserved between zebrafish and amniotes.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2010.12.036.

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