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Conditions that influence the response to Fgf during otic placode induction

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Abstract

Despite the vital importance of Fgf for otic induction, previous attempts to study otic induction through Fgf misexpression have yielded widely varying and contradictory results. There are also discrepancies regarding the ability of Fgf to induce otic tissue in ectopic locations, raising questions about the sufficiency of Fgf and the degree to which other local factors enhance or restrict otic potential. Using heat shock-inducible transgenes to misexpress Fgf3 or Fgf8 in zebrafish, we found that the stage, distribution and level of misexpression strongly influence the response to Fgf. Fgf misexpression during gastrulation can inhibit or promote otic development, depending on context, whereas misexpression after gastrulation leads to expansion of otic markers throughout preplacodal ectoderm surrounding the head. Elevated Fgf also expands expression of the putative competence factor Foxi1, which is required for Fgf to expand other otic markers. Misexpression of downstream factors Pax2a or Pax8 also expands otic markers but cannot bypass the requirement for Fgf or Foxi1. Co-misexpression of Pax2/8 with Fgf8 potentiates formation of ectopic otic vesicles expressing a full range of otic markers. These findings document the variables critically affecting the response to Fgf and clarify the roles of *foxi1* and *pax2/8* in the otic response.

Keywords

zebrafish; Fgf; Pax2/5/8; Foxi1; cranial placodes; otic competence; heat shock

INTRODUCTION

The vertebrate inner ear develops from a simple epithelial thickening called the otic placode. In all vertebrate species examined to date, the otic placode is induced from uncommitted ectoderm lateral to the developing hindbrain in response to localized Fgf signaling (Reviewed by Ladher et al., 2010; Ohyama et al., 2007; Schimmang, 2007). In zebrafish embryos, for example, *fgf3* and *fgf8* are expressed in the hindbrain primordium during gastrulation and serve as the principal inducers of otic development (Léger and Brand, 2002; Liu et al., 2003; Maroon et al., 2002; Phillips et al., 2001). Subotic mesoderm also expresses

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fgf3 and *fgf8* and contributes to induction and maintenance of the otic placode (Mendonsa and Riley, 1999; Nechiporuk et al., 2007; Nikaido et al, 2007). Disruption of *fgf3* and *fgf8* blocks the earliest known steps in otic development. Moreover, application of the Fgf-inhibitor SU5402 after the onset of otic induction shows that Fgf signaling must continue through mid-somitogenesis stages to maintain otic fate (Léger and Brand, 2002).

Although there is widespread acceptance that Fgf is required for otic induction, there have been contradictory findings regarding the sufficiency of Fgf. In zebrafish embryos, application of Fgf-coated beads can moderately expand the endogenous otic domain but does not lead to production of otic tissue in ectopic locations (Léger and Brand, 2002). Similar findings have been found following global activation of a heat shock-inducible transgene expressing *fgf8* (Hans et al., 2007). In contrast, injection of plasmid expression-vectors at the 8-cell stage to achieve mosaic misexpression of *fgf3* or *fgf8* can expand endogenous otic domains and induce ectopic otic placodes in cranial ectoderm from the level of anterior somites to the front of the head (Phillips et al., 2004). A similar range of outcomes has been reported following Fgf-misexpression in chick and *Xenopus*. In chick Fgf19 alone cannot induce otic markers in explants of prospective otic ectoderm (Ladher et al., 2000), whereas applying Fgf-coated beads to intact embryos can induce expression of a subset of early otic markers, albeit only in regions near the endogenous otic placode (Adamska et al., 2001). There are also reports that Fgf can impair otic development: In one such study, Fgf misexpression reduced the size of the otic vesicle while stimulating production of microvesicles expressing lens markers (Domíngues-Frutos et al., 2009). In another study, electroporation of Fgf-expression vectors initially expanded the otic domain of *Pax2* but blocked all subsequent stages of otic development (Freter et al., 2008). In contrast, viral misexpression of Fgf3 can induce formation of ectopic otic vesicles expressing a full range of otic markers (Vendrell et al., 2000), and cultured explants of head ectoderm show that the entire preplacodal ectoderm surrounding the head is competent to express early otic markers in response to exogenous Fgf2 (Martin and Groves, 2006). In *Xenopus*, Fgf2-coated beads can induce formation of ectopic otic vesicles in a wide region between the eyes and anterior somites (Lombardo and Slack, 1998). The reason for the different outcomes in these experiments is not clear, but the varied techniques used likely produce marked differences in the stage, duration, spatial distribution, and amount of Fgf signaling. Any or all of these variables could influence the response to Fgf signaling.

Members of the Pax2/5/8 family of transcription factors are important mediators of Fgf signaling during otic induction. Expression of *pax8* marks the earliest known response to Fgf during late gastrulation and is critical for setting the size of the otic placode (Pfeffer et al., 1998; Phillips et al., 2001). Knockdown or loss of *pax8* reduces the size of the otic placode by nearly half (Ikenega et al., 2011; Mackereth et al, 2005). *pax2a* and *pax2b* expression normally begin during early somitogenesis stages and are partially redundant with *pax8* (Hans et al., 2004; Mackereth et al., 2005). Knockdown of all *Pax2/8* function leads to loss of otic fate by 24 hpf, indicating that these genes are needed to maintain otic fate. Whether Pax2/8 function is sufficient as a downstream response to Fgf has not been previously examined.

In addition to Fgf signaling, the transcription factor Foxi1 is required for induction of *pax8* in prospective otic tissue (Hans et al., 2004; Solomon et al., 2003; Solomon et al., 2004). Although otic expression of *pax2a* and *pax2b* is induced independently of Foxi1, their expression domain is much smaller in *foxi1* mutants. Despite the importance of Foxi1, the functional relationship between Fgf and Foxi1 remains unclear. For example, there are discrepancies as to whether Fgf inhibits or enhances *foxi1* expression (Hans et al., 2007; Nechiporuk et al., 2007; Phillips et al., 2004), possibly reflecting differences in misexpression technique. Additionally, because *pax2a/b* expression depends on Fgf but not Foxi1, appropriate misexpression of Fgf might be expected to expand the domain of *pax2a* and bypass the need for Foxi1.

Here we used heat-shock inducible transgenes to examine key parameters that influence the ability of Fgf to induce otic development. The effects of transient misexpression of Fgf were dependent on the stage and level of misexpression. Global transient activation of *hs:fgf3* or *hs:fgf8* at mid-late gastrula stages (7–8 hpf) severely impaired otic induction, in part by disrupting formation of the principal signaling centers in the hindbrain. Additionally, mosaic studies showed that high-level misexpression blocks otic fate cell-autonomously, whereas low to moderate levels promote otic development. At later stages high-level Fgf misexpression, either global or local, was no longer inhibitory but instead caused a dramatic expansion in the expression of otic markers into preplacodal ectoderm surrounding the anterior neural plate. At all stages examined, Fgf misexpression upregulated *foxi1* expression in ectoderm abutting the anterior neural plate. Moreover, the ability of Fgf to expand otic tissue required *foxi1*. Misexpression of *hs:pax2a* or *hs:pax8* also expanded endogenous otic domains but was not sufficient to bypass the requirement for Fgf or Foxi1. Co-misexpression of Fgf with *pax2a* or *pax8* led to production of ectopic otic tissue in a broad range of cranial ectoderm rostral to somites. Our data document the extent to which even small changes in the timing, distribution and level of Fgf signaling and its downstream effectors can influence otic induction. Furthermore, the data clarify functional relationships between Fgf, *foxi1* and *pax2/8* genes.

MATERIALS & METHODS

Strains and developmental conditions

Wild type embryos were derived from AB line (Eugene, OR). Transgenic lines used in this study include *Tg(hsp70:fgf8a)*^{x17} (Millimaki et al., 2010), *Tg(hsp70:fgf3)*^{x27} and *Tg(hsp70:pax2a)*^{x23} (Sweet et al., 2011), *Tg(hsp70:pax8)*^{x22} (this publication) and *Tg(brn3c:gfp)*^{s356t} (Xiao et al., 2005). For convenience, these transgenes are referred to in the remainder of the text as *hs:fgf8*, *hs:fgf3*, *hs:pax8*, *hs:pax2a* and *brn3c:gfp* respectively. Except for some experimental conditions noted below, embryos were developed under standard conditions at 28.5°C in fish water containing methylene blue and were staged based on standard protocols (Kimmel et al., 1995).

Misexpression

For standard misexpression studies, embryos heterozygous for heat shock inducible transgenes were incubated in a water bath at 39°C for 30 minutes at time points described in

the results. Variations of the heat shock regimen included use of different temperatures and/or use of homozygous transgenic embryos, as noted in the text. After heat shock, embryos were incubated at 33°C in order to maintain elevated transgene expression for a longer period. Additionally, incubation at 33°C after heat shock eliminates problems with cell death sometimes observed when transgenic embryos are returned to lower temperatures following heat shock). Except where noted, phenotypes caused by trasngene activation were assessed in at least 20 embryos per experiment. The phenotypes described herein were fully penetrant unless otherwise stated.

Cell transplantation

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

In situ hybridization

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

Morpholinos

For gene knockdown experiments, 5 ng of morpholino oligomer directed against *foix1* (*foxi1*-MO) (Gene Tools, Inc.) was injected into embryos at one-cell stage. The *foxi1*-MO sequence has been previously published (Solomon et al., 2003).

SU5402 treatment

SU5402 was dissolved in DMSO to make a stock solution of 20mM. Embryos are incubated with their chorions intact in a working concentration solution of 30μM SU5402 starting from 10.5 hpf, and then fixed at 13 hpf to examine the changes in *pax8* or *pax2a* expression.

RESULTS

The temporal, spatial and genetic factors influencing the response to Fgf during otic induction are not fully established. To explore Fgf-responsiveness in more detail, we used heat shock inducible transgenic lines to misexpress *fgf8* or *fgf3* (Millimaki et al., 2010; Sweet et al., 2011) at various developmental stages and expression levels. Except where noted, our standard conditions for misexpression involved heat shocking heterozygous carriers at 39°C for 30 minutes (see Materials and Methods). Under these conditions, *hs:fgf* transcript levels peak by the end of the heat shock period, remain elevated for 90 minutes and then gradually decline over the next 2 hours. Expression of the Fgf-target genes *erm* and *spry4* is slightly elevated by the end of the heat shock, increases to maximal expression one hour later and remains elevated for 4–5 hours after heat shock (Fig. 1C and data not shown).

We began by testing the effects of misexpression of *fgf8* or *fgf3* at various developmental stages. Activation of *hs:fgf3* or *hs:fgf8* during late blastula/early gastrula stages (5 hpf or earlier) resulted in complete dorsalization of the embryo and was not informative (not

shown). Activation of *hs:fgf3* or *hs:fgf8* at early to mid-gastrula stages (6–8 hpf) caused only partial dorsalization but severely impaired otic induction or blocked otic induction altogether (Table 1, Fig. 2A-F, M). Activation of *hs:fgf8* or *hs:fgf3* near the end of gastrulation (9–9.5 hpf), when otic markers are normally first detected, had no effect on otic development (Fig. 2M, Table 1). In contrast, activation of *hs:fgf8* or *hs:fgf3* after gastrulation (10–11 hpf) caused a dramatic enlargement of endogenous otic domains, though it did not induce formation of ectopic otic tissue anterior to the midbrain-hindbrain border (Table 1, Fig. 2G-L, M). Activating these transgenes at 12 hpf or 14 hpf also expanded otic tissue, though to a lesser degree, and activation at 16 hpf or later had no effect (Fig. 2M, Table 1). Similar results were previously reported using another *hs:fgf8* line (Hans et al., 2007), though the transgenic lines used here appeared to show markedly stronger effects than the previous line. How Fgf misexpression causes such dramatic stage-dependent differences in otic development is not well understood.

Distinct mechanisms of otic-impairment by early Fgf misexpression

The observation that Fgf misexpression at mid gastrula stage blocks otic development is paradoxical because this is the stage when otic induction is thought to begin during normal development. We considered two possible explanations for this impairment: First, we hypothesized that early global activation of *hs:fgf* disrupts endogenous signaling centers needed to induce and maintain otic development. In support, global activation of *hs:fgf8* or *hs:fgf3* at 7 hpf prevented proper expression of endogenous *fgf8* and *fgf3* in the hindbrain through at least 9.5 hpf (Fig. 3C-F). This change is likely sufficient to disrupt otic development because, even if the transient pulse of transgenic Fgf were sufficient to initiate otic development, otic fate could not be maintained at later stages without endogenous signaling sources. Similar results were observed following heat shock at 8 hpf (not shown).

Second, because otic development normally occurs in cells near (but not within) domains of Fgf expression, we hypothesized that excess Fgf signaling might cell-autonomously block otic fate. To test this we generated mosaic embryos by transplanting *hs:fgf8* transgenic cells into non-transgenic host embryos. The level of transgene-promoter activity can be regulated by adjusting the temperature from 35°C to 39°C (Adám et al., 2000; compare different responses in Figs. 1B and C). In one set of experiments, mosaic embryos were heat shocked under standard conditions (39°C for 30 minutes) at 8 hpf. This treatment caused cell-autonomous impairment of *pax8* expression in transgenic cells within the otic region at 11 hpf (Fig. 4B, C). In these same embryos, the domain of *pax8* expression was expanded non-autonomously in adjacent host cells (Fig. 4B, C). The same results were obtained following transgene activation at 38°C (not shown). In contrast, a lower level of activation of *hs:fgf8* at 37°C did not repress *pax8* expression in transgenic cells (Fig. 4E, F). In this case, too, the domain of *pax8* expression expanded in adjacent host cells (Fig. 4E, F). These results indicate that high levels of Fgf inhibit otic fate cell-autonomously, whereas low to moderate levels promote otic fate. Together, these data show that strong early misexpression of Fgf impairs otic induction by at least two mechanisms: It acts directly by cell-autonomously blocking otic fate, and it acts indirectly by preventing establishment of endogenous Fgf-signaling centers.

Effects of Fgf misexpression at later stages

To distinguish autonomous from non-autonomous effects at later stages, we next examined the effects of mosaic activation of *hs:fgf8* at 11 hpf. In agreement with the effects of global activation of *hs:fgf8* at later stages (Fig. 2G-M), activating the transgene at 11 hpf in mosaic embryos, even ones containing relatively few *hs:fgf8/+* cells, also expanded the otic domain of *pax2a* (Fig. 4G, H). Unlike misexpression at earlier stages, however, strong activation at 11 hpf did not cell-autonomously block otic differentiation within transgenic cell. Thus, once the otic development has been initiated, strong misexpression of Fgf reinforces otic fate and efficiently expands the endogenous otic primordium.

Despite enlargement of endogenous otic domains following Fgf misexpression at later stages, it is noteworthy that there were no signs of ectopic otic induction under standard misexpression conditions. This agrees with previous findings obtained with an earlier transgenic line (Hans et al., 2007). However, these results conflict with our previous findings that Fgf misexpression from injected plasmid vectors can induce otic placodes in ectopic locations around the front of the head (Phillips et al., 2004). Because injected plasmid frequently integrates into the genome as large concatemers, it is possible that the vectors used in our earlier study elicited stronger Fgf signaling than the transgenes utilized here. To boost the level of expression from our transgenic lines, *hs:fgf3/+* and *hs:fgf8/+* heterozygotes were heat shocked for 60 minutes beginning at 10 hpf. This resulted in dramatic upregulation of the otic competence factor *foxi1* in preplacodal ectoderm surrounding the front of the head (Fig. 5B), mimicking the effects of plasmid-injection (Phillips et al., 2004). Additionally, the otic/epibranchial marker *sox3* was ectopically expressed throughout the anterior preplacodal ectoderm (Fig. 5F). However, these conditions did not result in ectopic expression of *pax8* (Fig. 5J). To further increase Fgf expression levels, we generated *hs:fgf8/hs:fgf8* homozygotes and heat shocked them for 60 minutes beginning at 10 hpf. This resulted in significant, though spotty, ectopic expression of *pax8* in anterior preplacodal ectoderm, as well as marked lateral expansion of endogenous otic domains (Fig. 5K). Domains of *foxi1* and *sox3* were also expanded (Fig. 5C, G). Note that the domain of *sox3* was smaller in *hs:fgf8/hs:fgf8* homozygotes compared to *hs:fgf8/+* heterozygotes (Fig. 5F, G), consistent with previous finding that *sox3* exhibits distinct thresholds for activation by moderate Fgf levels and downregulation by high Fgf levels (Bhat et al., 2011; Nikaido et al., 2007; Padanad and Riley, 2011; Sun et al., 2007). Similar results were obtained by heat shocking *hs:fgf3/hs:fgf3* homozygotes for 60 minutes at 10 hpf (Fig. 5D, H, L). Analysis of additional early otic markers confirmed that strong global misexpression of *hs:fgf8* induced ectopic expression of *pax2a* and, to a lesser extent, *fgf24* (Fig. 5M-P). In contrast, expression of *cldna* was not induced ectopically (Fig. 5Q, R). Moreover, none of these transgenic embryos produced ectopic otic vesicles at later stages. Heat shocking *hs:fgf8/hs:fgf8* homozygotes at 12 hpf or 14 hpf gave similar but weaker responses compared to activation at 10 hpf (not shown). Activation at 16 hpf was not effective, confirming that otic competence is gradually lost during mid-somitogenesis stages (Groves and Bronner-Fraser, 2000; Hans et al., 2007). Together, these data show that transient high-level misexpression of Fgf can induce expression of many early otic markers in anterior preplacodal ectoderm, but the conditions used here are not sufficient to sustain the full program of otic development in ectopic locations.

To achieve maximal Fgf misexpression in a more localized manner, we generated mosaic embryos containing scattered *hs:fgf8/hs:fgf8* cells and heat shocked them for 60 minutes at 10 hpf. The majority (57/62) of mosaic embryos showed patches of robust *pax8* expression in anterior preplacodal ectoderm in regions near transgenic cells (Fig. 5S-V). Interestingly, transgenic cells themselves tended not to express *pax8* (arrows in Fig. 5S-U), similar to results obtained with plasmid-injection (Phillips et al., 2004). This suggests that high-level misexpression of Fgf8 can to some extent cell-autonomously impair otic differentiation in anterior preplacodal ectoderm, possibly accounting for the less robust ectopic expression of *pax8* seen after global misexpression of Fgf8 (Fig. 5K, L). To more fully analyze the effects of mosaic misexpression, we took advantage of the variable distribution of transgenic cells to assess whether different regions of anterior preplacodal ectoderm are equally responsive to localized Fgf8 signaling. To facilitate quantitative analysis, we divided the anterior preplacodal ectoderm into five equal domains along the AP axis. As summarized in Fig. 5V, the highest frequency of ectopic *pax8* expression was observed in the first two (anterior-most) regions, with right or left sides showing *pax8* expression in up to 75% of mosaic embryos. The third (middle) region showed *pax8* expression less than half as often, affecting no more than 31% of mosaic embryos on the right or left side (Fig. 5V). In no case did we observe ectopic *pax8* expression in the last two (posterior-most) regions (n=0/62), despite the fact that transgenic cells were often observed there. Nevertheless, it is unlikely these regions lack otic-competence because global Fgf misexpression was able to induce ectopic *pax8* throughout the anterior preplacodal ectoderm (Fig. 5K, L). It is possible that signals emitted by the midbrain-hindbrain border or other nearby tissues normally restrict otic development but are disrupted by global Fgf misexpression. Together, these data show that the entire preplacodal region is competent to express otic markers in response to Fgf, but responsiveness is not uniform, with some regions appearing somewhat resistant. Presumably, other signals or intrinsic factors can locally modulate the response to Fgf, as hypothesized by others (McCabe and Bronner-Fraser, 2009; Schlosser, 2006).

Misexpression of *pax2/8* expands the otic field

Previous studies have shown that *pax2/8* genes are important mediators of Fgf signaling and are required for normal induction and maintenance of otic tissue (Hans et al., 2004; Mackereth et al., 2005). We therefore generated heat shock inducible transgenic lines to test the effects of misexpression of *pax8* or *pax2a* (Sweet et al., 2011, and this work). These transgenes have similar effects on embryonic patterning, though in the experiments reported here *hs:pax8* was activated under standard conditions whereas *hs:pax2a* was activated by heat shocking at 38°C for 30 minutes (see Materials and Methods).

Activation of *hs:pax8* at mid to late gastrula stage (8.5 hpf) caused expansion of early otic markers into anterior preplacodal ectoderm to the level of the midbrain-hindbrain border (*pax2a*) or to the level of the eye (*fgf24*, *cldna*) (Fig. 6B, E, H). When these embryos were examined at 30 hpf, diffuse ectopic staining of *cldna* was observed in ectoderm just anterior and posterior to the otic vesicle (Fig. 6K). However, no ectopic otic vesicles were ever observed under these conditions. Similar results were obtained after misexpression of *pax8* at tailbud stage (10 hpf), except that *cldna* expression at 30 hpf was less diffuse, often appearing in microvesicles near the endogenous otic vesicle (Table 2, and data not shown).

Activation of *hs:pax2a* at 8.5 hpf or 10 hpf led to similar expansion of early otic markers (Fig. 6C, F, I). In this line, however, production of *cldna*-positive microvesicles was more common and was observed after heat shock at either 8.5 or 10 hpf (Fig. 6L, Table 2). In rare cases, ectopic microvesicles were observed beyond the midbrain-hindbrain border up to the level of the eye (Fig. 6L, Table 2). The microvesicles produced under these conditions were not normal otic structures, however, because they did not express other otic patterning genes such as *pax2a*, *dlx3b*, *otx1* or *atoh1a* (not shown). These data indicate that misexpression of *pax2/8* genes can expand the endogenous domain of early otic markers but is usually not sufficient to induce otic development elsewhere in the preplacodal ectoderm. Additionally, these genes are not sufficient to induce a full program of otic differentiation even in cases where extra-otic microvesicles were observed.

Misexpression of *pax2/8* cannot bypass the need for Fgf signaling

The extent to which Pax2a and Pax8 can mediate the full effect of Fgf signaling is not clear. We therefore tested whether activation of *hs:pax2a* or *hs:pax8* can bypass the need for Fgf during otic induction. Embryos carrying *hs:pax8* or *hs:pax2a* were heat shocked at 10 hpf, just after the onset of otic induction, and afterwards Fgf signaling was blocked using the pharmacological inhibitor SU5402. Under these conditions, otic development was completely abolished by 13 hpf (Fig. 7D-F). These results show that *pax2/8* genes are not sufficient to expand or maintain otic development in the absence of Fgf signaling, suggesting that additional Fgf-target genes are essential for otic induction.

Effects of co-misexpression of Fgf8 and Pax8/Pax2a

Given the lag between Fgf misexpression and activation of endogenous *pax8* and *pax2a* genes, we speculated that co-misexpression of transgenic Fgf8 with transgenic Pax2a/8 might accelerate and enhance early steps inotic development and thereby stabilize production of ectopic otic vesicles. In support, co-activation of *hs:fgf8* with *hs:pax2a* or *hs:pax8* at 10 hpf (in embryos heterozygous for relevant transgenes) strongly enhanced production of ectopic microvesicles compared to activation of individual transgenes (Fig. 8A, B and Table 2). For example, there was a three-fold increase in the number of microvesicles produced in *hs:fgf8-hs:pax2a* embryos compared to *hs:pax2a* alone (Table 2). Moreover, 90% (9/10) of double transgenic embryos produced ectopic microvesicles in the anterior head and/or adjacent to anterior somites (Fig 8B, D, F, H, I and Table 2). Similarly, 60% (9/15) of *hs:fgf8-hs:pax8* double transgenic embryos produced ectopic otic vesicles in these regions (Fig. 8A, C, E, G, and Table 2). Another difference between single vs. double transgenic embryos was in the range of otic markers expressed within microvesicles. While microvesicles induced by *hs:pax8* or *hs:pax2a* alone expressed only *cldna*, microvesicles produced in *hs:fgf8-hs:pax8* or *hs:fgf8-hs:pax2a* embryos expressed a full range of otic vesicle markers including *cldna*, *dlx3b*, *otx1*, *pax2a* and *atoh1a* at 30 hpf (Fig. 8C-H, and data not shown). Additionally, ectopic vesicles were observed to express *brn3c:gfp* (Fig. 8I), indicating the presense of sensory hair cells (Xiao et a., 2005). To better understand the effects of co-misexpression, we heat shocked *hs:fgf8-hs:pax2a* double transgenic embryos at 10 hpf and analyzed expression of early otic markers at 13 hpf. Double transgenic embryos showed a number of changes in gene expression compared to single transgenic embryos. First, expression of *cldna* was more robust in anterior preplacodal ectoderm in double

transgenic embryos (Fig. 9A-D). Second, scattered patches *pax2a*-positive of cells were observed in anterior preplacodal ectoderm only in double transgenic embryos (Fig. 9E-H), despite significant downregulation of *pax2a* in the optic stalk (Fig. 9H). Third, expression of *fgf24* in anterior preplacodal ectoderm was stronger in double-transgenic embryos (Fig. 9I-L). Likewise, upregulation of the Fgf-target gene *spry4* was also more pronounced in preplacodal ectoderm in double transgenic embryos (Fig. 9M-P). Together, these results indicate that co-misexpression of *hs:fgf8* with *hs:pax2a* or *hs:pax8* can synergistically promote stable induction of ectopic otic placodes and vesicles expressing a host of otic patterning genes.

An indispensable role for *foxi1*

It was previously established that expression of *pax8* requires *foxi1* in the endogenous otic placode as well as in ectopic locations following Fgf misexpression (Hans et al., 2004; Solomon et al., 2003; Solomon et al, 2004). However, Fgf is still able to induce residual *pax2a*-positive otic placodes in the absence of *foxi1*. We therefore tested whether maximal misexpression of Fgf8 or Fgf3 could expand the otic domain of *pax2a* in *foxi1* morphants. As expected, the otic domain of *pax8* was eliminated under these conditions (Fig. 10A-D). Surprisingly, the otic domain of *pax2a* was no larger in *foxi1* morphants after Fgf misexpression compared to non-transgenic *foxi1* morphants (Fig. 10H-J). We also observed no ectopic *pax2a* expression under these conditions. Thus, despite the presence of a small domain of Fgf-dependent/Fox1- independent otic precursors, competence to respond to Fgf in other regions is absolutely dependent on *foxi1*.

We next tested whether the requirement for *foxi1* could be bypassed by misexpressing its downstream target, *pax8*. We focused on *pax8* because this gene is especially critical for mediating the effects of f*oxi1* in establishing a normally sized otic placode (Ikenega et al., 2011; Mackereth et al., 2005). Despite the importance of *pax8* for this function, activation of *hs:pax8* alone was not sufficient to expand the otic domain in *foxi1* morphants (Fig. 10E, K). In contrast, co-activation of *hs:pax8* and *hs:fgf8* expanded the otic domain of *pax2a* up to the level of the midbrain-hindbrain border (Fig. 10L), though there was still no otic expression of endogenous *pax8* (Fig. 10F). Thus, Pax8 acting in concert with Fgf can partially compensate for loss of *foxi1* to affect the size of the otic domain.

DISCUSSION

Previous studies investigating the effects of Fgf misexpression on otic induction have yielded widely varying, often contradictory results (Adamska et al., 2001; Domíngues-Frutos et al, 2009; Freter et al., 2008; Hans et al., 2007; Ladher et al., 2000; Léger and Brand, 2002; Lombardo and Slack, 1998; Martin and Groves, 2006; Phillips et al., 2004; Vendrell et al., 2000). We have identified a number of variables that critically influence how prospective otic cells respond to Fgf. First, sensitivity to Fgf varies according to developmental stage. Embryos are particularly vulnerable to inhibitory effects of Fgf over-expression during gastrulation stages, partly reflecting disruption of endogenous signaling centers. Second, the spatial distribution of Fgf expression strongly affects the outcome of Fgf signaling, with high levels blocking otic development within expressing cells while

promoting otic development in neighboring cells. Third, delivering sufficiently high levels of Fgf just after gastrulation expands expression of early otic markers throughout preplacodal ectoderm surrounding the head. The ability of Fgf to expand otic development absolutely requires Foxi1, though our results suggest that Foxi1 acts downstream of Fgf rather than in an independent parallel pathway. Finally, misexpressing Pax2a or Pax8 expands otic development and potentiates the effects of misexpressing Fgf, leading to formation of enlarged and ectopic otic vesicles that express a full array of otic patterning genes. Nevertheless, Pax2/8 misexpression cannot bypass the need for either Fgf or Foxi1. Together these findings provide important insights into the conditional requirements for otic induction and potentially reconcile discrepancies in the literature regarding the effects of Fgf misexpression.

Response to Fgf is conditional

Otic placodes, like all other cranial placodes, develop from a contiguous zone of preplacodal ectoderm surrounding the anterior neural plate. Although preplacodal ectoderm is clearly multipotent, cells in different regions appear to have distinct biases reflecting their unique expression profiles of various transcription factors (McCabe and Bronner-Fraser, 2009; Schlosser, 2006). Nevertheless, sufficiently high levels of Fgf signaling can overcome regional biases to induce expression of early otic markers (*pax8*, *pax2a*, *fgf24* and *sox3*) throughout the preplacodal ectoderm. At slightly lower Fgf levels, *pax2/8* and *fgf24* genes are not induced ectopically, yet expression of *sox3* is expanded even more than with higher Fgf. This pattern mimics the gene expression profiles seen in otic and epibranchial placodes, respectively, which are induced in abutting domains by a lateral gradient of Fgf (Bhat and Riley, 2011; Padanad and Riley, 2011).

After initial otic induction, Fgf signaling continues from endogenous signaling centers and is initially required to maintain otic fate (Léger and Brand, 2002), and later to pattern the otic vesicle (Hammond and Whitfield, 2011; Kwak et al., 2002; Lecaudey et al., 2007). Our misexpression studies do not address these later functions because transgene activity is only transient. This likely explains why even maximal trasngene activity was not sufficient to stably induce morphological development of ectopic otic vesicles. Serial heat shock can sometimes prolong the effects of Fgf misexpression (Sweet et al., 2011), but in the current study maximal Fgf misexpression induced such robust expression of *spry4*, and presumably other feedback inhibitors, that secondary heat shocks were relatively ineffective (our unpublished observations). However, co-misexpression of *hs:fgf8* with either *hs:pax8* or *hs:pax2a* frequently led to formation of ectopic otic vesicles expressing a full array of otic markers. Although endogenous *pax2/8* genes are induced ectopically by Fgf, activation of *hs:pax2/8* transgenes avoids the lag-time normally required for this response. We speculate that such co-activation triggers a self-reinforcing feedback loop that stabilizes otic development. Consistent with this notion, co-activation of *hs:fgf8* and *hs:pax2a* leads to greater ectopic expression of *fgf24* in anterior preplacodal ectoderm, which likely prolongs Fgf signaling and helps maintain *pax2a* expression. Additionally, Pax2/8 function might protect otic cells from potential inhibitory effects of Fgf over-expression. In the endogenous otic field, for example, Fgf over-expression no longer cell-autonomously inhibits otic fate once expression of *pax8* and *pax2a* have been established. A similar protective mechanism

might explain why co-misexpression of Pax2/8 with Fgf stabilizes formation of ectopic otic placodes in anterior preplacodal ectoderm.

The mechanism by which Fgf over-expression cell-autonomously inhibits otic development remains unclear. Because Fgf acts as a DV morphogen during gastrulation, it is possible that excess Fgf inhibits otic fate by specifying more dorsal ectodermal fates. However, we detect no enhanced expression of characteristic markers of neural crest (*foxd3*) or neural plate (*sox19* or *krox20*) (data not shown). Another common role for Fgf is maintenance of stem cell pluripotency (Lanner and Rossant, 2010), raising the possibility that excess Fgf exerts a general block to differentiation. If so, it is not clear why inhibition is limited to cells that directly express Fgf, since immediate neighbors presumably also experience high Fgf signaling. Some Fgf ligands, including Fgf3, can regulate mitosis and differentiation by being imported directly into the nucleus without prior secretion (Kiefer et al., 1994; Kiefer and Dickson, 1995). However, no similar activity has been reported for Fgf8, raising the possibility that a more general mechanism mediates cell-autonomous inhibition by multiple Fgf ligands. This remains an important unresolved question.

Foxi1 and otic competence

Foxi1 function is complex. It is initially expressed throughout nonneural ectoderm in response to Bmp (Hans et al., 2004; Solomon et al., 2003) and serves as a general competence factor for all preplacodal ectoderm (Kwon et al., 2010). In a distinct subsequent process, *foxi1* downregulates in most of its original domain but upregulates in otic and epibranchial precursors as they begin to experience elevated Fgf signaling. In this latter process, *foxi1* is thought to act as a spatially localized competence factor required for the otic/epibranchial response to Fgf (Hans et al., 2004; Solomon et al., 2003; Solomon et al., 2004). However, we find that Fgf misexpression is sufficient to upregulate *foxi1* throughout ectoderm surrounding the head. Because *foxi1* is Fgf-responsive, the *foxi1* domain might not be the principal means of spatially restricting otic/epibranchial competence; rather it is the availability of sufficiently high levels of Fgf. In this context it would be more appropriate to consider *foxi1* an essential early mediator of Fgf during otic induction rather than an independent competence factor that localizes the response to Fgf. Additional evidence for this view comes from analysis of *foxi1* mutants and morphants. Although loss of *foxi1* ablates otic expression of *pax8*, a small domain of *pax2a* still forms in response to Fgf (Hans et al., 2004; Solomon et al., 2004). However, not even maximal Fgf over-expression can enlarge this domain in the absence of *foxi1*. Thus, *foxi1* is not required to position the endogenous domain of otic competence, but instead *foxi1* is required downstream of Fgf to expand otic development beyond this restricted domain. Interestingly, it has been noted that upregulation of *foxi1* in the otic/epibranchial domain does not require Fgf (Solomon et al., 2004). However, this appears to reflect partial redundancy between the Fgf and Pdgf pathways since blocking both pathways with pharmaceutical inhibitors severely impairs development of preplacodal ectoderm blocks local upregulation of *foxi1* (Kwon et al. 2010; and our unpublished observations).

Pax8 appears to be a key mediator of Foxi1 in expanding the initial domain of otic development. Knockdown of *pax8* substantially reduces the size of the otic placode (Ikegena

et al., 2011; Mackeret et al., 2005), and co-misexpression of Pax8 and Fgf8 dramatically expands otic development in the absence of *foxi1* (Fig. 10L). On the other hand, Pax8 plus Fgf8 were unable to expand otic development in the absence of *foxi1*, indicating that other Foxi1-target genes also contribute to this function.

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Highlights

- Fgf misexpression can inhibit or promote otic development, depending on context.
- Optimal Fgf expands expression of early otic markers into anterior cranial ectoderm.
- Misexpression of Pax2a/8 expands some otic markers but cannot bypass Fgf or Foxi1.
- Comisexpression of Pax2a/8 with Fgf8 potentiates formation of ectopic otic vesicles.

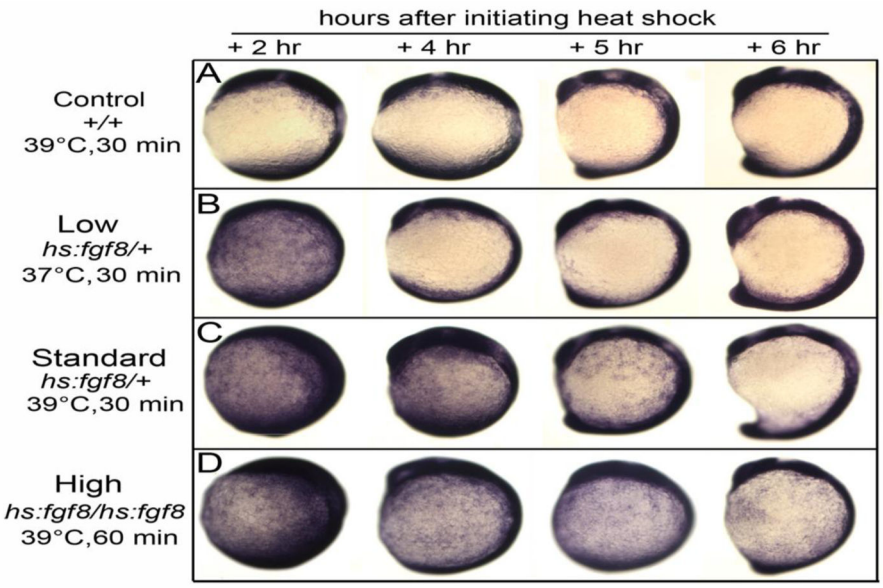

Figure 1. Expression of *erm* in response to differential activation of *hs:fgf8*

(A-D) Embryos were heat shocked at 10 hpf under conditions indicated to the left and fixed at times indicated across the top to examine expression of the Fgf-target gene *erm*. Images show lateral views with dorsal to the right and anterior up.

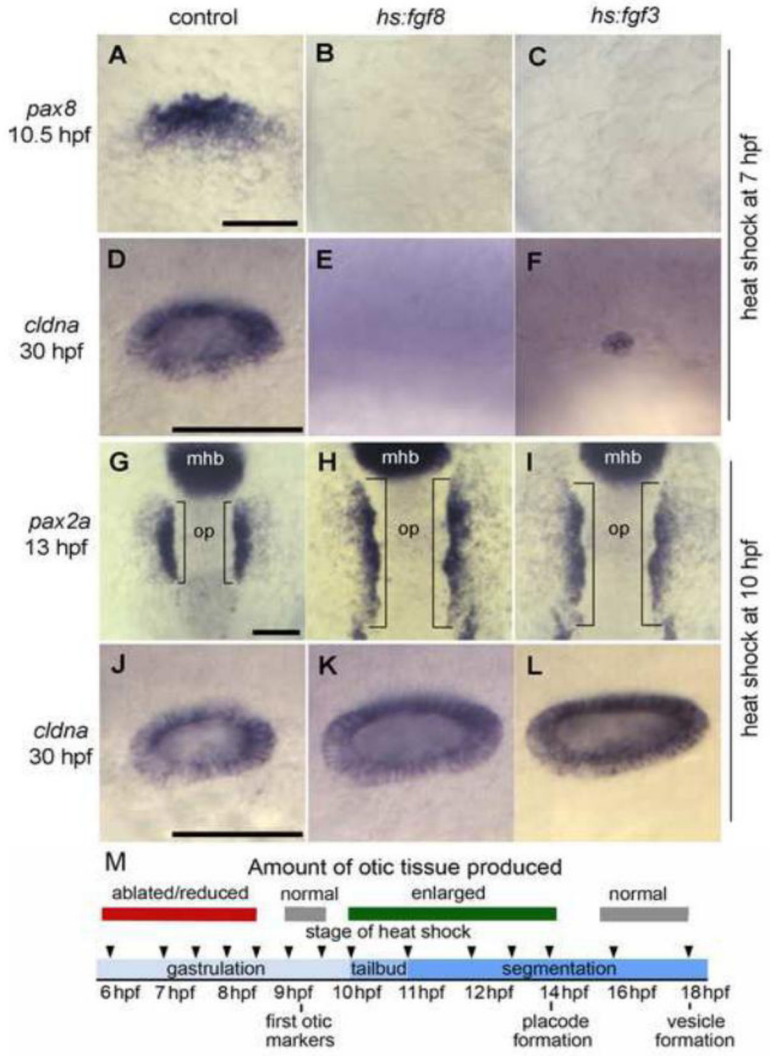

Figure 2. Stage-dependent effects of Fgf misexpression

(A-F) Embryos were heat shocked at 7 hpf and fixed at 10.5 hpf to examine expression of *pax8* in the otic primordium (A-C) or 30 hpf to examine expression of *cldna* in the otic vesicle (D-F). **(G-L)** Embryos were heat shocked at 10 hpf and fixed at 13 hpf to examine otic expression of *pax2a* (G-I) or at 30 hpf to examine expression of *cldna* (J-L). Genotypes of wild-type controls and heterozygous transgenic embryos are indicated across the top of the figure. Images show lateral views with anterior to the left (A-F, J-L); dorsal views with anterior to the top (G-I). Scale bar, 50 μm. (M) Summary of the effects of activating *hs:fgf8* at various developmental stages.

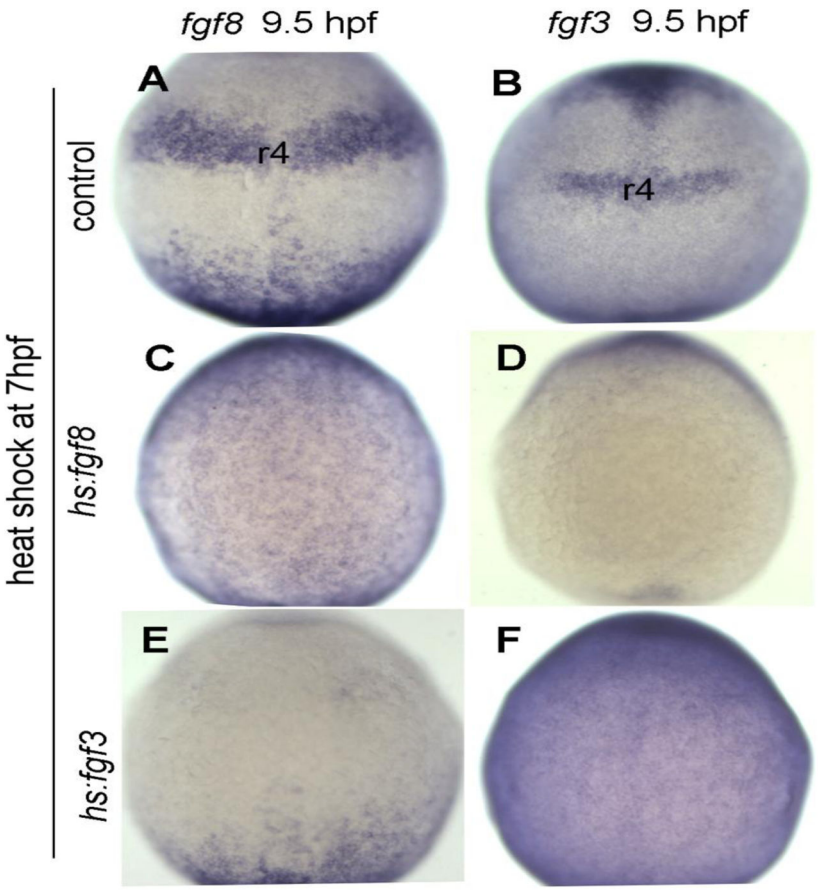

Figure 3. Misexpression of Fgf during gastrulation perturbs endogenous signaling centers

Expression of *fgf8* and *fgf3* in rhombomere 4 (r4) of hindbrain at 9.5 hpf in control embryos **(A, B)**, *hs:fgf8* transgenic embryos **(C, D)** and *hs:fgf3* transgenic embryos **(E, F)** respectively. Embryos were heat shocked at 7 hpf. Note that *fgf8* expression is still globally elevated in *hs:fgf8*/+ embryos whereas *fgf3* is globally elevated in *hs:fgf3*/+ embryos, but *fgf* genes are not detectably upregulated in the r4 region. All images show dorsal views with anterior to the top.

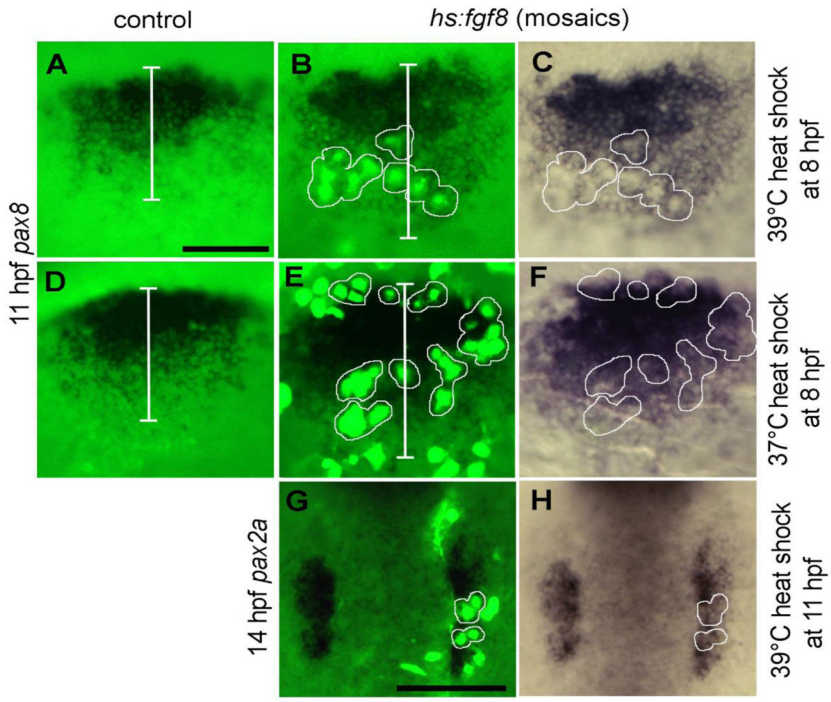

Figure 4. Effects of mosaic misexpression of Fgf8

(A-F) Lateral views (anterior to the left) showing expression of *pax8* at 11 hpf in the otic domain in control embryos (A, D) or *hs:fgf8*/+ mosaic embryos (B, C, E, F) heat shocked at 39°C (A-C) or 37°C (D-F) at 8 hpf. Clusters of transgenic cells (green) are encircled with white borders to facilitate comparison of fluorescent images (B, E) with bright field images of the same specimens (C, F). White bars (A, B, D and E) mark the ML width of the otic domain. Note that transgenic cells express *pax8* following heat shock at 37°C but not at 39°C, yet the otic domain is laterally expanded at both temperatures. **(G, H)** Dorsal views (anterior to the top) showing expression of *pax2a* at 14 hpf in a mosaic embryo heat shocked at 39°C at 11 hpf. Transgenic cells (green, with white borders) express *pax2a* and the otic domain is lengthened along the AP axis. Scale bar, 150 μm.

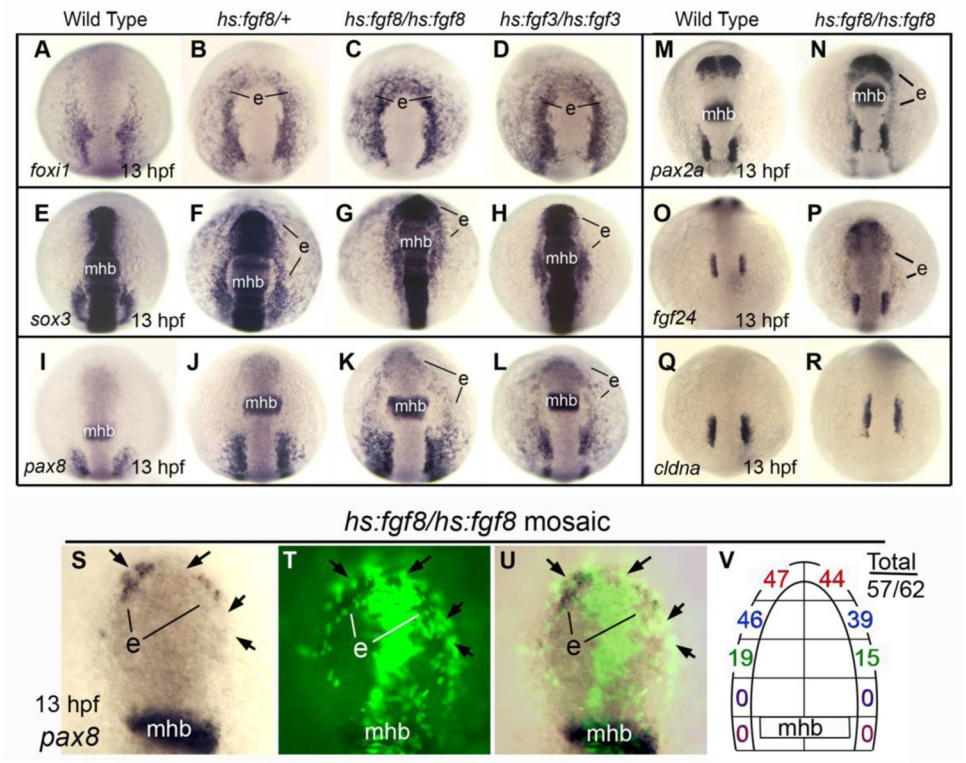

Figure 5. High level Fgf induces ectopic expression of otic markers in anterior preplacodal ectoderm after gastrulation

(A-R) Dorsal views (anterior up) of embryos heat shocked at 39°C for 1 hour starting at 10 hpf and then fixed at 13 hpf to examine expression of *foxi1* (A-D), *sox3* (E-H), *pax8* (I-J), *pax2a*, (M, N), *fgf24* (O, P) and *cldna* (Q, R). Genotypes of embryos are indicated across the top of the figure. The midbrain-hindbrain border (mhb) and regions showing ectopic expression (e) are indicated. **(S-U)** A representative *hs:fgf8/hs:fgf8* mosaic embryo that was heat shocked at 39°C for 1 hour at 10 hpf and fixed at 13 hpf to examine *pax8* expression. Images show the same specimen viewed under bright field (S), fluorescence (T), and an overlay (U). Positions of *hs:fgf8/hs:fgf8* transgenic cells (green, black arrows), the midbrain-hindbrain border (mhb), and ectopic patches of *pax8* expression (e) are indicated. **(V)** A summary diagram showing the number of *hs:fgf8/hs:fgf8* mosaic embryos with ectopic expression of *pax8* in different regions of the preplacodal ectoderm. The total number of mosaic embryos showing any ectopic *pax8*/the total number examined is also indicated.

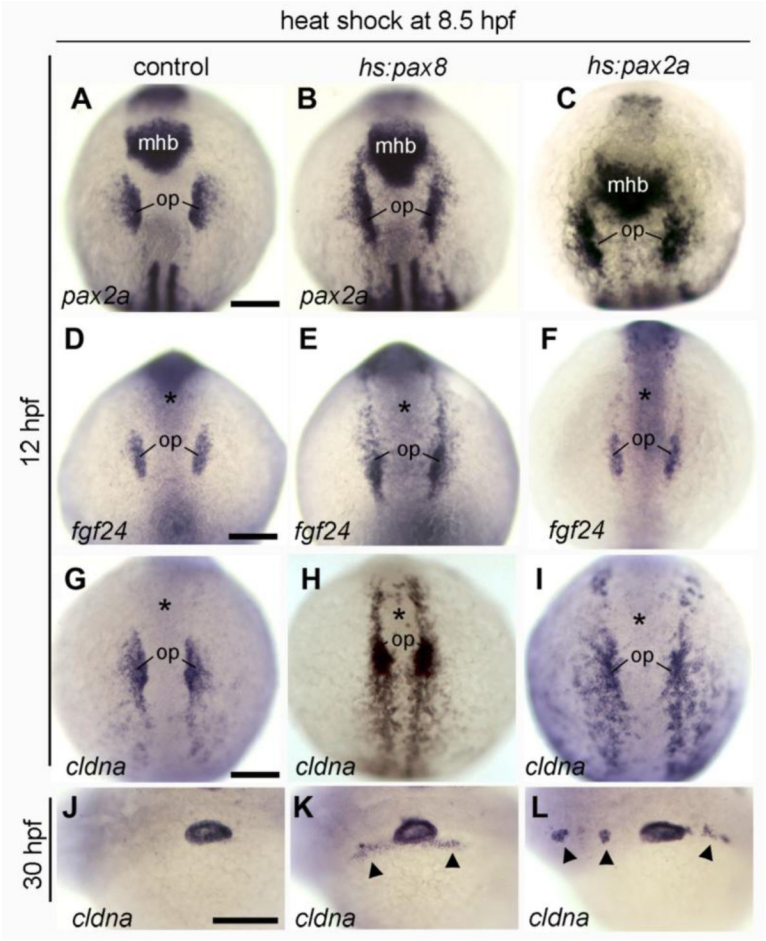

Figure 6. Expansion of otic markers following activation of *hs:pax8* or *hs:pax2a*

(A-L) Dorsal views (anterior up) or lateral views (anterior to the left) of embryos heat shocked at 8.5 hpf and fixed at 12 hpf to examine expression of *pax2a* (A-C), *fgf24* (D-F) and *cldna* (G-I), or embryos were fixed at 30 hpf to examine expression of *cldna* in the otic vesicle (J-L). Genotypes of wild-type or heterozygous transgenic embryos are indicated across the top of the figure. Expression in the otic placode (op) and midbrain-hindbrain border (mhb) is indicated. Alternatively, the position of the midbrain-hindbrain border is marked by an asterisk in (D-I). Arrowheads in K, L mark regions with ectopic expression of *cldna*. Scale bar, 150 μm.

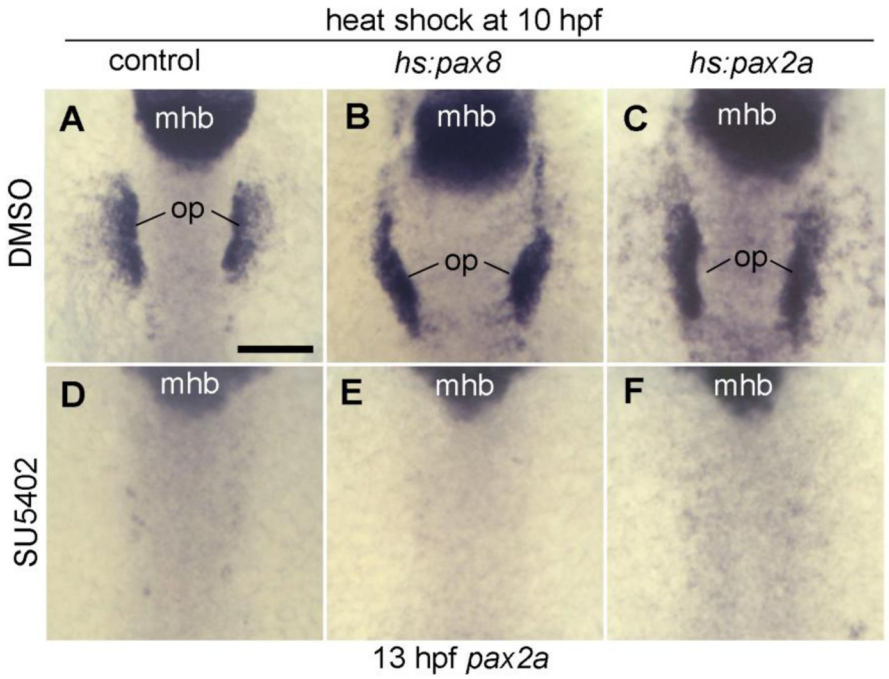

Figure 7. Pax2/8 misexpression cannot bypass the need for Fgf

(A-F) Dorsal views (anterior up) of *pax2a* expression at 13 hpf. Embryos were heat shocked at 10 hpf and then incubated in water containing 0.15% DMSO or 30 μM SU5402 and 0.15% DMSO. Genotypes of wild-type and heterozygous transgenic embryos are indicated across the top of the figure. Positions of midbrain-hindbrain boundary (mhb) and otic placode (op) are indicated. Scale bar, 150 μm.

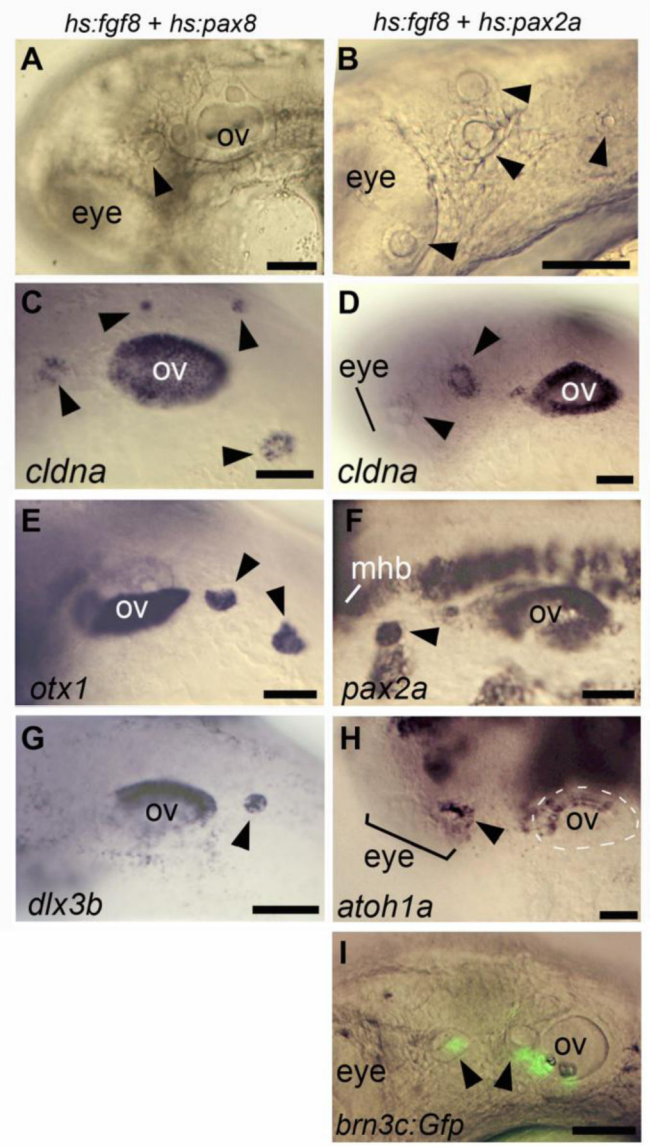

Figure 8. Formation of ectopic otic vesicles following co-misexpression of Fgf*8* with Pax2a or Pax8

(A-L) Lateral views (anterior to the left) of embryos at 30 hpf, following heat shock at 10 hpf. Images show live embryos (A, B, L) or fixed specimens showing expression of *cldna* (C, D), *otx1b* (E), *pax2a* (F), *dlx3b* (G), or *atoh1a* (H). The specimen in (L) also carries *brn3c:Gfp* transgene to mark sensory hair cells. Embryos heterozygous for the indicated inducible transgenes are labeled across the top of the figure. Positions of the eye, midbrain-hindbrain border (mhb), endogenous otic vesicle (ov) and ectopic otic vesicles (arrowheads) are indicated. Scale bar, 50 μm.

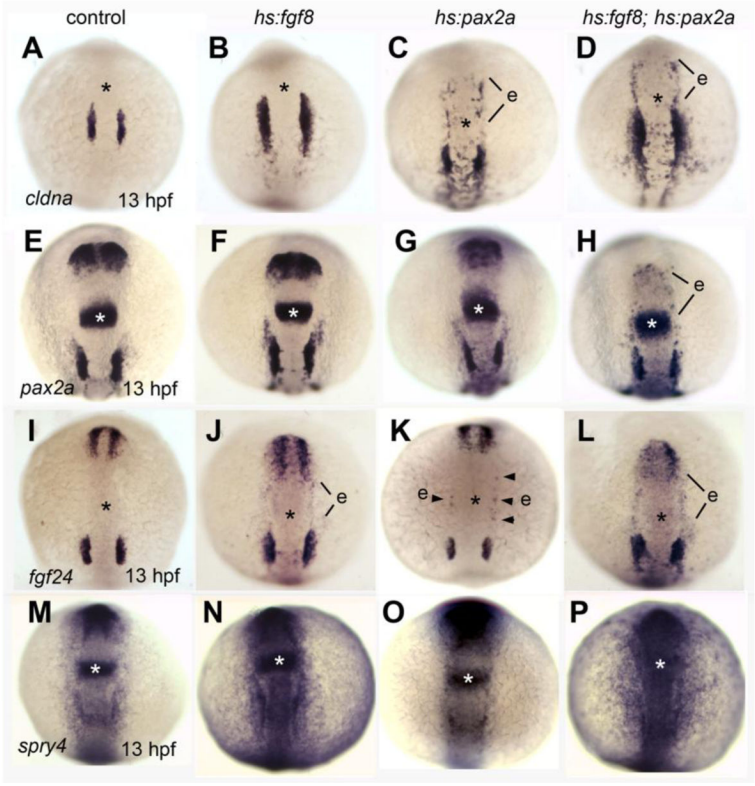

Figure 9. Effects of co-misexpression of Fgf8 with Pax2a on early otic development

(A-P) Dorsal views (anterior up) of embryos heat shocked at 10 hpf and fixed at 13 hpf to examine expression of *cldna* (A-D), *pax2a* (E-H), *fgf24* (I-L) and *spry4* (M-P). Genotypes of wild-type and heterozygous transgenic embryos are indicated across the top of the figure. The position of the midbrain-hindbrain border is marked with an asterisk. Regions with ectopic gene expression (e) are indicated.

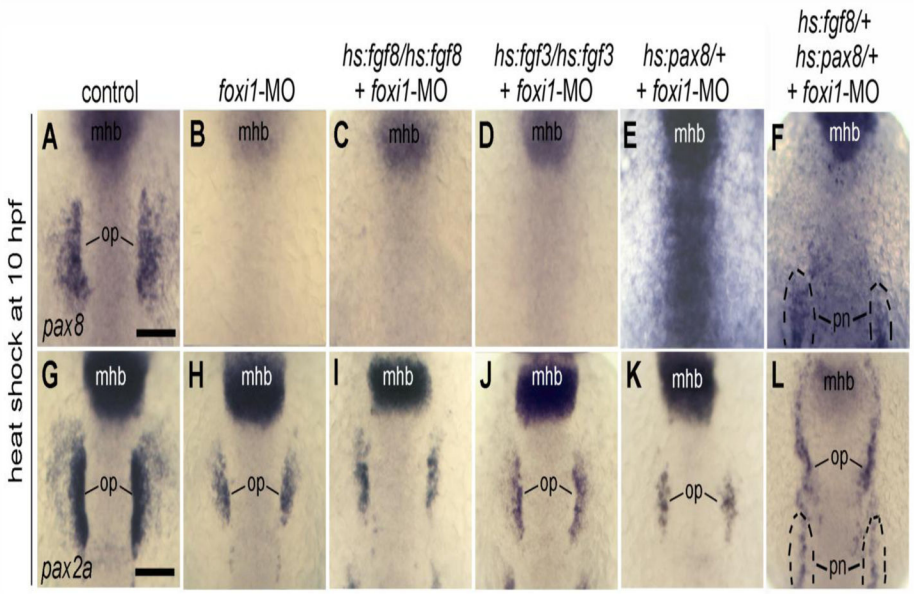

Figure 10. Misexpression of Fgf or Pax8 cannot expand otic development without Foxi1

(A-F) Expression of *pax8* at 13 hpf. **(G-L)** Expression of *pax2a* at 13 hpf. Embryos were heat shocked at 10 hpf, and most embryos were injected at the one-cell stage with *foxi1*-MO (B-F, H-L) as indicated across the top of the figure. Genotypes of transgenic embryos, including *hs:fgf8/hs:fgf8* (C, I), *hs:fgf3/hs:fgf3* (D, J), *hs:pax8/+* (E, K), and *hs:fgf8/+; hs:pax8/+* (F, L) are indicated across the top of the figure. Positions of midbrain-hindbrain boundary (mhb), pronerphros (pn) and otic placode (op) are indicated. Note that the pronerphric domain of *pax2a* is also expanded anteriorly in double trasngenic embryos (L). All images show dorsal views with anterior to the top. Scale bar, 150 μm.

Table 1

Stage-dependent effects on otic development following misexpression of *fgf* and/or *pax* genes.

Trasngene	Heat Shock	Stage	Placodal Domain	Vesicle at 30 hpf
hs:fgf8/+	39°C, 30 min	6 hpf	ablated	ablated
		7 hpf	ablated	ablated
		8 hpf	ablated	ablated
		9 hpf	normal	normal
		10 hpf	enlarged	enlarged
		11 hpf	enlarged	enlarged
		12 hpf	enlarged	enlarged
		14 hpf	enlarged	normal
	39°C, 60 min	10 hpf	enlarged	enlarged
	38°C, 30 min	8 hpf	ablated	ablated
	37°C, 30 min	8 hpf	reduced	reduced
	35°C, 18 hours	6 hpf	normal	normal
hs:fgf8/hs:fgf8	39°C, 30 min	10 hpf	enlarged	enlarged
	39°C, 60 mine	10 hpf	enlarged, ectopic	enlarged
hs:fgf3/+	39°C, 30 min	6 hpf	ablated	ablated
		7 hpf	ablated	ablated
		8 hpf	strongly reduced	strongly reduced
		9 hpf	normal	normal
		10 hpf	enlarged	enlarged
		11 hpf	enlarged	enlarged
hs:fgf3/hs:fgf3	39°C, 60 min	10 hpf	enlarged, ectopic	enlarged
hs:pax8/+	39°C, 30 min	8 hpf	enlarged	enlarged
		10 hpf	enlarged	enlarged
hs:pax2a/+	38°C, 30 min	8 hpf	enlarged, ectopic	enlarged, ectopic
		10 hpf	enlarged, ectopic	enlarged, ectopic
hs:fgf8/+; hs:pax8/+	39°C, 30 min	10 hpf	enlarged, ectopic	enlarged, ectopic
hs:fgf8/+ hs:pax2a/+	38°C, 30 min	10 hpf	enlarged, ectopic	enlarged, ectopic

Table 2

Production of microvesicles following global misexpression of *hs:fgf8*, *hs:pax2a* or *hs:pax8* beginning at 10 hpf.

Transgene	No. of embryos	Mean no. of microvesicles per embryo*	Fraction of embryos with ectopic microvesicles†
hs:fgf8	17	0	0/17
hs:pax2a	10	6.7 ± 2	2/10
hs:pax8	12	4.4 ± 2	0/12
hs:fgf8 + *hs:pax2a*	10	17.4 ± 3.7	9/10
hs:fgf8 + *hs:pax8*	15	9.7 ± 2.3	9/15

*Mean ± SD of the total number of microvesicles.

†Refers to microvesicles forming anterior to the midbrain-hindbrain border or posterior to the first somite.