Zebrafish Foxi1 provides a neuronal ground state during inner ear induction preceding the Dlx3b/4b-regulated sensory lineage

Stefan Hans*, Anne Irmscher and Michael Brand*

SUMMARY
Vertebrate inner ear development is a complex process that involves the induction of a common territory for otic and epibranchial precursors and their subsequent segregation into otic and epibranchial cell fates. In zebrafish, the otic-epibranchial progenitor domain (OEPD) is induced by Fgf signaling in a Foxi1- and Dlx3b/4b-dependent manner, but the functional differences of Foxi1 and Dlx3b/4b in subsequent cell fate specifications within the developing inner ear are poorly understood. Based on pioneer tracking (PioTrack), a novel Cre-dependent genetic lineage tracing method, and genetic data, we show that the competence to embark on a neuronal or sensory fate is provided sequentially and very early during otic placode induction. Loss of Foxi1 prevents neuronal precursor formation without affecting hair cell specification, whereas loss of Dlx3b/4b inhibits hair cell but not neuronal precursor formation. Consistently, in Dlx3b/4b- and Sox9a-deficient mutants almost all otic epithelial fates are absent, including sensory hair cells, and the remaining otic cells adopt a neuronal fate. Furthermore, the progenitors of the anterior lateral line ganglia also arise from the OEPD in a Foxi1-dependent manner but are unaffected in the absence of Dlx3b/4b or in b380 mutants. Thus, in addition to otic fate Foxi1 provides neuronal competence during OEPD induction prior to and independently of the Dlx3b/4b-mediated sensory fate of the developing inner ear.

KEY WORDS: Inner ear, Neurogenesis, Competence, Foxi1, Dlx3b/4b, Genetic lineage tracing, Cre/lox, PioTrack, Zebrafish

INTRODUCTION
The vertebrate inner ear mediates hearing and balance through a complex arrangement of mechanosensory hair cells, nonsensory supporting cells and sensory neurons. All of these cell types derive from the otic placode, a transient ectodermal thickening adjacent to the developing hindbrain (Barald and Kelley, 2004; Fritzsch et al., 2006). Otic placode formation is a multistep process initiated by the establishment of the preplacodal region, which surrounds the anterior neural plate and contains precursors for all sensory placodes (Streit, 2007). Subsequently, the otic-epibranchial progenitor domain (OEPD), a common territory for otic and epibranchial precursors, is specified (Ladher et al., 2010; Chen and Streit, 2012). In zebrafish, the OEPD also appears to contain the progenitors of the anterior lateral line ganglion (McCarroll et al., 2012). Studies in various species have shown that OEPD formation is triggered by Fibroblast growth factor (Fgf) ligands secreted by the hindbrain and subjacent mesendoderm (Phillips et al., 2001; Léger and Brand, 2002; Maroon et al., 2002; Alvarez et al., 2003; Wright and Mansour, 2003; Ladher et al., 2005). In response to these signals, cells express Pax8 and Pax2, two members of the Pax2/5/8 transcription factor family, which are crucial regulators of OEPD formation and proper inner ear development (Brand et al., 1996; Pfaffer et al., 1998; Hans et al., 2004; Mackereth et al., 2005; Bouchard et al., 2010; Freter et al., 2012). The subsequent segregation of otic and epibranchial progenitors is mediated by Wnt signaling in a Pax2-dependent manner (Freter et al., 2008; McCarroll et al., 2012). With respect to otic fate, a two-phase model has been proposed to summarize genetic interactions during otic induction in zebrafish (Hans et al., 2004; Solomon et al., 2004). According to this model, the forkhead transcription factor Foxi1 enables expression of Pax8 during the early phase, and the homeodomain transcription factors Dlx3b and Dlx4b (Dlx3b/4b) provide competence to activate Pax2a during the second phase (Hans et al., 2004; Solomon et al., 2004). Previous work has shown that dlx3b, dlx4b and foxi1 are regulated initially independently in a BMP-dependent manner in the same region at late gastrula stages and whereas foxi1 is progressively restricted to the presumptive OEPD, dlx3b and dlx4b are maintained in a stripe corresponding to cells of the preplacodal region (Akimenko et al., 1994; Ellies et al., 1997; Nissen et al., 2003; Solomon et al., 2003; Solomon et al., 2004; Hans et al., 2007). Subsequently, downregulation of foxi1 in a Pax2a- and Pax8-dependent manner is required for proper otic development, whereas dlx3b and dlx4b are maintained in the cells of the future otic placode (Akimenko et al., 1994; Padanad and Riley, 2011). Loss of Foxi1 or Dlx3b/4b results in compromised otic induction and development of smaller otic vesicles (Solomon and Fritz, 2002; Liu et al., 2003; Nissen et al., 2003; Solomon et al., 2003), and combined loss of both factors eliminates all indications of otic specification (Hans et al., 2004; Solomon et al., 2004). However, the functional differences of Foxi1 and Dlx3b/4b during otic induction have not been addressed thus far and remain elusive.

After placode formation, otic tissue develops into the otic vesicle where sensory neurons and mechanosensory hair cells are born. The neuronal precursors delaminate as neuroblasts from an anterior-ventral position in the otic vesicle and give rise to the eighth cranial or statoacoustic ganglion, whereas hair cells are generated in the sensory epithelia of the otic vesicle (Haddon and Lewis, 1996;
It is well established that neuroblast and hair cell formation require the activation of the proneural proteins Neurogenin 1 (Neurog1) and Atonal homolog 1 (Atoh1) (Ma et al., 1998; Bermingham et al., 1999; Andermann et al., 2002; Fritzsch et al., 2010). However, despite an understanding of proneural gene function and extensive research on otic placode induction, upstream events, including the underlying regulation of Atoh1 and Neurog1 expression in sensory and neuronal precursors, are just beginning to be understood. In zebrafish, it was shown that Dlx3b/4b is required for proper expression of atoh1a and atoh1b and subsequent formation of mechanosensory hair cells (Millimaki et al., 2007). However, clonal analyses have indicated only a limited relationship between neurons and hair cells with regional differences within the developing inner ear (Satoh and Fekete, 2005; Abello and Alsinas, 2007; Sapède et al., 2012). Currently, it is unknown whether lineage commitment followed by proneural activation of Neurog1 and Atoh1 occurs in separate neuronal and sensory domains, or in a common neurogenic domain.

Here, we demonstrate that the competence to enter a neuronal or sensory fate is provided sequentially by Foxi1 and Dlx3b/4b very early during inner ear development. Initially, Foxi1, a known otic competence factor, induces the OEPD and provides competence to enter a neuronal fate. Consequently, inactivation of Foxi1 does not only result in compromised otic vesicle formation, but in a loss of all neuronal OEPD derivatives, including the statoacoustic, anterior lateral line and epibranchial ganglia, which has been reported previously (Lee et al., 2003). Subsequently, Dlx3b/4b, which is able to provide otic fate in the absence of Foxi1, restricts neuronal and promotes sensory fate.

**RESULTS**

**Early pax2a-positive OEPD cells give rise to delaminated neuroblasts and the anterior-ventral part of the otic vesicle**

Previous fate-mapping experiments using Kaede and caged fluorescein-dextran have shown that the Pax2-positive OEPD contributes to the otic vesicle, the epibranchial and presumably the anterior lateral line ganglia (McCarroll et al., 2012). Similarly, genetic lineage tracing in mice using the Cre/loxP recombination system revealed that OEPD Pax2-expressing cells give rise to the otic placode, as well as to epidermis (Ohyama and Groves, 2004). Recently, we showed that Cre/loxP is also highly efficient in zebrafish (Hans et al., 2009; Hans et al., 2011; Kroehne et al., 2011). In common genetic lineage-tracing analyses, reporter expression is usually driven by a constitutive promoter and directly linked to Cre-mediated recombination. Consequently, all Cre-positive cells activate the reporter and indicate the cell fates of the entire Cre domain at later stages (Fig. 1A,B). By contrast, we devised a novel Cre-mediated lineage-tracing method called pioneer tracking (PioTrack) that allows fate mapping specifically of the first Cre-expressing cells of a nascent Cre domain, if the domain subsequently expands by de novo Cre expression in neighboring cells. PioTrack employs a conditional promoter, which can disconnect Cre-mediated recombination from reporter activation. We used the zebrafish temperature-inducible hsp701 promoter, which is expressed strongly and ubiquitously only during heat treatment (Halloran et al., 2000). Application of heat during the early stages of a nascent Cre domain results in reporter expression in only those cells that have undergone recombination, and persistence of the reporter protein reveals the fates of these cells at later stages (Fig. 1C). Application of heat at later stages, after Cre is expressed throughout the entire domain, activates reporter expression and indicates cell fates of the entire Cre domain in a manner similar to common genetic lineage tracing (Fig. 1D). To use PioTrack in earlyotic development, we generated the transgenic line Tg(pax2a:CreER<sup>T2</sup>)<sup>β31</sup> driving CreER<sup>T2</sup> in the OEPD, using a pax2a promoter fragment that faithfully recapitulates OEPD expression and has been used in previous studies (Picker et al., 2002; Hans et al., 2009; McCarroll et al., 2012). To confirm the utility of Tg(pax2a:CreER<sup>T2</sup>)<sup>β31</sup>, we performed in situ hybridizations with pax2a and CreER<sup>T2</sup> probes (supplementary material Fig. S1). Compared with pax2a, CreER<sup>T2</sup> is absent in the midbrain-hindbrain boundary and ectopic expression is found in rhombomeres 3 and 5 during early segmentation stages as previously reported (Picker et al., 2002; Hans et al., 2009). By contrast, CreER<sup>T2</sup> expression in the OEPD closely recapitulates the endogenous pax2a expression in a temporal and spatial manner (supplementary material Fig. S1A-D,F-I) as confirmed by double color in situ hybridization (Fig. 1E-G'). Only after otic placode formation does pax2a and CreER<sup>T2</sup> expression diverge when CreER<sup>T2</sup> is not downregulated in Tg(pax2a:CreER<sup>T2</sup>)<sup>β31</sup> anteriorly to the otic placode (supplementary material Fig. S1E,J). We crossed Tg(pax2a:CreER<sup>T2</sup>)<sup>β31</sup> with the conditional red-to-green reporter Tg(hsp701:loxP-DsRed-loxP-EGFP) (Kroehne et al., 2011), applied tamoxifen to elicit immediate Cre-mediated recombination as soon
as CreER<sup>2</sup> is expressed, divided the embryos into different groups and provided heat treatments at various developmental stages (Fig. 1H). Using this strategy, we find that enhanced green fluorescent protein (EGFP)-labeled cells end up as delaminated neuroblasts and in an anterior-ventral position within the otic vesicle after heat treatments at early OEPD stages, including the 3- and the 6-somite stages (Fig. 1I,J). Heat treatments at late OEPD stages, including the 9-somite stage, enlarge the domain, but no labeled cells populate the posterior-dorsal region (Fig. 1K). By contrast, EGFP-labeled cells are present throughout the otic vesicle after heat treatment at placodal or vesicle stages (Fig. 1L; data not shown). Taken together, our results show that early OEPD pax2a-positive cells give rise predominantly to the anterior-ventral part of the otic vesicle, including the neurogenic region, but do not contribute randomly to the entire otic vesicle.

**Fig. 1. Pioneer tracking reveals that early OEPD pax2a-positive cells predominantly contribute to the anterior-ventral part of the otic vesicle, including the neurogenic region.** (A) Removal of a loxP-flanked transcriptional STOP cassette (red) in the presence of Cre (purple) activates reporter expression (yellow). (B) In a common genetic lineage-tracing setup, Cre expression and reporter activation are directly linked by the constitutive promoter driving reporter expression. Consequently, the reporter is active in all Cre-positive cells even at later stages when Cre expression has vanished. (C) By contrast, use of the conditional temperature-inducible hsp70l promoter uncouples Cre expression and reporter activation. Early activation (red thunderbolt) restricts reporter expression to cells that experience recombination within a nascent Cre domain and allows fate mapping of these cells due to reporter protein persistence at later stages. (D) Late activation (red thunderbolt) of the conditional promoter reveals the fate of the entire Cre domain at later stages similar to a constitutive promoter. (E-G) CreER<sup>2</sup> (blue) recapitulates the dynamic, endogenous pax2a expression (red) during OEPD stages (3-, 4-, 6-somite) in transgenic Tg(pax2a:CreER<sup>2</sup>)<sup>21</sup> embryos shown by two-color in situ hybridization. (E’-G’) High magnification views of the OEPD region of embryos shown in E-G, respectively. (H) Schematic of the experimental outline. The progeny of the indicated cross were exposed to tamoxifen (TAM) to elicit immediate Cre-mediated recombination as soon as CreER<sup>2</sup> is active. Subsequently, the offspring were divided into different groups, exposed to a single heat treatment at various developmental stages (3, 6, 9, 14-somites (so)) and analyzed at 24 hpf. (I,J) EGFP-labeled cells are found in an anterior-ventral position within the otic vesicle after heat shock at the 3- or the 6-somite stage in Tg(pax2a:CreER<sup>2</sup>)<sup>21</sup>; Tg(hsp70l:loxP-DsRed-loxP-EGFP) double transgenic embryos. (K) Heat shock at the 9-somite stage expands the EGFP-positive domain without labeling posterior-dorsal positions within the otic vesicle. (L) EGFP-labeled cells can be detected throughout the otic vesicle after heat shock at placodal stages. E-G are dorsal views with anterior to the top at 3-, 4-, 5- and 12-somite stages. I-L are lateral views with anterior to the left at 24 hpf. Scale bars: in L, 90 μm for E-G; in L, 30 μm for E’-G’; in L, 40 μm for I-L.

**Independent regulation of sensory and neuronal lineages by Dlx3b/4b and Foxi1**

The finding that early pax2a-positive OEPD cells are spatially restricted within the developing inner ear suggests that neuronal fate might already be specified within the OEPD very early on. In zebrafish, the transcription factors Foxi1 and Dlx3b/4b play pivotal roles during otic induction, and Dlx3b/4b is essential for sensory lineage development (Nissen et al., 2003; Solomon et al., 2003;
Millimaki et al., 2007). To test whether Dlx3b/4b is also required for the neuronal lineage during otic development, we examined neurogenic differentiation (neurod) and cadherin 6 (cdh6) expression (Andermann et al., 2002; Liu et al., 2006). In control embryos at 24 hpf, neurod is expressed in the anterior-ventral region of the otic vesicle and in delaminated neuroblasts, whereas cdh6 is expressed in delaminated neuroblasts only (Fig. 2A,E). At this stage, both neurod and cdh6 also label progenitors of the anterior lateral line ganglia (gALL) that are closely associated with the delaminated neuroblasts of the otic vesicle (Andermann et al., 2002; Liu et al., 2006). In the absence of Dlx3b/4b, neurod and cdh6 expression are slightly increased or unaffected (Fig. 2D,H; Table 1), rather than being reduced, despite smaller otic vesicles as indicated by starmaker (stm) expression, a marker of the entire epithelium of the otic vesicle (Söllner et al., 2003). By contrast, removal of Foxi1 activity (Fig. 2C,G), which also results in smaller otic vesicles, severely reduces or even abolishes expression of both neurod and cdh6 in a manner similar to the removal of Neurog1 (Fig. 2B,F), which is required for the development of all zebrafish cranial ganglia, including the statoacoustic and anterior lateral line ganglia (Andermann et al., 2002). At vesicle stages, homeo box (H6 family) 3 (hmx3) is also expressed in delaminated neuroblasts and in an anterior-ventral position of the otic vesicle; however, in contrast to neurod, hmx3 is already initiated in the anterior portion of the developing otic placode by the 12-somite stage (15 hpf), prior to expression of Neurog1 (Adamska et al., 2000). Compared with control embryos, loss of Neurog1 or Dlx3b/4b activity does not affect placodal hmx3 expression, whereas loss of Foxi1 completely abolishes hmx3 at placodal stages (Fig. 2I-L). We also readdressed the functions of Foxi1 and Dlx3b/4b in sensory lineage development using myosin VIIAa (myo7aa), a marker of sensory hair cells (Ernest et al., 2000), and atonal homolog 1b (atoh1b), a proneural gene required for hair cell formation (Adolf et al., 2004; Millimaki et al., 2007). In 24 hpf control embryos, myo7aa is expressed in discrete anterior and posterior domains of the otic vesicle corresponding to the prospective utricular and saccular maculae, which are indicated at the 12-somite stage by atoh1b expression (Fig. 2M,Q). Both myo7aa and atoh1b expression are present in the absence of Neurog1 or Foxi1, which, however, frequently results in the formation of only one sensory patch (Fig. 2N,O,R,S). By contrast, and as previously reported (Millimaki et al., 2007), removal of Dlx3b/4b activity results in the complete loss of myo7aa and atoh1b (Fig. 2P,T). We also examined T-box 1 (tbx1), which is expressed in the non-neurogenic otic territory during placodal and vesicle stages (Piotrowski et al., 2003; Radosevic et al., 2011). We found that Dlx3b/4b-depleted embryos at the 12-somite stage also show a complete absence of tbx1 compared with control embryos or embryos lacking Neurog1 or Foxi1 activity (Fig. 2U-X).

The observation that absence of Foxi1 frequently results in smaller otic placodes or vesicles containing only one sensory patch suggested that Foxi1 might regulate hair cell formation, either directly or indirectly, and we thus analyzed sensory lineage development in foxi1 mutants in more detail. In control siblings, a broad and robust atoh1b expression can be detected during early OEPD stages, and over time this single domain resolves into two smaller patches (Millimaki et al., 2007), which give rise to the prospective utricular and saccular maculae (supplementary material Fig. S2A,D,G). By contrast, all foxi1 mutants show reduced atoh1b expression at OEPD stages although the degree of reduction is highly variable (supplementary material Fig. S2B,C). Subsequently, a strong correlation of otic placode or vesicle size with sensory patch number can be observed with small or medium-sized placodes or vesicles displaying one or two sensory patches, respectively (supplementary material Fig. S2E,F,H,I). Thus, the neuronal lineage of the inner ear is regulated by Foxi1. By contrast, the sensory lineage is regulated by Dlx3b/4b and only indirectly by Foxi1;
frequently, loss of Foxi1 reduces otic induction resulting in smaller atoh1b domains that do not separate into two domains during subsequent otic development.

**Persistent OEPD-dependent neurogenesis in Dlx3b/4b- and Sox9a-deficient b380 mutants**

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in a discrete domain as in control siblings, but rather in a dispersed band of cells in b380 mutants (Fig. 4I,J). To demonstrate that the underlying pathways leading to the expression of neurod, cdh6 and hmx3 are equivalent in control siblings and b380 mutant embryos, we compromised the function of Neurog1 or Foxi1. We find that, similar to control embryos, loss of Neurog1 activity in b380 mutant embryos completely abolishes neurod and cdh6 but not hmx3 expression at 24 hpf or 12-somite stages, respectively (Fig. 4C,G,K). Consistently, depletion of Foxi1 in b380 mutant embryos results in the absence of neurod, cdh6 and hmx3 in the same manner as depletion of Foxi1 in control embryos at 24 hpf or the 12-somite stage (Fig. 4D,H,L). These results show that, in the absence of Dlx3b/4b and Sox9a, OEPD-dependent neurogenesis occurs and that the genetic pathways underlying OEPD-dependent neuronal development in b380 mutants are identical to those in control embryos.

**All neuronal OEPD derivatives are formed in Dlx3b/4b- and Sox9a-deficient b380 mutants**

Because previous studies have shown that the OEPD contributes to the statoacoustic, epibranchial and presumably anterior lateral line ganglia (McCarroll et al., 2012), we analyzed the identity of the neuronal progenitors in b380 mutants. At 24 hpf, the proneural gene neurogenin1 (neurog1) is expressed in a distinct pattern delineating the neurogenic placodes with strong expression in the progenitors of the anterior lateral line ganglion and in a subset of cells within the otic vesicle that will delaminate to form the statoacoustic ganglion (Andermann et al., 2002). In comparison with control or Dlx3b/4b-depleted embryos, neurog1 is expressed in the progenitors of the anterior lateral line ganglion in b380 mutants and a second strong expression domain is present despite the absence of the otic vesicle (Fig. 5A-C). To distinguish anterior lateral line and statoacoustic ganglion progenitors, we examined T-cell leukemia, homeobox 3b (tlx3b) and cadherin10 (cdh10) expression (Langenau et al., 2002; Liu et al., 2006). Whereas tlx3b is exclusively expressed in trigeminal and anterior lateral line ganglion progenitors, as well as in the developing hindbrain (Langenau et al., 2002), cdh10 is initially expressed in anterior lateral line ganglion progenitors, but is restricted to statoacoustic ganglion progenitors at 24 hpf (Liu et al., 2006). In b380 mutants, expression of cdh10 is reduced in comparison with control or Dlx3b/4b-depleted embryos at 24 hpf, whereas tlx3b appears increased in the absence of Dlx3b/4b as well.
as in b380 mutants compared with control embryos at 24 hpf (Fig. 5D-I). To identify epibranchial ganglion progenitors, we examined the expression of paired-like homeobox 2a (phox2a) and paired-like homeobox 2bb (phox2bb) (Guo et al., 1999). At 24 hpf, expression of phox2a is initiated in the progenitors of the geniculate ganglion (gVII) in Dlx3b/4b-depleted embryos, b380 mutants and control siblings (Fig. 5J-L). In comparison with control siblings at 48 hpf, phox2bb expression revealed that the petrosal (gIX) and nodose ganglion (gX) progenitors are also generated in a similar manner in the absence of Dlx3b/4b activity or in b380 mutants (Fig. 5M-O). Taken together, our results show that all neuronal derivatives of the OEPD, the statoacoustic, anterior lateral line and epibranchial ganglia progenitors, are generated in the absence of Dlx3b/4b or in Dlx3b/4b- and Sox9a-deficient b380 mutants.

**DISCUSSION**

Our results provide important insights into the acquisition of neuronal competence during inner ear development. In zebrafish, fate mapping of early OEPD cells using live imaging of a fluorescent reporter is challenging because endogenous pax2a expression is highly dynamic. The new conditional Cre recombination procedure we used here, PioTrack, importantly allows labeling of the first Cre-expressing cells of a nascent Cre domain owing to a conditional reporter that disconnects Cre-mediated recombination and reporter activation. The zebrafish temperature-inducible hsp70I promoter is inactive at normal temperatures and is only strongly and ubiquitously expressed during heat treatment (Halloran et al., 2000). Consequently, cells that have undergone recombination show reporter expression after heat treatment, and subsequent reporter persistence reveals the fate of these cells at later stages. By contrast, neighboring cells that initiate Cre expression after heat treatment undergo Cre-mediated recombination, but do not show reporter activity unless a second heat treatment is administered (Fig. 1). Furthermore, Cre-mediated recombination is a stochastic event, meaning that recombination does not happen in all Cre-expressing cells at the same time, and the probability of a recombination event increases as the concentration of Cre protein builds up over time (Nagy, 2000). Hence, our observation that EGFP-labeled cells are present throughout the otic vesicle only after heat treatment at placodal or later stages is due to delayed recombination and does not indicate that dorsal and posterior otic cells are added after otic placode formation. Using Kaede and caged fluo1orscein-dextran to label small groups of cells of the pax2a-positive OEPD showed that cells contributing to the otic vesicle are distributed throughout the pax2a-positive OEPD with some bias of anterolateral cells labeling the statoacoustic/anterior lateral line ganglia (McCarroll et al., 2012), which partly conflicts with our data. However, in our analysis, onset of pax2a expression regulated by Pax8 (Hans et al., 2004; Mackereth et al., 2005) correlates with the spatial distribution of labeled cells within the otic vesicle. Given that heterogeneous levels of Pax2a and Pax8 are found in the OEPD and high Pax2a and Pax8 levels correlate with otic fate (McCarroll et al., 2012), we assume that, in the Kaede and caged fluorescence-dextran approach, neighboring cells with differential Pax2a and Pax8 levels have been labeled that subsequently contribute to the entire otic vesicle. In this respect, our results are also consistent with the previous finding that high Pax2a and Pax8 levels correlate with otic fate (McCarroll et al., 2012), because early OEPD pax2a-positive cells always contribute to the anterior lateral line ganglia progenitors or otic lineage but never enter an epibranchial fate.

Previous studies in chick have shown that neural specification takes place only in the anterior region of the placode (Adam et al., 1998; Abel6 et al., 2010), and our data indicate that the crucial determinative event occurs earlier, concurrent with OEPD induction. Previsously the Foxi1-Pax8 pathway has been suggested to act as an early ‘jumpstart’ mediating the initial Fgf-dependent otic induction that occurs over a much shorter time period in zebrafish than in mice (Hans et al., 2004). We now show that in addition to otic induction, Foxi1 also provides competence to embark on a neuronal fate. Loss of Foxi1 results in a loss of pax8 and in a patchy and variable onset of pax2a expression in the OEPD, which might explain why foxi1 mutants develop small otic vesicles with no or just one otolith, whereas others have two small lumina each containing a single otolith (Nissen et al., 2003; Solomon et al., 2003). In contrast to this variable phenotype, compromised neurogenesis was robustly observed in all foxi1 mutants examined (Table 1). Size reduction of the otic vesicle cannot alone explain this finding, because neurogenesis is not impaired in the absence of Dlx3b/4b activity, which causes a similar otic vesicle size reduction. Furthermore, other OEPD-derived neuronal derivatives, i.e. the progenitors of the anterior lateral line ganglion, are also absent in foxi1 mutants. Taken together with previous findings showing that Foxi1 is required to specify OEPD-derived epibranchial progenitors (Lee et al., 2003), we propose that Foxi1 converts naive cells of the preplacodal region into the OEPD and provides neuronal competence for all OEPD-derived neuronal progenitors (Fig. 6). Expression analysis showed that at the end of gastrulation foxi1 and dlx3b, a marker of the preplacodal region, share the same medial border, but foxi1 extends further laterally (Solomon et al., 2003), supporting results that cells
outside of the preplacodal region also contribute to epibranchial ganglia (Padanad and Riley, 2011; McCarroll et al., 2012). Subsequently, Dlx3b/4b, which is able to initiate otic induction in the absence of Foxi1, represses neuronal and imposes sensory fate by the activation of atoh1b. Consistently, it has been shown that neuronal differentiation is repressed after overexpression of Dlx3 (Pieper et al., 2012). However, although sensory hair cell formation is completely abolished and neuronal fate is increased in Dlx3b/4b-depleted embryos, nonsensory epithelial cells are still present and give rise to a size-reduced otic vesicle. This indicates that additional factors are required to repress Foxi1-mediated neuronal competence, and Sox9 is a likely candidate. Zebrafish sox9a and sox9b genes are duplicate orthologs of the human SOX9 gene and both genes are expressed in the otic placode (Chiang et al., 2001). In b380 mutants, sox9a is deleted and additional removal of Sox9b activity completely blocks all signs of otic specification and formation of the residual epithelial ball (Liu et al., 2003). It is unknown which OEPD-derived neuronal fate is still present in Sox9b-depleted b380 mutants. We now show that all neuronal progenitors of the OEPD, the statoacoustic, anterior lateral line and epibranchial ganglia, are established in b380 mutants. However, the number of statoacoustic ganglion progenitors is reduced whereas anterior lateral line progenitors are increased, indicating that in the absence of properly formed otic placode/vesicle, OEPD-derived cells embark on an anterior lateral line progenitor fate. Nevertheless, neurod expression within the otic vesicle, which is confined to an anterior-ventral position in wild-type embryos, is present throughout the remaining epithelial ball in b380 mutants, showing that all otic cells adopt a neuronal fate in this genotype. Cell death as well as proliferation might also play an important role for proper neuronal progenitor formation, because Pax2 has been shown to play an important role in proliferation in the OEPD (Freter et al., 2012).

We also conclude that Foxi1 has no direct role in sensory hair cell formation, although absence of Foxi1 frequently results in smaller otic vesicles containing only one sensory patch. foxi1 mutants display a highly variable otic phenotype that is smaller otic vesicles containing only one sensory patch. We also conclude that Foxi1 has no direct role in sensory hair cell formation, although absence of Foxi1 frequently results in smaller otic vesicles containing only one sensory patch. foxi1 mutants display a highly variable otic phenotype that is smaller otic vesicles containing only one sensory patch.

It will be interesting to learn whether there is a similar developmental pattern in mammals. Mouse Foxi1 is expressed and required at a much later stage of inner ear development (Hulander et al., 2003), but expression of two other mouse Foxi family members, Foxi2 and Foxi3, coincide spatiotemporally with the period of otic induction. In particular, Foxi3 is expressed in a broad ectodermal region before and during otic induction and is subsequently downregulated in the developing placode similar to Foxi1 downregulation in zebrafish (Ohyama and Groves, 2004a). Thus, analysis of Foxi3 mutant mice will be necessary to learn whether this developmental pattern extends to mammals. Furthermore, how neuronal competence downstream of Foxi1 is executed remains elusive. The SoxB1 transcription factor Sox3 is expressed early in the OEPD region of chick and zebrafish and regulates the formation of the sensory-neural domain of the otic vesicle (Sun et al., 2007; Dee et al., 2008; Abelló et al., 2010). Furthermore, in zebrafish, Foxi1, but not Dlx3b/4b, is required for proper sox3 expression, which is also unchanged in b380 mutant embryos (Sun et al., 2007). Although a recent study reports no perceptible deficiency in neuronal precursor formation in the absence of Sox3 (Padanad and Riley, 2011), the finding that SoxB1 proteins control neural differentiation by directly regulating Neurog1 expression makes Sox3 a promising candidate (Okuda et al., 2010).

**Fig. 6. Model of the early events providing competence to adopt a neuronal or sensory fate during inner ear development**. After formation of the preplacodal region (PPR), Foxi1 initiates OEPD induction in the posterior PPR and further laterally provides competence to embark on a neuronal fate (blue). Subsequently, Dlx3b/4b, which is able to initiate otic induction in the absence of Foxi1, represses neuronal and imposes a sensory fate (red).


Early inner ear development is revealed through analysis of the zebrafish pax2.1 promoter in transgenic lines. Development 129, 3227-3239.


Fig. S1. Expression of CreERT2 recapitulates the endogenous pax2a expression during OEPD development. (A–J) The temporal and spatial expression of CreERT2 (F–I) within the OEPD is identical to pax2a (A–D) in Tg(pax2a:CreERT2)^a2i transgenic embryos. Subsequently, pax2a (E) is maintained exclusively in the otic placode (bracket) but is downregulated in non-incorporated cells anteriorly to it (arrow), whereas CreERT2 (J) is sustained in both regions. Dorsal views with anterior to the top at the 1-, 3-, 5-, 8- and 12-somite (so) stages, as indicated. Scale bar: 100 μm.

Fig. S2. Hair cell formation is highly variably in foxi1 mutants, which is foreshadowed by atoh1b expression at OEPD stages. (A–I) Blue: Expression of atoh1b (A–F) and myo7aa (G–I) in control (A,D,G) and foxi1 mutant embryos (B,C,E,F,H,I). Red: Expression of pax2a and stm reveal size of the otic placode and vesicle, respectively. A–C are dorsal views with anterior to the top at the 3-somite stage. D–F are dorsolateral views with anterior to the left at the 12-somite stage. G–I are lateral views with anterior to the left at 24 hpf. Arrowheads indicate the position of the sensory patches. Scale bar: 35 μm.
Fig. S3. Expression of *DsRed* in a gene trap in which the coding sequence of *DsRed* is inserted into the *pax8* locus, recapitulates the endogenous *pax8* expression during otic development. (A-H) Blue: Expression of *DsRed* (A-D) and *pax8* (E-H) in *pax8nia03Gt* transgenic embryos. Red: Expression of *pax2a*. Dorsal views with anterior to the top at the 3-, 5-, 12-somite (so) stage and 24 hpf, as indicated. Scale bar: 100 µm.