Zebrafish *atoh1* genes: classic proneural activity in the inner ear and regulation by Fgf and Notch

Bonny B. Millimaki*, Elly M. Sweet*, Mary S. Dhason and Bruce B. Riley†

Hair cells of the inner ear develop from an equivalence group marked by expression of the proneural gene *Atoh1*. In mouse, *Atoh1* is necessary for hair cell differentiation, but its role in specifying the equivalence group (proneural function) has been questioned and little is known about its upstream activators. We have addressed these issues in zebrafish. Two zebrafish homologs, *atoh1a* and *atoh1b*, are together necessary for hair cell development. These genes crossregulate each other but are differentially required during distinct developmental periods, first in the preotic placode and later in the otic vesicle. Interactions with the Notch pathway confirm that *atoh1* genes have early proneural function. Fgf3 and Fgf8 are upstream activators of *atoh1* genes during both phases, and *foxi1*, *pax8* and *dlx* genes regulate *atoh1b* in the preplacode. A model is presented in which zebrafish *atoh1* genes operate in a complex network leading to hair cell development.

KEY WORDS: Hair cells, Proneural genes, Fgf, Delta-Notch, Pax2-5-8, Foxi1, Dlx, Msx, *no isthmus* (*pax2a*), *mind bomb*

INTRODUCTION

Sensory epithelia of the vertebrate inner ear consist of two cell types, hair cells and support cells. Both are produced from a prosensory equivalence group initially marked by expression of *Atoh1*, a homolog of the *Drosophila* proneural gene *atonal* (*ato*) (Bermingham et al., 1999). As the equivalence group develops, a few cells upregulate *Atoh1* expression and complete differentiation as hair cells. The rest lose expression of *Atoh1* and become support cells. As the principal regulator of hair cell differentiation, *Atoh1* has received great attention in recent years in both basic and applied research (Shailam et al., 1999; Lanford et al., 2000; Zheng and Gao, 2000; Itoh and Chitnis, 2001; Chen et al., 2002; Wang et al., 2002; Woods et al., 2004; Fritzsch et al., 2005; Izumikawa et al., 2005; Kelley, 2006). However, despite extensive analysis of *Atoh1*, a number of fundamental issues still need to be resolved. Most notably, there are conflicting reports as to the precise role(s) of *Atoh1* in otic development. Although *Atoh1* is maintained only in hair cells, it may function earlier to specify the equivalence group itself – a definitive proneural function. Accordingly, disruption of mouse *Atoh1* (*Math1*) ablates all hair cells and support cells in the cochlea (Woods et al., 2004). However, the persistence of cells expressing some early markers of sensory epithelia has been interpreted to mean that mouse *Atoh1* is not required for specifying the equivalence group per se, but instead only promotes the final stages of hair cell development (Bermingham et al., 1999; Chen et al., 2002; Fritzsch et al., 2005). Additionally, a key aspect of prosensory development does not require *Atoh1*: prospective sensory cells begin to express $p27^{kip1}$ and exit the cell cycle before expression of *Atoh1*, and this process still occurs in *Atoh1* mutants. However, $p27^{kip1}$ expression and cell cycle withdrawal could be regulated independently from equivalence group specification. Indeed, sensory epithelia still form in $p27^{kip1}$ mutants, despite the failure of cells to properly exit the cell cycle (Chen and Segil, 1999). This leaves open the question of when the equivalence group forms and whether *Atoh1* acts early or late in the process.

Work on *Drosophila ato* provides a useful paradigm for testing vertebrate *Atoh1* function (Fig. 1). *ato* is initially expressed in a broad pattern (the equivalence group) well before cell fate specification (Jarman et al., 1995). The equivalence group then restricts its own size through activation of Delta-Notch (Dl-N) signaling (Baker et al., 1996; Baker and Yu, 1997). In this process, N-dependent downregulation of *ato* breaks the equivalence group into discrete 'intermediate groups' of *ato*-expressing cells separated by non-expressing cells that are excluded from the sensory structure. Subsequently, the balance of ato and N activity selects between alternate fates within intermediate groups (lateral inhibition). Because *ato* is required for *Dl* expression, *ato* mutants fail to activate N-mediated restriction of *ato*, resulting in retention of a broad field of *ato*-expressing cells that are otherwise blocked from further development (Jarman et al., 1995; Baker and Yu, 1997). Similarly, *N* mutants also fail to restrict *ato* expression, but in this case all cells differentiate as sensory cells (Baker et al., 1996). Paradoxically, during the prosensory phase of development elevating N activity by expressing N intracellular domain (NICD) enhances *ato* expression (Baker and Yu, 1997). This involves a poorly characterized branch of the N pathway not requiring Su(H) (Ligoxygakis et al., 1998). During subsequent phases of development, NICD activates the canonical N pathway and abolishes *ato* expression. This work provides clear predictions for how vertebrate *Atoh1* might function, assuming it acts as a classic proneural gene. By contrast, terminal differentiation factors such as NeuroD are insensitive to N activity and are not required for cell fate specification (Chitnis and Kintner, 1996) (reviewed by Brunet and Ghysen, 1999; Hassan and Bellen, 2000).

A crucial determinant of proneural gene function is the regulatory context in which it operates (Niwa et al., 2004). Activation of *ato* requires combinatorial signaling and specific regional identity genes such as *eyeless* (*Pax6*), which also modify the sensory fate specified by *ato* (Niwa et al., 2004). The factors that induce *Atoh1* in the ear and cooperate in its function are largely unknown. *Sox2* is expressed broadly in the early otic vesicle in mouse and is required for induction of *Atoh1* several days later (Kiernan et al., 2005b). The lag in *Atoh1* expression suggests that Sox2 works combinatorially with other factors to initiate prosensory development. A number of signaling molecules have also been implicated in sensory epithelium development (Pirvola et al., 2002; Stevens et al., 2003; Daudet and

Biology Department, Texas A&M University, College Station, TX 77843-3258, USA.

*These authors contributed equally to this work
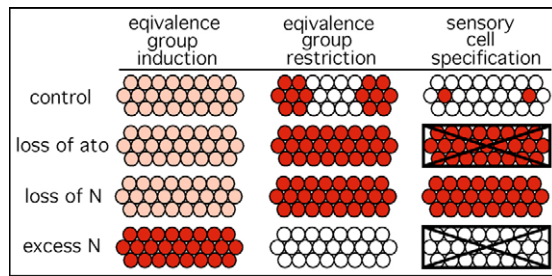†Author for correspondence (e-mail: briley@mail.bio.tamu.edu)

Fig. 1. *Drosophila ato* as a paradigm for proneural regulation and function. Red circles represent cells expressing *ato* at high level; pink circles represent cells expressing *ato* at low level. Stage-dependent refinement of the expression pattern is altered in distinctive ways by perturbing *ato* or *N* function. In addition, loss of *ato* or excess *N* blocks specification of sensory cells (crossed-out fields of cells).

Lewis, 2005; Brooker et al., 2006; Kiernan et al., 2006; Pujades et al., 2006), but their relationships to *Atoh1* expression remain unknown. Identifying the upstream activators of *Atoh1* is essential for understanding the regulatory network leading to formation and maintenance of hair cells.

Here we investigate the role of zebrafish *atoh1* genes, *atoh1a* and *atoh1b*, in hair cell development. Gene knockdown shows these genes play essential roles during successive stages of hair cell development, beginning in the preotic placode. Interactions with the Delta-Notch pathway strongly support a classic proneural role for *atoh1*. We also show that Fgf and members of the Pax2-5-8 family of transcription factors are required for induction or maintenance of *atoh1* expression. These data reveal a complex gene network in which *atoh1* genes play vital roles at multiple stages of sensory epithelium development.

MATERIALS AND METHODS
Strains and developmental conditions
The wild-type strain was derived from the AB line (Eugene, OR). The *mib*[ta52b] and *noi*[tu29a] mutations are probably null alleles (Lun and Brand, 1998; Itoh et al., 2003). *b380* is a deletion of *dlx3b* and *dlx4b* and mutants are easily identified after 11 hours post-fertilization (hpf) by lack of somitic segmentation (Fritz et al., 1996). The *hsp70-dnSu(H)* line was developed by Latimer et al. (Latimer et al., 2005), and the *hsp70-Gal4* and *UAS-NICD* lines were developed by Scheer and Campos-Ortega (Scheer and Campos-Ortega, 1999). About 25% of embryos were affected by dnSu(H) and NICD in these lines, respectively. Embryos were developed in fish water containing methylene blue at 28.5°C and staged according to standard protocols (Kimmel et al., 1995). At least 30 embryos were observed for each time point, except where noted.

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

Immunofluorescence
Antibody staining was performed as described by Riley et al. (Riley et al., 1999). Primary antibodies: Pax2 (Covance, diluted 1:100), acetylated tubulin (Sigma T-6793, diluted 1:100). Secondary antibodies: Alexa 546-conjugated goat anti-rabbit IgG (Molecular Probes A-11010, diluted 1:50) or Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes A-11001, diluted 1:50).

Misexpression
The *atoh1a* plasmid was obtained from Reinhard Köster. To misexpress *atoh1a* under the control of the cytomegalovirus promoter, 30-90 pg plasmid was injected into one-cell embryos. For RNA misexpression, wild-type mRNA was synthesized in vitro using mMessage mMachine kit (Ambion). A total of 60-80 pg mRNA was injected into one-cell embryos, or was co-injected with *atoh1a*;*atoh1b* double MO.

Morpholinos
Morpholino oligomers (MOs) were obtained from Gene Tools, Inc. For most experiments, 5 ng morpholino was injected into one-cell embryos. MOs for *dlx3b*, *dlx4b*, *fgf3*, *foxi1*, *pax2b* and *pax8* were described previously (Solomon and Fritz, 2002; Mackereth et al., 2005). Additional MO sequences are as follows: *atoh1b* MO 5′-TCATTGCTTGTGTA-GAAATGCATAT-3′; *atoh1a* MO1 5′-TCTGTTGGTTTGTGCTTTTG-GGAGG-3′; *atoh1a* MO2 5′-AAAGTTTGTGGCTATGGATACAGGG-3′; *atoh1a* MO3 5′-ATCCATTCTGTTGGTTTGTGCTTTT-3′. *atoh1a* MO3 was used for most experiments. The phenotypes caused by injection of *atoh1a* and/or *atoh1b* MOs affected 90-100% of embryos, except where noted.

SU5402 inhibitor treatment
SU5402 was dissolved in DMSO to prepare a 40 mmol/l stock solution. Embryos were treated in their chorions with 50 μmol/l SU5402 (10-14 hpf), 80 μmol/l (12-18 hpf), or 100 μmol/l (18-24 hpf). Controls were incubated in an equal concentration of DMSO to that of treated embryos. To terminate treatment, embryos were washed several times and either allowed to develop further or fixed and processed immediately.

RESULTS
Requirement of *atoh1* genes for hair cell development
It was shown previously that zebrafish *atoh1a* (formerly *zath1*) is expressed in hair cells in the inner ear and lateral line (Itoh and Chitnis, 2001; Whitfield et al., 2002). We designed three different MOs to block translation of *atoh1a*, all of which affected hair cell development. While two of these MOs caused varying degrees of non-specific cell death in the neural tube, the third was effective at a dose that had no discernable toxicity and was therefore used for the remainder of this study. Injection of *atoh1a* MO strongly impairs formation of hair cells in the inner ear (Fig. 2U). Tether cells, an early-forming hair cell required for otolith localization (Riley et al., 1997), were not affected in *atoh1a* morphants, and otoliths formed normally (Fig. 2G). Tether cells, named for their precocious kinocilia, initially formed in pairs at both ends of the nascent otic vesicle and later adopted the morphology of fully developed hair cells by 22 hpf. Normally, later-forming hair cells begin to accumulate soon after 24 hpf. However, later-forming hair cells were profoundly impaired in all *atoh1a* morphants, as additional hair cells were not evident until 48 hpf (Fig. 2I,U, and data not shown).

Adolf et al. (Adolf et al., 2004) recently described a second zebrafish *atonal* homolog, *atoh1b*, that we hypothesized might also play a role in hair cell development. By contrast to *atoh1a* MO, injection of *atoh1b* MO ablated tether cells in both the utricle and saccule (Fig. 2B) in all specimens. Later-forming hair cells were still produced, albeit more slowly than normal (Fig. 2F,U). A single otolith was produced but initially formed as an untethered mass due to the absence of tether cells (Fig. 2D). Otoliths eventually bound to utricular hair cell cilia after 30 hpf (not shown).

Co-injection of *atoh1a* MO and *atoh1b* MO ablated all hair cells in the inner ear in >90% of specimens (Fig. 2J,U). This was confirmed using phalloidin to mark stereocilia and anti-actetylated tubulin staining of kinocilia (not shown). A single untethered otolith was produced (Fig. 2H), reflecting loss of tether cells. Hair cells did begin to form by 48 hpf in *atoh1a*;*atoh1b* double morphants (Fig. 2K,U), probably reflecting diminishing capacity of the MOs to knock down *atoh1* function at later stages. Thus, *atoh1* function is essential for hair cell formation in zebrafish, as in mouse. Moreover,
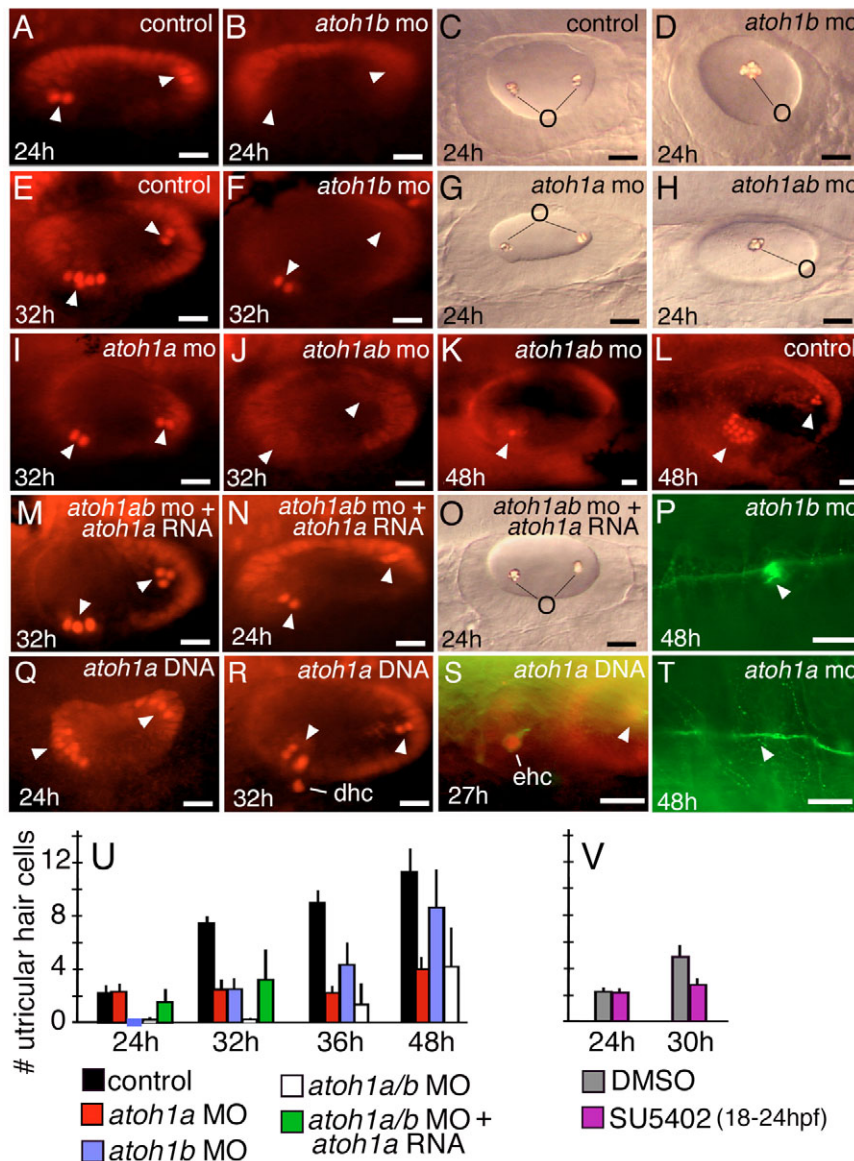
Fig. 2. Requirement for *atoh1* in hair cells in the ear and lateral line. All panels show dorsolateral views with anterior to the left and dorsal up. (**A,B,E,F,I-N,Q-S**) Pax2 antibody staining of otic hair cells (arrowheads) at the indicated times in control embryos (A,E,L), *atoh1a* morphant (I), *atoh1b* morphants (B,F), *atoh1a;atoh1b* double morphants (J,K), *atoh1a;atoh1b* double morphant co-injected with *atoh1a* mRNA (M,N) and embryos injected with *atoh1a* plasmid (Q-S). *atoh1a* plasmid stimulates production of supernumerary hair cells at 24 hpf (Q), but these are not maintained at 32 hpf (R), and instead displaced hair cells appear ventrally within subjacent mesenchyme, leaving gaps in the hair cell layer. An ectopic hair cell is revealed anterior to the otic vesicle by co-staining with Pax2a (red) and acetylated-tubulin (green) (S). (**C,D,G,H,O**) Otoliths produced in control (C), *atoh1a* morphant (G), *atoh1b* morphant (D) *atoh1a;atoh1b* double morphant (H) and *atoh1a;atoh1b* double morphant co-injected with *atoh1a* RNA (O). (**P,T**) Acetylated-tubulin staining of the lateral line and neuromasts (arrowheads) in *atoh1b* morphant (P) and *atoh1a* morphant (T) at 48 hpf. (**U,V**) The mean (± standard deviation) of Pax2-postive hair cells present in the utricle at the indicated times and under the indicated conditions. Sample sizes ranged from 15-35 embryos per time point. Scale bar: 15 μm. dhc, displaced hair cells; ehc, ectopic hair cell; o, otolith.

the data support a model in which *atoh1b* preferentially regulates development of tether cells, whereas *atoh1a* regulates later-forming hair cells.

Neuromasts of the lateral line were also ablated by knocking down *atoh1a* (Fig. 2T). However, knocking down *atoh1b* had no effect on neuromasts (Fig. 2P). These data are consistent with findings that neuromasts express and require *atoh1a* but not *atoh1b* (Itoh and Chitnis, 2001; Sarrazin et al., 2006) (B.B.R., unpublished).

Misexpression of *atoh1a*

To test whether the effects of *atoh1* MOs on hair cell development could be rescued, *atoh1a;atoh1b* double morphants were co-injected with 80 pg of *atoh1a* mRNA. More than half of these co-injected embryos produced tether cells, tethered otoliths and later-forming hair cells (Fig. 2M-O), indicating substantial rescue from the effects of the MOs. These data show that loss of hair cells in *atoh1* morphants is a specific consequence of disrupting *atoh1* function.

Injecting 80 pg of *atoh1a* mRNA (with or without MOs) did not lead to formation of excess or ectopic hair cells. This is in contrast to mouse, in which misexpression of *atoh1* promotes formation of ectopic hair cells in tissues immediately surrounding endogenous

sensory epithelia (Zheng and Gao, 2000; Woods et al., 2004; Izumikawa et al., 2005). Because injected mRNA may not be stable enough to strongly affect later stages of otic development, we injected zebrafish embryos with plasmid DNA to misexpress *atoh1a* under the control of the powerful and ubiquitously expressed cytomegalovirus promoter. Injection of 90 pg of *atoh1a* plasmid caused axial truncation in up to 30% of embryos, whereas injection of 30 or 60 pg did not alter overall embryonic morphology (not shown). Embryos injected with 60 or 90 pg of *atoh1a* plasmid often showed expanded sensory patches at 24 hpf (Fig. 2Q). By 30 hpf, however, many supernumerary hair cells were lost, whereas isolated Pax2-positive cells appeared sporadically in the subjacent mesenchyme (Fig. 2R). The latter are likely to be dying hair cells, as suggested by general elevation of Acridine Orange staining (not shown). We showed in another study that dying hair cells are often extruded from the otic vesicle to the underlying mesenchyme (Kwak et al., 2006). This also occurs in *mind bomb* (*mib*) mutants, which form supernumerary hair cells that are later extruded as they undergo apoptosis (Haddon et al., 1999). It is possible that excess hair cells die because forced expression of *atoh1a* bypasses vital processes required for hair cell maintenance. We also cannot exclude the

possibility of non-specific toxicity associated with concentrated plasmid injection. In addition to changes in the otic vesicle, about one-third of embryos injected with *atoh1a* plasmid also formed ectopic Pax2a-positive cells in the surface ectoderm just anterior or posterior to the otic vesicle. Double labeling with acetylated tubulin antibody confirmed that some of these cells were hair cells (Fig. 2S). Although ectopic hair cells formed at the level of the lateral line, *pax2a* expression indicated that these were not lateral line neuromasts. These data show that in zebrafish, as in mouse, *atoh1* misexpression can induce excess and ectopic hair cells, but only in regions close to the endogenous hair cell domains. This is consistent with findings that basic helix-loop-helix proteins work combinatorially with other transcription factors, such as Hox and Pax proteins, with regional expression that establishes restricted zones of competence (Niwa et al., 2004) (reviewed by Westerman et al., 2003).

Expression of *atoh1a* and *atoh1b* during normal development

Otic expression of *atoh1a* began at 14 hpf in two domains in the otic placode, marking the primordia of the utricular and saccular sensory epithelia (Fig. 3A). As hair cells began to differentiate, *atoh1a* expression upregulated in the hair cell layer, but weak expression was also detected in the basal cell layer. The latter may represent nascent hair cells in the earliest stages of differentiation (Fig. 3C). Expression continued in the sensory maculae through at least 48 hpf. Expression was also seen in the sensory cristae by 48 hpf (not shown).

Expression of *atoh1b* began much earlier, marking the medial edge of the preotic placode by 10.5 hpf (Fig. 7A,B). This pattern resolved into two discrete patches by 14 hpf, encompassing the future sensory epithelia (Fig. 3D). At this stage, expression of *atoh1b* overlapped with that of *atoh1a*, but *atoh1b* was expressed at a higher level (compare Fig. 3A,D). By 22 hpf, *atoh1b* expression diminished and marked only a subset of the *atoh1a*

domain (Fig. 3E,F). These differences in temporal expression are consistent with the notion that *atoh1b* acts early in otic development, whereas *atoh1a* predominates during later development of sensory epithelia.

Autoregulation and crossregulation of *atoh1* gene expression

Because proneural genes often regulate their own expression, we examined expression of *atoh1a* and *atoh1b* in embryos knocked down for either or both functions. In *atoh1b* morphants, preplacodal expression of *atoh1b* was not altered (not shown). However, *atoh1b* expression failed to become restricted to two sensory primordia in the otic placode at 14 hpf (compare Fig. 3D,P). Expression of *atoh1b* ceased by 16 hpf in *atoh1b* morphants (Fig. 3Q and data not shown), indicating that *atoh1b* is required to maintain its own transcription. Interestingly, macular expression of *atoh1b* returned after 24 hpf (Fig. 3R).

atoh1a was not expressed in *atoh1b* morphants until around 20 hpf and was limited to the utricular (anterior) macula (Fig. 3M,N). By 30 hpf, *atoh1b* morphants showed *atoh1a* expression in both utricular and saccular maculae, although the level of expression was lower than normal (Fig. 3O). These data show that *atoh1a* requires *atoh1b* for expression in the otic placode but not in the otic vesicle after 20 hpf. Once activated, *atoh1a* could be responsible for reactivation of *atoh1b* expression after 24 hpf (Fig. 3R).

In *atoh1a* morphants, *atoh1a* and *atoh1b* were expressed normally to 20 hpf (Fig. 3G,J, and data not shown). By 22 hpf, *atoh1a* morphants began to express *atoh1a* at higher than normal levels (Fig. 3H,I). Conversely, *atoh1b* expression was nearly extinguished by 22 hpf and could not be detected after 24 hpf (Fig. 3K,L). These data show that *atoh1a* is necessary to maintain *atoh1b* expression after 22 hpf and that *atoh1a* limits its own expression.

In *atoh1a;atoh1b* double morphants, *atoh1b* was expressed in an expanded domain at 14 hpf but was not maintained in the ear after 16 hpf (Fig. 3V-X and data not shown). Expression of *atoh1a* could
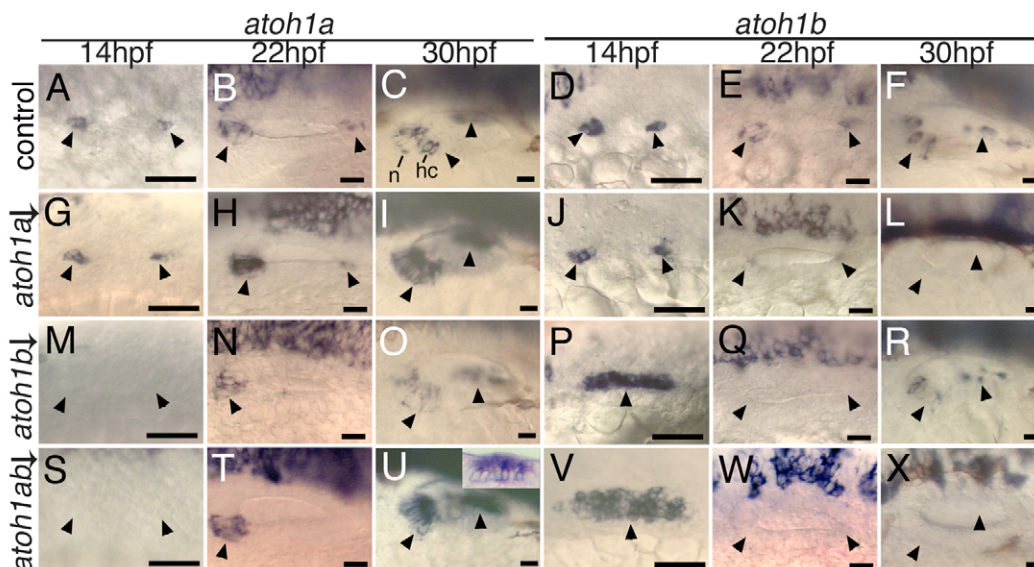

Fig. 3. Atoh1-dependent and -independent expression of *atoh1* genes. Dorsolateral views (anterior to left) showing expression of *atoh1a* (**A-C,G-I,M-O,S-U**) and *atoh1b* (**D-F,J-L,P-R,V-X**) in control (A-F) *atoh1a* morphant (G-L), *atoh1b* morphant (M-R) and *atoh1a;atoh1b* double morphant (S-X) embryos at the indicated times. Expression of *atoh1a* at 32 hpf in mature hair cells and putative nascent hair cells is indicated in C. Arrowheads indicate observed or expected domains of otic expression. Inset in U shows a parasagittal section through the anterior *atoh1a* expression domain. Scale bar: 15 μm. hc, mature hair cells; n, nascent hair cells.

not be detected until 22 hpf, after which it was expressed at higher than normal levels (Fig. 3S-U). Sections showed that the epithelium had only a single layer of columnar cells that expressed high levels of *atoh1a* (Fig. 3U, inset).

Taken together, these data show that *atoh1b* acts early to establish and refine the sensory equivalence group and to induce early expression of *atoh1a*, while *atoh1a* is required later to maintain expression of *atoh1b* and to limit its own expression. The requirement for *atoh1b* to restrict its own expression domain at such an early stage is consistent with the possibility that it acts as a classic proneural gene (Fig. 1). The data also confirm that *atoh1b* is required for differentiation of tether cells, whereas *atoh1a* is required for later-forming hair cells.

Involvement of *atoh1* genes in Delta-Notch signaling

Proneural genes often limit their own expression by transcriptional activation of Delta (Dl), which in turn stimulates Notch (N) and thereby inhibits subsequent proneural gene expression (Baker and Yu, 1997; Parks et al., 1997). In support of this, knocking down *atoh1b* strongly inhibited expression of *dlA* and *dlD* in the ear at 14 hpf (Fig. 4C,D, and data not shown). Similarly, knocking down *atoh1a* diminished *dlA* and *dlD* expression at 22 hpf (Fig. 4A,B, and data not shown). Thus, *atoh1* genes are required for normal activation of *delta* gene expression.

To further investigate the role of Dl-N feedback, we examined *atoh1* function in *mind bomb* (*mib*) mutants. The *mib* gene encodes an E3 ubiquitin ligase essential for Dl-N signaling (Itoh et al., 2003). *mib* mutants produced an enlarged domain of both *atoh1a* and *atoh1b* at 14 hpf, mimicking the failure to restrict expression seen in *atoh1b* morphants (Fig. 4G,H). Because both *atoh1* genes remain fully active in *mib* mutants, *delta* gene expression is also greatly expanded and all cells in the equivalence group complete differentiation as hair cells (Haddon et al., 1999; Riley et al., 1999) (Fig. 4E,I). However, injection of *atoh1a* MO and *atoh1b* MO into *mib* mutants fully suppressed these latter defects, blocking *delta* gene expression and ablating all hair cells in all specimens (Fig. 4F,J). These data further support a role for *atoh1* genes as upstream activators of Dl-N signaling, which normally acts to limit and refine *atoh1* expression and function.

To test the temporal requirements for the canonical N pathway, we used a transgenic line to express a dominant-negative form of Su(H) [dnSu(H)] under the control of *hsp70* promoter (Wettstein et al., 1997; Shoji et al., 1998; Latimer et al., 2005). This promoter induces high-level transcription within 15 minutes following heat shock, providing a pulse of protein accumulation lasting several hours (Scheer et al., 2002). Heat shock induction of dnSu(H) at 8 hpf did not alter *atoh1b* expression or hair cell development (not shown). However, heat shock at 10 hpf caused the initially broad domain of *atoh1b* to be maintained to at least 13.5 hpf, about 2 hours longer than normal (Fig. 5B). By 14.5 hpf, expression became restricted to two discrete domains that were larger than normal (Fig. 5E). This domain restriction presumably reflects resumption of Dl-N signaling as the pulse of dnSu(H) subsides. However, the enlarged domains showed no further reduction after 14.5 hpf and went on to form supernumerary hair cells (Fig. 5H). Heat shock at 12 hpf (after equivalence-group restriction had already begun) also resulted in maintenance of two large domains and production of excess hair cells (Fig. 5C,F,I). Heat shock at 14 hpf had little effect on *atoh1b* expression or hair cell formation (not shown). These data show that equivalence group restriction can still occur after 13.5 hpf, but then *atoh1b* expression stabilizes by 14.5 hpf regardless of domain size, defining an interval during which cell fates are specified.

To test how N gain of function affects *atoh1* gene expression (as in Fig. 1), we used a heat shock-inducible Gal4-UAS system to drive expression of N intracellular domain (NICD) (Scheer and Campos-Ortega, 1999). In this system, heat shock induced sustained NICD expression for at least 17 hours (Scheer et al., 2002). Heat shock induction of NICD at 9 or 10 hpf did not prevent induction of *atoh1b* in the preotic placode (Fig. 6B). However, *atoh1b* expression was lost by 12 hpf (Fig. 6D). In addition, *atoh1a* was never activated and no hair cells were produced (not shown). Heat shock induction of NICD at 18 hpf also rapidly extinguished *atoh1* expression and blocked hair cell formation (not shown). We also examined the effects of NICD in *atoh1b* morphants, which usually have no functional equivalence group until 20 hpf, when *atoh1a* is first expressed. In *atoh1b* morphants, activation of NICD at 18 hpf induced *atoh1a* by 19 hpf, 1 hour earlier than without NICD (Fig. 6E-G). Expression then subsided by 20 hpf and no hair cells were produced (Fig. 6H, and data not shown). Thus, NICD initially stimulates, or at least does not block, upregulation of *atoh1* genes as the equivalence group forms but then rapidly extinguishes *atoh1* expression at all later stages.

In summary, the relationship between *atoh1* function and the Dl-N pathway is consistent with all predictions of the fly *ato* paradigm (Fig. 1). Moreover, *atoh1*-dependent restriction of the equivalence group precedes fate specification by several hours. These findings strongly support a classic proneural mechanism of action for zebrafish *atoh1* genes.
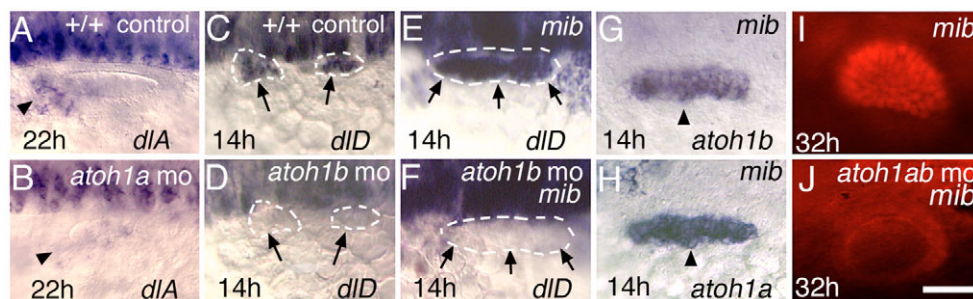

Fig. 4. Interactions between *atoh1* and the Delta-Notch pathway. (A,B) Expression of *dlA* at 22 hpf in a control embryo (A) and *atoh1a* morphant (B). **(C-F)** Expression of *dlD* at 14 hpf in a control embryo (C), *atoh1b* morphant (D), *mib* mutant (E) and *mib* mutant-*atoh1b* morphant (F). **(G,H)** *mib* mutants show expanded otic domains of *atoh1b* (G) and *atoh1a* (H) at 14 hpf. **(I,J)** Pax2 antibody staining at 32 hpf reveals supernumerary hair cells in a *mib* mutant (I) but no hair cells in a *mib* mutant co-injected with *atoh1a* MO and *atoh1b* MO (J). Arrowheads and arrows indicate otic regions. All images are dorsolateral views with anterior to the left. Scale bars: 30 μm in A,E,I-P; 15 μm in B-D,F-H.
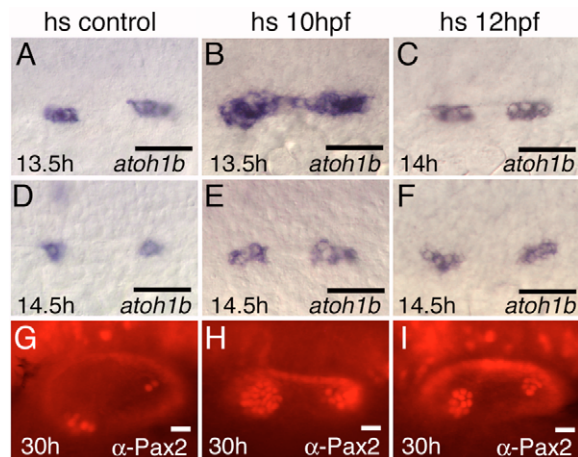
Fig. 5. Heat-shock induction of dnSu(H). Expression of *atoh1b* at 13.5 or 14 hpf (**A-C**), *atoh1b* at 14.5 hpf (**D-F**) and Pax2 at 30 hpf (**G-I**), as seen in control embryos heat shocked at 10 hpf (A,D,G) or *hsp70-dnSu(H)* transgenic embryos heat shocked at 10 hpf (B,E,H) or 12 hpf (C,F,I). Images show lateral views with anterior to the left. Scale bar: 15 μm.

Fig. 6. Heat-shock induction of NICD. (**A-D**) Expression of *atoh1b* and *ngn1* at 11 hpf (A,B) and 12 hpf (C,D) in control embryos (A,C) or NICD-positive embryos (B,D) heat shocked at 9 hpf. Loss of *ngn1* expression, which is non-overlapping with *atoh1b*, confirms effective NICD-induction. (**E-H**) Expression of *atoh1a* at 19 hpf (E,F) and 20 hpf (G,H) in *atoh1b* morphants without NICD (E,G) or with NICD (F,H) heat shocked at 18 hpf. Otic vesicles are outlined. Arrowheads mark otic expression domains. All are lateral views with anterior to the left. Scale bar: 15 μm. tg, trigeminal ganglion.

Regulation of *atoh1b* in preotic cells

Expression of *pax8* is the earliest known marker of otic placode induction (Pfeffer et al., 1998). *atoh1b* is expressed in a subset of *pax8*-expressing cells in the preotic placode (Fig. 7A,B), raising the possibility that *pax8* is required for early activation of *atoh1b*. Knocking down *pax8* reduced the size of the preotic domain of *atoh1b* (Fig. 7F), but the level of expression appeared normal. We next asked whether factors that act upstream of or parallel to *pax8* might also regulate *atoh1b*. Induction of *pax8* requires Foxi1 autonomously within the preplacodal ectoderm, as well as stimulation by Fgf3 and Fgf8 secreted from adjacent hindbrain tissue (Phillips et al., 2001; Maroon et al., 2002; Leger and Brand, 2002; Liu et al., 2003; Solomon et al., 2003; Hans et al., 2004). Knocking down *foxi1* caused severe reduction of *atoh1b* expression (Fig. 7G). To test the role of Fgf, embryos were treated with the Fgf signaling inhibitor SU5402. Induction of *atoh1b* was blocked in embryos treated from 10-14 hpf (not shown). When SU5402 was added beginning at 10.5 hpf, after the onset of *atoh1b* expression, expression of *atoh1b* was lost in all specimens by 12.5 hpf (Fig. 7D). Expression of *atoh1a* was also blocked (Fig. 7I), consistent with a requirement for *atoh1b* in *atoh1a* induction. Embryos co-injected with *fgf3* MO and *fgf8* MO also did not express *atoh1* genes (not shown). Thus, Foxi1 and Fgf signaling are required to initiate and maintain expression of *atoh1b* in the preotic placode, and Pax8 is needed to produce a normal-sized domain.

Distal-less genes *dlx3b* and *dlx4b* also regulate early otic development but in a distinct pathway, acting parallel to *foxi1-fgf-pax8*. Loss of *dlx3b* and *dlx4b* does not block induction of *pax8* but subsequent steps in otic development fail (Solomon and Fritz, 2002; Liu et al., 2003; Hans et al., 2004). Accordingly, neither *atoh1a* nor *atoh1b* were expressed in *dlx3b*;*dlx4b* morphants during placodal development (Fig. 7E,J). Similarly, *b380* mutants, which are deleted for *dlx3b* and *dlx4b* (Fritz et al., 1996), also failed to express *atoh1* genes in the otic placode (not shown). Later in development, *dlx3b*;*dlx4b* morphants produced small otic vesicles containing only anterior (utricular) sensory patches. Tether cells did not form, consistent with loss of early *atoh1b*, but later hair cells began to form after 24 hpf (not shown) in association with belated expression of
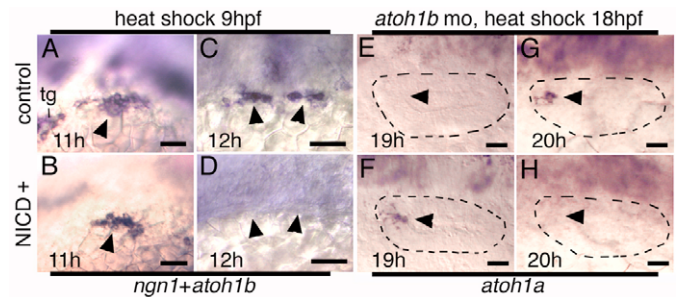
atoh1a (Fig. 7O). Dlx proteins could act directly on *atoh1b* transcription or indirectly by regulating competence to respond properly to Fgf after initial otic induction, as suggested by recent studies (Hans et al., 2004; Solomon et al., 2004).

Pax2 and Pax8 proteins maintain *atoh1b*

Pax8 normally cooperates with closely related proteins Pax2a and Pax2b to maintain the otic placode (Hans et al., 2004; Mackereth et al., 2005). Knockdown of *pax8* and *pax2b* in embryos homozygous for a null mutation in *pax2a* (*noi* mutants) (Lun and Brand, 1998) causes progressive loss of otic tissue and no vesicles are produced. Accordingly such embryos do not express *atoh1a* or *atoh1b* in the otic region (not shown). Reducing the MO concentration by half allows the majority of *pax2a-pax2b-pax8*-deficient embryos to produce small otic vesicles. In 100% of these specimens, *atoh1a* was expressed at a high level in a nearly normal number of cells at the anterior end of the otic vesicle, whereas *atoh1b* expression was barely detectable in any specimen (Fig. 7K,L). Partial knockdown of *pax8* and *pax2b* in wild-type embryos resulted in a moderately diminished otic vesicle expressing normal levels of both *atoh1a* and *atoh1b* (Fig. 7M,N), although *atoh1b* was typically expressed in only one or two cells. These data show that full expression of *atoh1b* requires Pax8 and Pax2 functions. By contrast, *atoh1a* expression is not strictly dependent on Pax2 or Pax8 function.

Continuing requirements for Fgf

As the otic vesicle forms, *fgf3* and *fgf8* begin to be expressed in domains encompassing the sensory epithelia (Leger and Brand, 2002). To test whether Fgf signaling regulates *atoh1* expression after placode formation, embryos were treated with SU5402 for various intervals at successively later stages of development. Treatment from 12-18 hpf did not affect *atoh1b* but reduced expression of *atoh1a* (Fig. 8A-D). When embryos were treated at 18 hpf for 1, 2, 4 or 6 hour intervals, expression of both *atoh1a* and *atoh1b* were strongly reduced but not eliminated (Fig. 8E-H). We hypothesized that the period of SU5402 insensitivity of *atoh1b* from 12 to 18 hpf reflects maintenance of *atoh1b* by autoregulation. Furthermore, as *atoh1a* and *atoh1b* help maintain each other at later stages, crossregulation
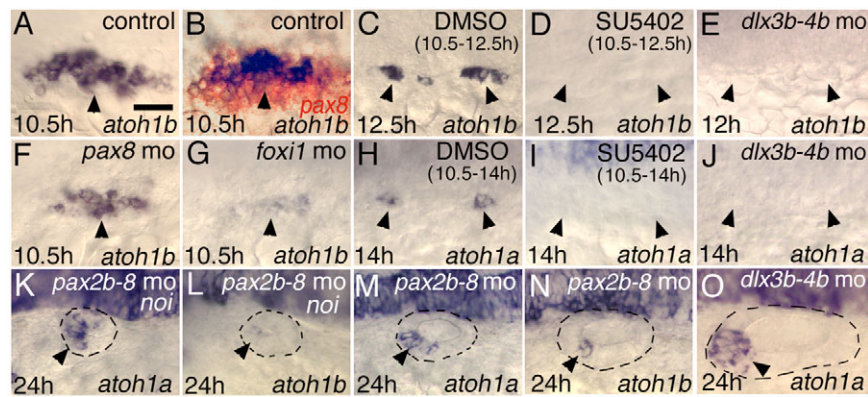
Fig. 7. Inducers of early *atoh1* expression. (**A,B,F,G**) Expression of *atoh1b* at 10.5 hpf in a control embryo (A,B), *pax8* morphant (F) and *foxi1* morphant (G). The specimen in B was double stained to reveal *pax8* expression (red). (**C,D**) Expression of *atoh1b* at 12.5 hpf in embryos treated from 10.5-12.5 hpf with DMSO alone (C) or SU5402 in DMSO (D). (**H,I**) Expression of *atoh1a* at 14 hpf in embryos treated from 10.5-14 hpf with DMSO alone (H) or SU5402 in DMSO (I). (**E,J,O**) *dlx3b;dlx4b* morphants showing expression of *atoh1b* at 12 hpf (E) or *atoh1a* at 14 hpf (J) or 24 hpf (O). (**K-N**) Expression at 24 hpf of *atoh1a* (K,M) and *atoh1b* (L,N) in *noi* mutants injected with *pax2b-pax8* MO (K,L), and in wild-type embryos injected with *pax2b-pax8* MO (M,N). All are dorsolateral views with anterior to the left. Arrowheads indicate observed or expected domains of otic expression. Scale bar: 30 μm in A,B,F,G,K-O; 10 μm in C-E,H-J.

could account for residual expression seen in SU5402-treated embryos. In support of this hypothesis, *atoh1b* morphants failed to express either *atoh1a* or *atoh1b* when treated with SU5402 from 18-22 hpf (Fig. 8J). We next tested the effects of SU5402 on hair cell formation. In embryos treated from 18-24 hpf, tether cells were produced normally (not shown). This was not unexpected, because tether cells are already present in the otic vesicle at 18 hpf and hence their specification cannot be blocked by this treatment. However, production of later-forming hair cells was strongly impaired during the 6 hour period following removal of the inhibitor (Fig. 8L, Fig. 2V). Presumably the severe reduction in *atoh1* expression seen at 24 hpf delays resumption of macular development. These data show that *atoh1* expression and hair cell development require ongoing Fgf signaling. This marks the first identification of a signaling molecule required to both induce and maintain *atoh1* expression in the vertebrate inner ear.

atoh1-dependent and -independent expression of macular genes

We next tested whether *atoh1* function affects *fgf* or *pax* gene expression. Otic expression of *fgf3* and *fgf8* was normal in *atoh1a;atoh1b* double morphants (Fig. 9A-D). Likewise, expression of *pax5* in the utricle, which is regulated by Fgf signaling (Kwak et al., 2002; Kwak et al., 2006), was also unaltered in *atoh1a;atoh1b* double morphants (Fig. 9F). By contrast, knockdown of both *atoh1a* and *atoh1b* strongly reduced the level of *pax2b* expression (Fig. 9H). *pax5* and *pax2b* are both required for normal development and maintenance of hair cells (Whitfield et al., 2002; Kwak et al., 2006), but only the latter was affected by *atoh1* function. Thus, expression of *fgf* genes and some downstream targets (*pax5*, *atoh1a*) continue in the macular region despite disruption of *atoh1* function and the absence of a sensory epithelium.

DISCUSSION

Our data support a model in which *atoh1a* and *atoh1b* act in a complex network leading to the establishment of a sensory equivalence group and subsequent differentiation of hair cells (Fig. 10). There are two distinct phases of *atoh1* function. In the first phase, *atoh1b* establishes a single prosensory domain during preplacodal development and subsequently activates Delta-Notch

feedback to split the domain into separate utricular and saccular primordia in the nascent otic placode by 12 hpf. Lateral inhibition and specification of tether cells occurs by 14 hpf, when *atoh1b* also activates expression of *atoh1a*. In the second phase, beginning soon after formation of the otic vesicle, *atoh1a* expression predominates in the maculae and maintains *atoh1b* in a subset of cells. Moreover, *atoh1a* is primarily responsible for specifying later-forming hair cells and activating Delta-Notch-mediated lateral inhibition.

Fgf signaling is an essential upstream activator of *atoh1* expression during both phases, although *atoh1b* becomes independent of Fgf after 12 hpf. This could reflect the onset of *atoh1b* autoregulation. A similar transition occurs with *Drosophila ato*, which becomes autoregulatory as it initiates domain restriction and lateral inhibition (Sun et al., 1998). Fgf may facilitate the transition to autoregulation of *atoh1b*, in a similar way to the role of Egfr and Map kinase activity in promoting autoregulation of *Drosophila ato* during sensory organ development (zur Lage et al., 2004). Unlike *atoh1b*, maintenance of *atoh1a* remains heavily dependent on Fgf but is not dependent on *atoh1* function after 20 hpf. Indeed, *atoh1a;atoh1b* morphants maintain higher than normal expression of *atoh1a*. This is probably because *fgf* genes continue to be expressed (Fig. 9) and promote *atoh1a* expression in the absence of N-mediated feedback inhibition.

The overlapping yet distinct functions of zebrafish *atoh1* genes probably reflects evolutionary 'subfunctionalization' (Force et al., 1999). Following a genome duplication thought to have occurred early in the teleost lineage, duplicate copies of genes often diverge in regulation to subdivide the ancestral function. Only *atoh1b* is required for development of tether cells, which are analogous to primary neurons. Because such precocious cell types are typical of anamniote embryos, this probably reflects an ancestral *atoh1* function. *atoh1a* has apparently lost regulatory elements required to respond to the *fgf-foxi1-pax* and *dlx* pathways involved in *atoh1b* induction. However, only *atoh1a* is essential for later hair cells, which continue to form well beyond embryonic development. This, too, is probably an ancestral *atoh1* function. Sensory epithelia continue to expand throughout life in teleosts, suggesting ongoing recruitment of new cells into the equivalence group. Fgf-dependent induction of *atoh1a* in adjacent cells might account for such
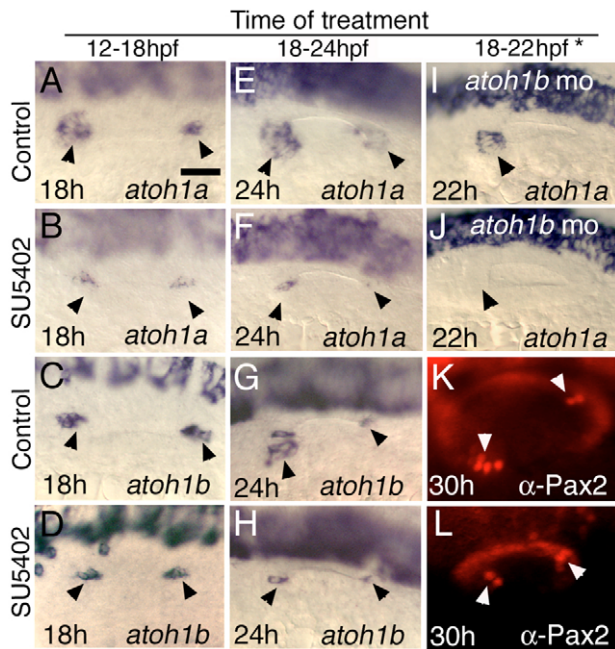
DEVELOPMENT

Fig. 8. Stage-dependent requirements for Fgf. Embryos were treated with DMSO (control) or DMSO plus SU5402 for the indicated time intervals. (**A-H**) Expression of *atoh1a* in control and SU5402-treated embryos at 18 hpf (A,B) and 24 hpf (E,F), and expression of *atoh1b* in control and SU5402-treated embryos at 18 hpf (C,D) and 24 hpf (G,H). (**I,J**) Expression of *atoh1a* at 22 hpf in *atoh1b* morphants treated with DMSO (I) and DMSO and SU5402 (J). (**K,L**) Pax2 staining of hair cells at 30 hpf in embryos treated with DMSO (K) or DMSO and SU5402 (L). *, treatment for K, L from 18-24 hpf. All images are dorsolateral views with anterior to the left. Black arrowheads indicate otic expression. White arrowheads indicate sensory epithelia. Scale bar: 30 μm.

recruitment, a function similar to the role of Egfr and *ato* in recruiting new sensory organ precursors in the *Drosophila* chordotonal organs (zur Lage et al., 1997). The two Atoh1 proteins probably retain similar DNA-binding properties, however, as misexpression of *atoh1a* can restore tether cell formation in *atoh1a*;*atoh1b* double morphants (Fig. 1M-O).

Zebrafish *atoh1* genes have proneural function

There have been differing opinions as to whether vertebrate *Atoh1* genes act as classic proneural genes or only as terminal differentiation factors (reviewed by Kelley, 2006). Specific comparisons between zebrafish *atoh1* genes and *Drosophila ato* (Fig. 1) reveal striking parallels. More generally, various authors have used four criteria to define proneural function (Brunet and

Ghysen, 1999; Hassan and Bellen, 2000; Westerman et al., 2003) that can be applied to zebrafish *atoh1* genes. First, proneural genes are expressed before sensory fate specification. *atoh1b* is induced broadly in the preotic placode at 10.5 hpf, whereas specification of tether cells (stabilization of *atoh1* expression) does not occur until 14 hpf. Second, proneural genes are subject to lateral inhibition (and the related process of domain restriction) via N-mediated repression. Zebrafish *atoh1* genes, once induced, are readily repressed by N activity. Moreover, both *atoh1* genes facilitate their own repression by autonomously activating *delta* expression. Third, proneural function is necessary for producing the equivalence group for the entire sensory structure. *atoh1a*;*atoh1b* morphants produce only a simple epithelium lacking hair cells; and while support cell markers are not known in zebrafish, it is important to note that the epithelium continues to express *atoh1a*. As loss of *atoh1* expression marks the first step in support cell specification, these cannot be support cells. Fourth, proneural function is sufficient to induce ectopic sensory development. Misexpression of *atoh1a* induces ectopic hair cells, although only in limited regions near the otic vesicle or endogenous sensory epithelia, as has been shown for *Atoh1* in mammals (Zheng and Gao, 2000; Woods et al., 2004; Izumikawa et al., 2005). Competence to respond appropriately to Atoh1 may require a unique combination of additional factors. The zone of competence could be influenced by *pax2-5-8* genes, which are co-regulated with *atoh1* genes by Fgf signaling. Other signaling pathways have also been implicated in this process. Misexpressing components of the Notch or Wnt pathways in chick can also induce ectopic sensory patches, but only in restricted regions near endogenous sensory patches (Stevens et al., 2003; Daudet and Lewis, 2005). Combinatorial signaling and restricted zones of competence also influence the functions of proneural genes in *Drosophila* (Westerman et al., 2003; Niwa et al., 2004). Thus, while many additional details need to be resolved, zebrafish *atoh1* genes meet all four criteria used to define proneural function.

Conserved mechanisms?

While mammals show no early phase of specification analogous to tether cell development, and sensory epithelia develop only during a limited stage of embryogenesis, some aspects of sensory development have been conserved. The clearest example is the role of N signaling. *Dll1* and *Jag2* encode N ligands that regulate the balance of hair cells and support cells in the mouse cochlea. Loss of *Jag2* causes a modest increase in hair cells (Lanford et al., 1999; Kiernan et al., 2005a), as does antisense knockdown of *N1* in cochlear cultures (Zine et al., 2000). Loss of *Dll1* causes a larger increase in hair cells (Brooker et al., 2006), and disrupting both *Dll1* and *Jag2* causes a dramatic increase in hair cells and a modest decrease in support cells (Kiernan et al., 2005a). The
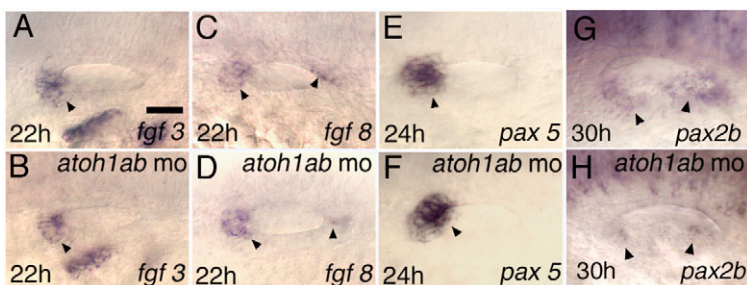

Fig. 9. Expression of macular genes. Expression of *fgf3* (**A,B**) and *fgf8* at 22 hpf (**C,D**), *pax5* at 24 hpf (**E,F**) and *pax2b* at 30 hpf (**G,H**) in control embryos (A,C,E,G) and *atoh1a*;*atoh1b* double morphants (B,D,F,H). All panels show dorsolateral views with anterior to the left and dorsal up. Arrowheads indicate expression in sensory epithelia. Scale bar, 30 μm.
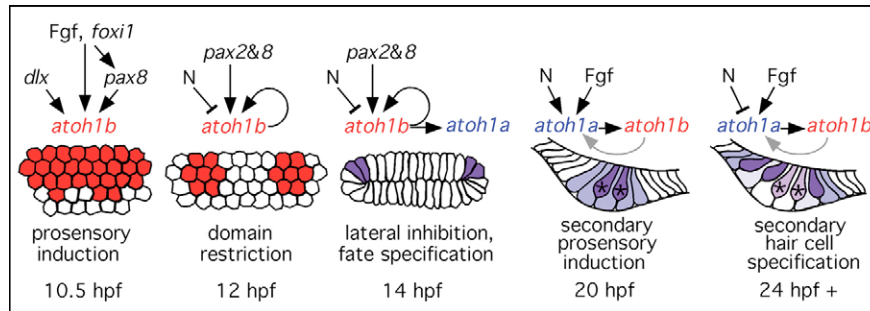
Fig. 10. Summary of *atoh1* regulation and function. *fgf-foxi1-pax8* and *dlx* pathways induce expression of *atoh1b* (red) in medial preotic cells, specifying the prosensory equivalence group. By 12 hpf, the domain is restricted into two intermediate groups by Dl-N activity, which is activated by *atoh1b* function. Tether cells are specified around 14 hpf as *atoh1a* is activated (blue, coexpression with *atoh1b*, purple). At 20 hpf, N and Fgf activate a wider domain of *atoh1a* associated with later-forming hair cells. Tether cells (asterisks) terminally differentiate. *atoh1a* is required to maintain or activate *atoh1b* in differentiating cells, and *atoh1b* helps maintain high levels of *atoh1a*. At 24 hpf and thereafter, later-forming hair cells begin to differentiate and coexpress *atoh1* genes, and N activity limits *atoh1* expression. Mature tether cells and hair cells downregulate *atoh1* expression.

number of support cells is greater than expected, because support cells continue to divide for longer than normal, partially offsetting earlier deficiencies. Although no phenotype comparable to zebrafish *mib* has been described in mouse, the mouse data nevertheless support the lateral inhibition model well. Residual support cell development probably reflects the activity of another N ligand, Jag1. *Jag1* is initially expressed throughout the prospective sensory region and later becomes restricted to support cells during differentiation. It has been proposed that Jag1 signaling between support cells augments lateral inhibitory signals from hair cells (Eddison et al., 2000). Indeed, partial loss of *Jag1* also leads to excess hair cell production (Zine et al., 2000; Kiernan et al., 2001). However, conditional knockouts of *Jag1* ablate much, although not all, of the sensory epithelia (Brooker et al., 2006; Kiernan et al., 2006). This supports a model in which the function of Jag1 changes with time, initially promoting the early inductive phase of N signaling and later augmenting lateral inhibition. While the mechanistic basis for the shift from inductive to repressive N signaling remains unknown, similar transitions occur in the regulation of *Drosophila ato* and zebrafish *atoh1a* (Baker and Yu, 1997) (Fig. 6F,H). It is not known whether mouse also shows N-dependent restriction of the initial equivalence group.

Fgf signaling may also play a conserved role in mammals. A number of Fgfs are expressed in the otic vesicle and developing sensory epithelia in mouse, but in most cases their role in hair cell formation is obscured by severe morphogenetic defects caused by specific gene knockouts. However, hypomorphic alleles of *Fgfr1* severely reduce hair cell production in the cochlea without blocking morphogenesis (Pirvola et al., 2002). Furthermore, Pirvola et al. (Pirvola et al., 2002) have proposed that Fgfs produced by inner hair cells in the Organ of Corti stimulate differentiation of later-forming outer hair cells through activation of Fgfr1.

A potential difference between mouse and zebrafish is the question of whether mouse *Atoh1* has proneural activity (reviewed by Kelley, 2006). This is especially evident when considering the mammalian cochlea, which is a highly derived structure that differs in important ways from the more primitive maculae and cristae. However, as summarized below, available data are complex and can be considered inconclusive. *Atoh1* is necessary for hair cell differentiation and is sufficient for inducing ectopic hair cells

(Bermingham et al., 1999; Zheng and Gao, 2000; Woods et al., 2004; Izumikawa et al., 2005). *Atoh1* is also subject to autoregulation (Helms et al., 2000), which in other species facilitates pattern refinement during lateral inhibition. Unfortunately, a direct link between lateral inhibition and *Atoh1* has not been shown in mouse. *Atoh1* is initially expressed in a broad domain that spans the full depth of the epithelium, approximately four to five cells thick (Bermingham et al., 1999; Lanford et al., 2000; Chen et al., 2002; Woods et al., 2004), but expression is not uniform and some cells appear to express little or no *Atoh1*. These data do not distinguish whether there is an earlier stage of low uniform *Atoh1* expression followed by rapid upregulation and pattern refinement or, alternatively, whether *Atoh1* marks only differentiating hair cells after fate specification. Several groups have concluded that mouse *Atoh1* lacks proneural activity based in part on the observation that sensory regions in *Atoh1* knockout mice contain a single layer of cells that morphologically resemble support cells (Bermingham et al., 1999). However, these cells express no definitive markers of mature support cells (Woods et al., 2005). Early non-restricted expression of *Jag1* occurs normally, but later expression normally associated with support cells is lost. Thus, support cell differentiation is disrupted, although it is not clear whether the defect lies in specification or maintenance. Another early marker of the sensory epithelium, $p27^{kip1}$, normally precedes *Atoh1* in expression and continues to be expressed in the prosensory region in *Atoh1* mutants (Chen et al., 2002). This has been interpreted to mean that cells of the equivalence group are specified but fail to differentiate. However, $p27^{kip1}$ plays no role in fate specification, and there are no independent indicators of when the equivalence group forms in mouse. While expression $p27^{kip1}$ is regulated partly by the same inductive signals that specify the equivalence group (Kiernan et al., 2006), upregulation of fate-specifying gene(s) need not follow precisely the same timecourse. Moreover, even if *Atoh1* were necessary for prosensory induction, loss of *Atoh1* would not be expected to block any of the initial transcriptional responses to inductive signals. Thus expression of $p27^{kip1}$ and *Atoh1* in the absence of *Atoh1* function (Bermingham et al., 1999; Chen et al., 2002; Fritzsch et al., 2005) could simply reflect ongoing parallel responses to common upstream activators in cells that are otherwise blocked at an early stage. Similarly, we have shown that several early markers of sensory epithelia in zebrafish (*atoh1a*, *pax5*) are co-

regulated by Fgfs and continue to be expressed in *atoh1a;atoh1b* morphants (Fig. 9). A similar situation has been documented in *Drosophila ato* mutants, which produce no photoreceptors in the eye but continue to coexpress genes normally preceding formation of the prosensory equivalence group, including *ato* and the N target gene *hairy* (Jarman et al., 1995). In summary, gene expression and genetic studies in mouse do not necessarily contradict the notion that *Atoh1* might have proneural activity, but key supportive data are also lacking. Resolving this issue will require assessment of precisely when fate specification occurs relative to expression of *Atoh1* and $p27^{kip1}$, how these genes are co-regulated, and the epistatic relationships between the various upstream factors, including Sox2, Jag1 and Fgf.

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