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 *atona1* (*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; Fritsch 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 makers of sensory epithelia has been interpreted to mean that mouse *Atoh1* is not required for specifying the equivalence group per se, but instead 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 *p27kip1* and exit the cell cycle before expression of *Atoh1*, and this process still occurs in *Atoh1* mutants. However, *p27kip1* expression and cell cycle withdrawal could be regulated independently from equivalence group specification. Indeed, sensory epithelia still form in *p27kip1* 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 (DI-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 DI 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

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

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

Accepted 31 October 2006
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 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<sup>m20</sup> and nemo<sup>m20</sup> mutations are probably null alleles (Lun and Brand, 1998; 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 unthethered mass due to the absence of tether cells (Fig. 2D). Otoliths eventually bound to the saccule (Fig. 2B) in all specimens. Later-forming hair cells were still profoundly impaired in all atoh1b morphants, as additional hair cells were not evident until 48 hpf (Fig. 2I,U, and data not shown).
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, atoh1a 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...
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 DI-N feedback, we examined atoh1 function in mind bomb (mib) mutants. The mib gene encodes an E3 ubiquitin ligase essential for DI-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 DI-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 mind bomb (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 DI-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 atoh1b 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. 6G). 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 DI-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,J-P; 15 μm in B-D,F-H.
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 might also regulate atoh1b. Induction of foxi1 (Phillips et al., 2001; Maroon et al., 2002; Leger and Brand, 2002) causes 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 atoh1b 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 (Fritz and Solomon, 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...
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 dIx 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 line 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, 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 Nl 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
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 aito 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, p27kip1, 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, p27kip1 plays no role in fate specification, and there are no independent indicators of when the equivalence group forms in mouse. While expression p27kip1 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 p27kip1 and Atoh1 in the absence of Atoh1 function (Bermingham et al., 1999; Chen et al., 2002; Fritzsche 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 aito* mutants, which produce no photoreceptors in the eye but continue to coexpress genes normally preceding formation of the prosensory equivalence group, including *aito* 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 how these genes are co-regulated, and the epistatic relationships between the various upstream factors, including Sox2, Jag1 and Fgf.

This work was supported by the National Institutes of Health, NICD grant RO1-DC039806. We thank Bruce Appel and Jim Fadool for supplying heat shock dnSu(H) and NICD lines, and Anne Lekien and Brian Perkins for helpful discussions of the data.

References


