Atoh1, an Essential Transcription Factor in Neurogenesis and Intestinal and Inner Ear Development: Function, Regulation, and Context Dependency

JOANNA MULVANEY¹ AND ALAIN DABDOUB¹

¹*Division of Otolaryngology, Department of Surgery, School of Medicine, University of California San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0666, USA*

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ABSTRACT

Atoh1 (also known as Math1, Hath1, and Cath1 in mouse, human, and chicken, respectively) is a proneural basic helix–loop–helix (bHLH) transcription factor that is required in a variety of developmental contexts. Atoh1 is involved in differentiation of neurons, secretory cells in the gut, and mechanoreceptors including auditory hair cells. Together with the two closely related bHLH genes, *Neurog1* and *NeuroD1*, Atoh1 regulates neurosensory development in the ear as well as neurogenesis in the cerebellum. Atoh1 activity in the cochlea is both necessary and sufficient to drive auditory hair cell differentiation, in keeping with its known role as a regulator of various genes that are markers of terminal differentiation. Atoh1 is known in other fields as an oncogene and a tumor suppressor involved in regulation of cell cycle control and apoptosis. Aberrant Atoh1 activity in adult tissue is implicated in cancer progression, specifically in medullablastoma and adenomatous polyposis carcinoma. We demonstrate through protein sequence comparison that Atoh1 contains conserved phosphorylation sites outside the bHLH domain, which may allow regulation through post-translational modification. With such diverse roles, tight regulation of Atoh1 at both the transcriptional and protein level is essential.

Keywords: Math1, bHLH, transcription factor, cochlea, organ of Corti, hair cells

Abbreviations used in this article: β Gal – β Galactosidase; APC – Adenomatous polyposis coli; ASC – Aschaete–Scute complex; AtEAM – Atoh1 E-box associated motif; Ato – Atonal; bHLH – Basic helix–loop–helix; EGL – External germinal layer; HC – Hair cell; Id – Inhibitor of differentiation and DNA binding; IHC – Inner hair cell; ISH – In situ hybridization; OC – Organ of Corti; OHC – Outer hair cell; SC – Supporting cell

Atoh1 IS A NEUROGENIC TRANSCRIPTION FACTOR

Atoh1 is a transcription factor with diverse roles during development and has been implicated as both an oncogene and a tumor suppressor. In addition to numerous reports demonstrating that Atoh1 is required for terminal differentiation of auditory hair cells (HCs) in the developing ear, Atoh1 also functions in early specification events during neurogenesis and has a role in cell cycle regulation in maintenance of the intestine. In order to elucidate how Atoh1 can induce expression of radically different gene sets depending on the tissue in which it is expressed, it is necessary to understand how Atoh1 protein functions, how Atoh1 activity is regulated, and where the results of its activity in different contexts diverge. Here, we review Atoh1 protein structure, its varied roles in developmental and homeostatic contexts, and how regulation of both transcription and protein activity allow for diversity of function.

Correspondence to: Alain Dabdoub · Division of Otolaryngology, Department of Surgery, School of Medicine · University of California San Diego · 9500 Gilman Drive, La Jolla, CA 92093-0666, USA. e-mail: adabdoub@ucsd.edu

Atoh1 IS THE VERTEBRATE HOMOLOGUE OF *DROSOPHILA MELANOGASTER* ATONAL

The well-characterized proneural genes of the Aschaete–Scute complex (ASC) are required for normal development of the fruitfly peripheral nervous system; however, a subset of sensory organs are unaffected by loss of ASC components (Dambly-Chaudiere 1987). These organs are the internal chordotonal organs, which include sensory bristles, hairs, and papillae. The ASC proteins are class II basic helix–loop–helix (bHLH) transcription factors that can form heterodimers with the ubiquitous transcription factor, Daughterless (Da) (Villares and Cabrera 1987). Loss of *da* results in a total loss of the peripheral nervous system, including the chordotonal organs, suggesting that there is another set of proneural bHLH transcription factors with the ability to form heterodimers with Da in addition to ASC (Caudy et al. 1988). The *D. melanogaster* gene *atonal* (*ato*) was identified as a candidate gene in 1993, cloned, and subsequently shown to code for a class II bHLH transcription factor necessary and sufficient for the formation of chordotonal organs (Jarman et al. 1993).

Atoh1 STRUCTURE AND FUNCTION

Murine *Atoh1* was cloned as a result of a screen for vertebrate *ato* homologues (Akazawa et al. 1995). A typical bHLH domain is 60 residues long, with a DNA-binding basic region plus a protein-binding region consisting of two α -helices separated by a variable loop region; the variable loop region allows heterodimeric interactions with class I members of the

bHLH family such as E47 (Aguado-Llera et al. 2010). The mouse *Atoh1* open reading frame consists of an intronless 1.053-kb coding region that produces a protein 351 amino acids long and 37 kDa in size (Figs. 1, A and 2). *Atoh1* shares 70% sequence identity with *atonal* in the bHLH domain. Outside of this domain, there is little similarity. In contrast to Atonal in which the bHLH domain is situated at the carboxy terminus, in Atoh1, the bHLH domain is in the middle of the protein (Fig. 2A). Atoh1 is rich in proline (35/351 residues), implying a protein-binding function, and 33% of the carboxy terminus (aa325–351) consists of serines, leading to the prediction that phosphorylation is integral to the protein function and regulation. The serines at sites 328 and 331 are specifically predicted to be targets for protein kinase C (Akazawa et al. 1995) (Fig. 2A).

Comparison of the Atoh1 peptide sequence in mouse, human, *Xenopus tropicalis*, zebrafish, and chicken (Fig. 2A) shows that, outside the bHLH region and the conserved serine-rich domain at the C terminus, there is reduced similarity (Fig. 2A). Several conserved phosphorylation sites have been identified in the C terminus region, and it has been shown that both the bHLH domain and the N terminus are essential for transcriptional activity (Aragaki et al. 2008). This suggests additional protein interactions outside of the bHLH domain and post-translational modifications. In addition, comparison of the N terminus in mouse, human, rat, cow, and dog (predicted) demonstrate that this region is well conserved within mammals, while comparison of mammalian Atoh1 homologues with their non-mammalian homologues shows little similarity (Fig. 2A). Comparison of the region extending from the methi-

FIG. 1. Gene structure of mouse Atoh1. A A schematic diagram of mouse *Atoh1*. The Atoh1 open reading frame (ORF), represented by an open box runs from position 1 to position 1053, consisting of a single exon. The two identified Atoh1 enhancer sites, represented by black boxes, are located approximately 3.4 kb 3' to the ORF. B The two enhancer sites, site (A) and site (B), are separated by 413 bp of

non-conserved sequence—the distance between enhancers (A) and (B) might influence enhancer activity. There is little sequence similarity between enhancer (A) and enhancer (B); enhancer (A) contains an E-box motif while enhancer (B) contains a Hairy preferred sequence, an E-box motif and an N box motif.

online marking the start of the protein to the last amino acid before the bHLH domain shows 82% match between mouse, human, rat, dog (predicted), and cow. Comparison of the same region of mouse Atoh1, zebrafish, and *X. tropicalis* gives a sequence similarity of 21%. Chicken was not included in the comparison because the sequence held in the NCBI database only includes 21 amino acids upstream of the bHLH domain.

The bHLH domain confers specificity of DNA binding and protein–protein interaction on Atoh1. Substituting the bHLH domain of Ascl1 (previously Mash1), the vertebrate ASC homologue, for the bHLH domain of Atoh1, induces differentiation of cells into alternative cell types (Nakada et al. 2004). Homology between *ato* and *Atoh1* (Fig. 2B) is such that, when *ato* is substituted for *Atoh1* in an *Atoh1* null mouse, the *Atoh1* phenotype is rescued, and over-expression of *Atoh1* in *ato* *Drosophila* mutants results in partial rescue of the loss of chordotonal neurons (Ben-Arie et al. 2000; Wang et al. 2002). Note that *atonal* is the only *Drosophila* class II bHLH that has been shown to entirely recapitulate the function of its mammalian orthologue. Work with *ato* and an *ato* homologue in the silk moth, *ato Bombyx mori* (*ato^{BM}*), suggests that the non-bHLH domains can play important roles. *At^{BM}* shares little identity with *ato* outside the bHLH domain; however, like *Atoh1*, when expressed in place of *ato* in the fly, it can partially rescue the *ato* phenotype (Yu et al. 2011). Further rescue experiments show that the bHLH domains of *ato* and *ato^{BM}* are not functionally equivalent, but the N terminus region of *ato^{BM}* can substitute for that of *ato* (Yu et al. 2011). This is a surprising result given that the N terminus sequences are so divergent; however, it does hint that the N terminus contains a structural element that is more important than the *ato*-mediated rescue of the *Atoh1* phenotype would suggest.

Class I (E proteins) and class II helix–loop–helix transcription factors can interact with E-box consensus sites. An E-box is a short hexameric DNA motif found in *cis*-acting gene regulatory regions that allows selective protein–DNA binding (Darlington et al. 2000; Kyriacou and Rosato 2000). Each class of bHLH (I–IV) has a preferred E-box sequence, and to add further specificity, nucleotides flanking the E-box select for individual bHLH proteins within each class (Powell et al. 2004; Powell et al. 2008). Like Atonal, Atoh1 binds directly to E-boxes (Jarman et al. 1993; Akazawa et al. 1995). Although Atoh1 can bind to E-boxes, it must form a heterodimer with a second bHLH transcription factor such as Da or E47 (E12) to activate target gene transcription. Typically, bHLH transcription factors bind to the E-box motif (5' CANNTG 3'). The basic domain of Atoh1 contains a unique palindromic motif that can bind to E-box

sequences, conferring specificity of DNA binding. Analysis of Atoh1 binding interactions during cerebellar development has identified the Atoh1 E-box-associated motif, AtEAM (5' G/A, C/A, CA, G/T, C/A, TG, G/T, C/T 3') (Chien et al. 1996; Klisch et al. 2011).

Identification of the AtEAM motif has facilitated genome-wide screening to find potential targets for Atoh1-mediated gene transcription (Klisch et al. 2011). Screening resulted in the identification of 601 genes that Atoh1 can potentially regulate (Klisch et al. 2011). Of the putative targets, 105 were involved in gene transcription; 33 in the cell cycle; 33 in chromosomal organization; 132 were metabolic genes; 15 were associated with Sonic Hedgehog (SHH) signaling, 12 with Notch signaling, 14 with TGF β signaling, 15 with Wnt signaling, and 11 with Jun N-terminal kinase (JNK)/mitogen-activating protein kinase (MAPK) signaling (Klisch et al. 2011). Overall, Atoh1 has been reported to be involved mainly in cell cycle maintenance and differentiation, as befits a proneural gene; however, the function of Atoh1 seems to vary among tissues and developmental contexts. In summary, Atoh1 is a transcription factor with diverse targets, but a high degree of specificity. Its DNA-binding basic domain binds specifically to the AtEAM and Atoh1 can associate with specific protein cofactors selected for by its helix–loop–helix domain and possibly its N terminus.

Atoh1 IS REQUIRED FOR HAIR CELL DEVELOPMENT

Atoh1 expression in the developing organ of Corti

Three methods have been used to characterize the spatiotemporal pattern of Atoh1 in the mouse inner ear: reporter-gene studies, in situ hybridization (ISH), and immunohistochemistry. There is some discrepancy among the reports, some of which can be explained by the limitations of each approach.

ISH for Atoh1 shows that expression starts in the prosensory epithelium of the basal turn of the cochlea between E12.5 and E13, spanning the epithelium from the ventral floor to the luminal roof (Lanford et al. 2000; Yang et al. 2010). At E13.5, a row of *Atoh1*-expressing cells can be detected running from the base to the mid-base (Matei et al. 2005) (illustrated in Fig. 3A). By E15, transcripts are detected along the length of the duct, with a bias towards the luminal surface (Fig. 3C). By E17, Atoh1 is expressed only in the single row of inner hair cells (IHC) and three rows of outer hair cells (OHC) (Fig. 3C). This expression continues to P0, but by P3, Atoh1 starts to be downregulated, proceeding in a basal to apical gradient (Lanford et al. 2000; Yang et al. 2010).

FIG. 3. A schematic diagram of the onset of Atoh1 expression in the mouse cochlea. **A** At E13.5, there is no detectable Atoh1 protein in the cochlea; however, mRNA expression has initiated at the base. Cross-section through the mid-base shows mRNA expression arises in the prosensory epithelia in a broad band spanning the depth of the epithelial layer. **B** At E14.5, Atoh1 protein is expressed in a single row of cells from the base to the mid-base of the cochlea. Cross-section through the mid-base shows Atoh1 protein is in one cell that will become an inner hair cell (IHC) but mRNA is expressed more broadly. **C** At E15.5, Atoh1 protein is in four rows of cells running

from the base to the mid-base, three rows from the mid-base narrowing to two rows of Atoh1-positive cells towards the apex. Cross-section through the mid-base shows Atoh1 protein is detected in three cells, with Atoh1 mRNA beginning to be restricted to cells closer to the luminal surface. **D** At E17.5, four rows of cells extend to the mid-base, three rows continue towards the apex that are Atoh1-protein-positive. Cross-section through the mid-base shows Atoh1 protein is found in one IHC and three OHCs. Atoh1 mRNA is now restricted to the HCs. *M* medial; *L* lateral; *IHC* inner hair cell; *OHCs* outer hair cells. Solid black line indicates level of cross-section.

Effectively, the expression starts in the base, spanning several cells through the depth of the epithelium, and spreads to the apex. Concurrent with the appearance of HCs, Atoh1 transcripts are restricted to first a single row of IHCs and then to the OHCs as each row arises. It appears from the ISH data that *Atoh1* is expressed not only in cells that will become HCs, but also in other cell types. Lineage tracing experiments provide evidence indicating that cells expressing Atoh1 can develop to form supporting cells in addition to HCs (Matei et al. 2005; Yang et al. 2010).

Atoh1 immunohistochemistry confirms that at E14.5 Atoh1 is expressed in the basal region of the cochlea in two discrete rows spanning the sensory epithelium and corresponding to IHCs and OHCs as they begin to differentiate. At E15.5, Atoh1 is restricted to the characteristic pattern of labeling one row of IHCs and three rows of OHCs in the basal region, two rows corresponding to the single row of IHCs and one row of OHC in the medial region, and one column of cells in the apical region (Chen et al. 2002).

Substituting the Atoh1-coding region for β -galactosidase (β Gal) provides a means of labeling cells where the Atoh1 promoter is active. Mice heterozygous for *Atoh1* and β Gal first show a short band of β Gal-positive cells at E12.5 in the prospective sensory epithelia (Bermingham et al. 1999). At E13.5, it is expressed in a short band of cells extending from the base of the cochlea (Matei et al. 2005). By E14.5, it is detected in the cochlea where it displays an apical to basal gradient of intensity of staining (Bermingham et al. 1999). By E18.5, β Gal activity is detected in four rows of hair cells in the base, narrowing through to a single row in the apex (Bermingham et al. 1999; Woods et al. 2004; Fritsch et al. 2005).

Expression of an EGFP driven by a fragment of the Atoh1 enhancer identifies a small number of GFP-positive cells in the otic vesicle at E11.5 (Matei et al. 2005). Reverse transcription polymerase chain reaction demonstrates increasing amounts of Atoh1 RNA in mouse sensory epithelium from E12.5 (Zine et al. 2001); however, ISH does not detect Atoh1 transcripts

in the presumptive organ of Corti (OC) until E13 (Lanford et al. 2000). It could be that transcript levels are too low for DIG-labeled riboprobes to be detected and visualized by alkaline phosphatase-conjugated antibodies; perhaps radiolabeled probes would give more information at these earlier time points. There is also some discrepancy between the ISH data and data obtained with an EGFP-reporter made by fusing Atoh1 3' enhancer to beta-globin basal promoter. Whereas Atoh1 RNA is detected spanning the putative sensory epithelium at the base from E13, becoming restricted to the HCs as they differentiate, in the EGFP-reporter mouse, Atoh1 is only expressed in cells that differentiate into HCs (Chen et al. 2002). Between E13.5 and E14.5, a single row of EGFP-positive cells (corresponding to presumptive IHCs) is detected on the medial border of the postmitotic nascent sensory epithelium (Chen et al. 2002) (Fig. 3B). As the cochlea matures, this row extends apically, and at around E14.5, a second row of GFP-positive cells begins to extend from the base. By E15.5, GFP is visible at the base in the characteristically patterned single row of IHCs and three rows of OHCs (Chen et al. 2002) (Fig. 3C). Comparison of RNA expression versus reporter-gene protein activity shows that RNA is first detected in a greater population than is fated to differentiate into hair cells and is gradually restricted to the HCs, whereas protein activity is confined to cells in the process of differentiating into HCs. It is predicted that there is at least one other enhancer, as yet unidentified, that could be modulating the expression detected by ISH that is not recapitulated by the EGFP-reporter. This could indicate that the dynamics of the changing pattern as development progresses involve interplay between Atoh1 and extrinsic factors and that Atoh1 can modulate its own expression. In brief, analyses of β Gal-insertion and GFP-reporter lines agree that expression of Atoh1 starts between E13 and E14 as four rows of cells that extend towards the apex serially as development progresses (Fig. 3), continuing until P4 at which time Atoh1 promoter/enhancer is no longer active. ISH analysis suggests that Atoh1 is produced in a broader pattern a day earlier than the onset of GFP expression, then refined over time to one row of IHCs and three rows of OHCs (Fig. 3).

Atoh1 and auditory hair cells

It has been demonstrated that Atoh1 is necessary for the development of auditory HCs. The cochlea of a homozygous Atoh1 mutant mouse lacks differentiated HCs. Histological analysis and electron micrographs of cochlear tissue throughout development and at P0 show that, within the zone usually assigned to the OC, HCs are absent (Bermingham et al. 1999; Pan et al.

2010). Further analysis using immunohistochemistry and ISH to identify individual cell types at P0 showed that not only are there no cells expressing HC markers such as myosin 6 and calretinin, but that there are also no cells expressing markers of the various organ of Corti supporting cells (SCs), including inner pillar cells (probed for Jagged1, Fgfr3, TC2, and p75^{NF}), Deiters' cells (probed for Jagged1, glial fibrillary acidic protein, TC2, and Fgfr3) and phalangeal cells (probed for Jagged1 and glial fibrillary acidic protein) (Woods et al. 2004). Although at P0 SCs are not found, examination of Atoh1 null cochleae at E16.5 demonstrates that there are cells in the region that would correspond to the OC that are immunoreactive for the SC markers Jagged1, Prox1, and Sox2 (Dabdoub et al. 2008). Given the caveat that there are no true SC markers, and that Jagged1, Sox2, and Prox1 are expressed first in prosensory cells and then in SCs, it is not clear whether the cells that are immunoreactive for these markers are cells that remain in the prosensory undifferentiated state or are differentiated SCs.

In the organ of Corti of Atoh1-null mice, Caspase 3 activity shows a wave of apoptosis between E14 and E18 that mimics the wild-type front of HC differentiation (Chen et al. 2002). It has been observed, however, that cells that look histologically similar to SCs are present at P0 (Bermingham et al. 1999), and the structure of the tissue is consistent with wild-type patterning at E16.5, E18.5, and P1 (Fritzsch et al. 2005; Dabdoub et al. 2008; Pan et al. 2010). It has been demonstrated that specification of the sensory epithelium is independent of Atoh1 (Chen et al. 2002; Dabdoub et al. 2008); this would mean that, during early development of the OC, SC markers such as Sox2, Prox1, and Jagged1 would be detected in Atoh1-null animals, as these factors are required for early patterning. Furthermore, it has been demonstrated that cells expressing Atoh1 can develop into either HCs or SCs (Matei et al. 2005; Yang et al. 2010), and examination of the homozygous β Gal knock-in mouse at E18.5 shows Atoh1 promoter activity in a greater population of sensory epithelial cells than would have differentiated into HCs (Fritzsch et al. 2005). Notch signaling has been shown to regulate the segregation of prosensory cells into either HC fate (Notch-signaling cells) or SC fate (Notch-receiving cells) through the process of lateral inhibition (Lanford et al. 1999). In the absence of Atoh1, it is possible that, after the initial specification of SCs, the cells begin to differentiate, but the lack of Atoh1 causes the lateral inhibition loop between Atoh1-expressing cells and Hes-expressing cells to break down. The reduced levels of Hes might cause SCs either to undergo apoptosis or to revert to flat epithelium (a layer of epithelium lacking characteristic organization of the

OC and immunoreactivity for markers of HCs and SCs).

A chimeric mouse was generated to circumvent the perinatal lethality of the homozygous null *Atoh1* mutant, with surprising results. The chimeric *Atoh1* null mouse exhibited normal patterning of the OC, with one row of IHCs and three rows of OHCs. Immunohistochemistry confirmed the presence of calretinin, myosin VIIa, and stereocilia (phalloidin staining) in all the alleged HCs (Du et al. 2007). The presence of β Gal in clusters of these HCs demonstrates that they contain no endogenous *Atoh1* and do not retain the ability to produce it. In addition, as is typical for HCs which are interspersed with supporting cells in the wild-type, there was no cell–cell contact between *Atoh1*-negative HCs and *Atoh1*-positive HCs (Du et al. 2007). These findings suggest that *Atoh1* is required only for very early events in HC differentiation and might induce secreted factors that can potentiate lateral inhibition in the absence of endogenous *Atoh1*. Bearing in mind that a degree of tissue organization persists in the *Atoh1* null cochlea at P1 (Fritzsch et al. 2005; Dabdoub et al. 2008; Pan et al. 2010), it is possible that these are HCs that have entered only initial stages of differentiation (Du et al. 2007). Furthermore, it was not possible to examine these putative HCs in mature cochleae as β Gal is only expressed in cells with active *Atoh1* promoter activity (*Atoh1* is not expressed after P4), so it is not known whether these cells retain their identity into adulthood or are functional (Du et al. 2007). Examination of the Pax2-Cre conditional knock out of *Atoh1* at P7 shows very few myosin VIIa-positive cells in receipt of the few remaining nerve fibers (Pan et al. 2010) while, at E18.5 and P0, there are no HCs. This implies that neural cells may supply extrinsic factors. Taken together, the chimeric mouse and the late-appearing HCs of the Pax2 conditional knock-out suggest some interesting possibilities for the mechanism by which expression of *Atoh1* is required for HC differentiation; it could be that, rather than directly inducing HC genes, it has a structural role in the regulatory network-controlling patterning.

In mammals, HCs of the vestibular system can regenerate and ectopic expression of *Atoh1* in mature animals results in supernumerary HCs (Forge et al. 1993; Lopez et al. 1997; Wang et al. 2010); however, *Atoh1* has limited ability to generate ectopic HCs in the mature cochlea. Several groups have successfully generated ectopic HCs by transfecting cochlear explants or by in utero electroporation with *Atoh1* under the control of a constitutively active promoter (Zheng and Gao 2000; Gubbels et al. 2008). Epithelial cells of the greater epithelial ridge readily take on a HC fate demonstrating that, even after HC differentiation has started, surrounding cells of the cochlea

retain the competence to take on a sensory identity (Zheng and Gao 2000). Attempts to produce ectopic HCs in mature animals and to regenerate HCs in vivo following cochlear injury by either ototoxic drugs or excessive noise have had limited success. One group has shown that adenovirus-mediated expression of *Atoh1* in mature guinea pig cochleae results in ectopic hair cells (Kawamoto et al. 2003) and infecting newly injured guinea pigs with an adenovirus carrying *Atoh1* leads to regeneration of HCs; however, this approach failed to restore HCs in animals with longer-term injuries or more damaged cochleae (Izumikawa et al. 2005, 2008). Replication of these results and further investigation into the requirement for SC survival may yield useful information.

Atoh1 IN DIFFERENTIATION, PROLIFERATION, AND APOPTOSIS OF NEURONS AND INTESTINAL CELLS

Atoh1 in neurogenesis

As a gene first cloned as a candidate proneural factor, *Atoh1* is well characterized as having a role in neurogenesis. Ectopic expression of *Atoh1* in the multipotent teratocarcinoma cell line P19 converts undifferentiated cells into neurons and downregulates the cell cycle (Farah et al. 2000). In vivo *Atoh1* is expressed and has been shown to be active in many regions of the developing nervous system including the dorsal neural tube, the rhombic lip, and the developing cerebellum. In dorsal neural tube, *Atoh1* is co-expressed with markers for dorsal commissural interneurons; cross-inhibition with Neurogenin1 allows differentiation into distinct populations of neuronal sub-types (Helms and Johnson 1998; Gowan et al. 2001). *Atoh1* also specifies the fate of the dorsal Lim1 neuronal lineage; in its absence, cells take on the fate of neighboring populations, roofplate, and dorsal Lim2 neurons (Bermingham et al. 2001; Gowan et al. 2001). *Atoh1* null mice lack rhombic lip derivatives that contribute to the proprioceptive, vestibular, and auditory sensory network and to the parafacial respiratory group/retrotrapezoid nucleus that controls breathing (Machold and Fishell 2005; Wang et al. 2005; Rose et al. 2009a; Rose et al. 2009b). *Atoh1* interacts with the transcription factor Tcf4 to induce differentiation of pontine neurons of the brainstem (Flora et al. 2007). In the cerebellum, *Atoh1* is required for specification of cerebellar granule cells (Ben-Arie et al. 1997) and is the earliest marker of developing granule cells in the external germinal layer (EGL) (Machold and Fishell 2005; Wang et al. 2005). SHH and Notch signaling have been shown to cross-regulate with *Atoh1* to allow differentiation of granule cells (Gazit et al. 2004).

SHH signaling regulates the size of the pool of granule cell precursors; in response to SHH, cells exit the cell cycle and differentiate (Flora et al. 2009). Induction of *Gli2* (a component of SHH) expression by Atoh1 renders cells responsive to SHH (Flora et al. 2009).

Atoh1 in intestine development and homeostasis

Atoh1 also has roles in homeostasis and dysplasia in the intestine. Mice null for Atoh1 lack goblet cells, and epithelial cells are maintained in a proliferative state (Yang et al. 2001). Atoh1 is required to specify a subgroup of secretory cells that give rise to paneth cells, goblet cells, and enteroendocrine cells through induction of the bHLH transcription factor Neurogenin 3 and the transcriptional repressor Gfi1 (Yang et al. 2001; Shroyer et al. 2005; Bjerknes and Cheng 2006). Overexpression of Atoh1 results in ectopic secretory cells (VanDussen and Samuelson 2010). Atoh1 can arrest the cell cycle during intestinal development by repressing the transcription factor RBP-J κ , which is implicated in Notch-mediated cell proliferation. It is also suggested that, independently of Notch, Atoh1 can regulate the cell cycle proliferation genes p57^{kip} and p27^{kip} (Kazanjian et al. 2010; Kim and Shivdasani 2011; Peignon et al. 2011). Although Atoh1 has been implicated in regulation of p27^{kip} in the gut and in medullablastoma (whether this regulation is direct is unclear), p27^{kip} is not regulated by Atoh1 in the inner ear (Chen et al. 2002).

Atoh1 in dysplasia

Atoh1 activity in the developing cerebellum maintains cells in a proliferative state whereas, in the intestine, it causes cells to exit the cell cycle and differentiate. It follows that uncontrolled proliferation can result from Atoh1 gain-of-function in developing cerebellum but from Atoh1 loss-of-function in intestine.

In medullablastomas arising from the EGL, Atoh1 is an oncogene. In medullablastomas strongly expressing Atoh1, markers of differentiation such as TuJ1, p27^{kip1}, NeuroD1, and Tag-1 are downregulated, whereas SHH target genes such as *Gli1* and *Gli2*, which control the cell cycle are upregulated. This pattern of expression suggests that Atoh1 is required to maintain cells at an early stage of specification in order to provide time for clonal expansion (Briggs et al. 2008; Zhao et al. 2008; Ayrault et al. 2010).

In other tissues, Atoh1 acts not as an oncogene, but as a tumor suppressor gene in adenomatous polyposis carcinoma (APC), Atoh1 is downregulated (Leow et al. 2004; Bossuyt et al. 2009b). Whereas in neural development Atoh1 makes cells receptive to signals

promoting proliferation, in gut development and homeostasis, Atoh1 represses proliferation. Atoh1 interacts with the N-methyltransferase, Dmmt, to antagonize tumor progression by (1) inducing differentiation into goblet cells, (2) halting proliferation, and (3) positively regulating apoptosis by modulating the JNK/MAPK pathway (Bossuyt et al. 2009b; VanDussen and Samuelson 2010).

Abnormal apoptotic response can also contribute to dysplasia. In APC carcinomas, ATOH1 acts through JNK/MAPK signaling to activate apoptosis, and in fly eyes, over-expression of *ato* leads to elevated apoptosis and an increase in the apoptotic marker Caspase 3 (Bossuyt et al. 2009a; Bossuyt et al. 2009b). Over-expression of Atoh1 in the brain results in elevated levels of apoptosis (Isaka et al. 1999), which is surprising given that in medullablastoma the apoptotic pathway is not activated. Atoh1-mediated cell death may also result from gene dosage effects rather than from specific targeting by Atoh1. Excess levels of Atoh1 in a cell may prompt the cell to enter the apoptotic pathway as a protective measure. Atoh1 activity (or lack thereof) in cancer is part of a multifactorial process. Epistasis and complementation can make it difficult to attribute cellular behaviors to specific genes. In the absence of NeuroD1 during cerebellar development, Atoh1 expression and proliferation are upregulated; however, because NeuroD1 is missing, the cells cannot differentiate and are forced to undergo apoptosis (Pan et al. 2009). Thus, it could be argued that, in some of the tumors where Atoh1 apparently causes cell death or proliferation, there is another factor required downstream that is also abnormally expressed.

In summary, Atoh1 expression is found in various parts of the developing embryo and exerts varying influence depending on the cell type and developmental stage. It has been shown that targets of Atoh1-mediated transcription are tissue-dependent (Lai et al. 2011). While there are some targets of Atoh1 that are common to all tissues, some are restricted to specific populations; for example, Atoh1 binds sites within enhancers of *Rassf4*, *Selm*, *Atoh1*, and *Grem2* in cerebellar tissue but does not drive their expression in the dorsal neural tube (Lai et al. 2011). Furthermore, targets validated in neural tissue are not necessarily transcribed in other developmental contexts; for example, only *Rab15* and *Selm* have as yet been identified as targets regulated in both the inner ear and neural tissue (Lai et al. 2011).

REGULATION OF Atoh1

Genetic regulation

In mice, two enhancer sites found 3.4 kb 3' of the *Atoh1* coding region have been identified (Helms and

Johnson 1998; Helms et al. 2000) (Fig. 1, B). These enhancers drive expression in the EGL of the cerebellum and in the ear at E14.5 and P0 (Helms et al. 2000). Enhancers A and B are 561 and 544 bp, respectively, separated by 300 bp.

There is no sequence similarity between A and B. Enhancer B contains an E-box selective for *Atoh1* (Ebert et al. 2003), which is essential for *Atoh1* expression in the neural tube. With the exception of dorsal neural tube expression, the two enhancers are redundant to each other. Interestingly, examination of *HATH1* regulatory sequences shows that the human and mouse enhancers share 92% identity at enhancer A and 87% at enhancer B; this is a greater similarity than that between the orthologous proteins (Helms et al. 2000).

Whether expression of *Atoh1* in vivo requires multiple combinations of different transcription factors or a single factor to effect changes in transcription, the size of enhancers A and B is suggestive of multiple protein-binding sites. Several proteins have been identified that directly interact with an *Atoh1* enhancer as demonstrated by chromatin immunoprecipitation, electrophoretic mobility shift assays, or luciferase activity, for example, *Zic1*, *cdx2*, *Hfn1a*, β -catenin, and *Hic1* (Akazawa et al. 1995; Ebert et al. 2003; Mutoh et al. 2006; Briggs et al. 2008; D'Angelo et al. 2010; Shi et al. 2010). *Atoh1*, *Sox2*, β -catenin, *Hfn1a*, and *Cdx2* upregulate *Atoh1* expression (Helms

et al. 2000; Ebert et al. 2003; Gazit et al. 2004; Mutoh et al. 2006; D'Angelo et al. 2010; Shi et al. 2010; Neves et al. 2011). *Hes1*, *Hes5*, *Sox2*, *Hic1*, *Ngn1*, *NeuroD1*, and *Zic1* repress *Atoh1* at the level of transcription (Akazawa et al. 1995; Gowan et al. 2001; Ebert et al. 2003; Qian et al. 2006; Briggs et al. 2008; Dabdoub et al. 2008; Pan et al. 2009; Jahan et al. 2010). Intriguingly, *Sox2* has the ability to both upregulate and downregulate *Atoh1* depending on context (Dabdoub et al. 2008; Neves et al. 2011). In human APC carcinomas, it has been shown that *ATOH1* is silenced through DNA methylation allowing tumors to escape cell cycle control and apoptosis (Bossuyt et al. 2009b).

Post-translational regulation

Activity of *Atoh1* is also modulated at the protein level through (1) formation of heterodimers that restrict its transcriptional activities, (2) competitive-binding to E-boxes, and (3) possibly post-translational modification. The abundance of serine residues in the C terminus implies that phosphorylation might be used to modulate activity (Fig. 4A). Inhibitors of differentiation and DNA binding (Ids) 1, 2, and 3 all negatively regulate *Atoh1* by competitive-binding to E proteins (Connerney et al. 2006; Jones et al. 2006) (Fig. 4B). *Atoh1* can bind to the transcription factor *Tcf4*, creating heterodimers that specifically induce pontine nuclear neuron differentiation. This interac-

FIG. 4. A schematic diagram illustrating some of the ways that *Atoh1* activity is modulated at the protein level. **A** Phosphorylation may prevent interaction of *Atoh1* with either DNA or with class I bHLH transcription factors such as E47, thereby preventing target gene transcription. **B** Ids, HLH proteins lacking the DNA binding basic region, may competitively bind E proteins displacing *Atoh1*. **C** *Atoh1*-mediated transcription is dependent on specific cofactors.

Atoh1 coexpressed in 3 T3 cells with *Tcf4*, a class I bHLH transcription factor, results in differentiation of pontine neurons, but if E47 is substituted for *Tcf4*, *Atoh1* does not promote transcription of pontine neuron genes. **D** Addition of E47 and *Atoh1* to HEK293 cells carrying an E-box luciferase reporter results in luminescence; adding the transcription factor *Hes1* abolishes luminescence, suggesting that *Hes1* competes with *Atoh1* in binding E proteins.

tion is highly specific; substituting other protein partners such as E47 caused cells to take an alternative fate (Flora et al. 2007) (Fig. 4C). Co-transfection of C3H10T1/2 cells with Atoh1, an E-box luciferase-reporter and E47 resulted in luciferase activity, but, on addition of the transcription factor Hes1, luminescence was abolished, implying that Hes1 out-competes Atoh1 in binding E proteins (Akazawa et al. 1995) (Fig. 4D).

In the gut, canonical Wnt signaling maintains equilibrium between β -catenin and Atoh1. In the presence of Wnt signaling, β -catenin is allowed to accumulate in the nucleus causing cell proliferation; Atoh1 is phosphorylated and targeted for ubiquitin-mediated degradation. When no Wnt signal is received, β -catenin is targeted for degradation in place of Atoh1; Atoh1 is allowed to accumulate in the nucleus, and cells exit the cell cycle and differentiate (Leow et al. 2004; Aragaki et al. 2008; Bossuyt et al. 2009b; VanDussen and Samuelson 2010; Peignon et al. 2011). In medullablastoma cells, addition of BMP2 and 4 leads to a down-regulation of Atoh1 (Zhao et al. 2008). In this case, Atoh1 may be targeted for degradation either through phosphorylation by BMP downstream effectors or by displacement of Atoh1 by Id proteins. Such interactions would be quite different from BMP's transcriptional activation of Atoh1 in the rhombic lip. In the cochlea, BMP signaling is required for specification of the sensory epithelium (Li et al. 2005), but, during HC differentiation, is upstream of Ids 1, 2, and 3, which antagonize Atoh1 activity (Kamaid et al. 2010).

In summary, Atoh1 has been shown to be regulated at both transcriptional and post-translational levels. Multiple regulators of Atoh1 transcription have been identified. Protein associations in tandem with competitive inhibition are proposed to modulate Atoh1 activity at the protein level, and it is likely that

phosphorylation-mediated degradation is employed to remove Atoh1 from the system; although, so far, direct evidence that Wnt signaling targets Atoh1 for destruction exists only for intestinal cells.

CONCLUSION AND PERSPECTIVES

Regulation of Atoh1 is exquisite, with multiple layers of control. As a transcription factor with such diverse targets, highly precise doses at clearly defined stages of development are crucial. Within the developing cochlear sensory epithelium, Sox2, Prox1, NeuroD1, Hes1, and Hes5 may repress Atoh1 transcription; it can be antagonized at the protein level by Ids, Hes1, and Hes5. Atoh1 can be targeted for degradation by GSK3 β in the gut and by BMP signaling during neural patterning. Although Atoh1 has been shown to be both necessary and sufficient to drive HC differentiation, interaction with co-factors is clearly necessary to explain why, in the gut, Atoh1 expression induced cells to take on a secretory lineage, as opposed to HCs or the variety of neuronal subtypes it can specify.

The molecular context within which Atoh1 is expressed is crucial to its activity. The proline-rich N terminus (Fig. 2A) suggests protein interactions in addition to those allowed by the HLH domain at its center. Atoh1 response to Wnt, BMP, and SHH signaling is also context-dependent. Table 1 summarizes the outcome of activating these pathways in different tissues, sometimes at the transcriptional level, sometimes at the protein level. BMP and Wnt signaling can both target Atoh1 for destruction. There are multiple conserved potential phosphorylation sites in

TABLE 1

Atoh1 is differently modulated by signaling pathways depending on developmental context

Signaling pathway	Tissue	Repression of Atoh1	Up regulation of Atoh1	Transcriptional regulation	Post translational regulation	Reference
Wnt	Gut	Yes	No	No	Yes	(Leow et al. 2004; Aragaki et al. 2008; Bossuyt et al. 2009b; VanDussen and Samuelson 2010; Peignon et al. 2011)
	Pontine neurons	No	Yes	Yes	No	(Flora et al. 2007)
	Chicken ear	No	Yes	?	No	(Stevens et al. 2003)
BMP	Rhombic lip	No	Yes	Yes	No	(Alder 1999; Lee et al. 2000; Wine-Lee et al. 2004)
	Granular neuron precursor expansion	Yes	No	No	Yes	(Zhao et al. 2008)
	Cochlea	Yes	No	No	Yes	(Li et al. 2005; Kamaid et al. 2010)
SHH	Granular neuron precursor expansion	Yes	Yes	Yes	No	(Kenney and Rowitch 2000; Romer et al. 2004; Briggs et al. 2008; Zhao et al. 2008)
	Cochlea	No	Yes	Yes	No	(Zhao et al. 2008; Hu et al. 2010)

the C terminus that may allow post-translation modification, thus facilitating regulation (Fig. 2A).

Damage to hair cells from any of multiple causes (age, toxic drugs, noise, and disease) is a common cause of hearing loss. Having identified Atoh1 as an important factor in HC differentiation there is much potential for exploring Atoh1-mediated HC regeneration. Non-mammals such as birds have the ability to regenerate HCs in the inner ear after injury by sound or drug (Cruz et al. 1987; Corwin and Cotanche 1988), but that is not the case in mammals. Ectopic expression of Atoh1 in mature cochleae has had limited success. This cannot be due to transcriptional regulation of Atoh1 since it is under the control of a constitutively active promoter. It is possible that the promoter used in the vector expressing Atoh1 has tissue specific limitations (Praetorius et al. 2010), but more likely that epigenetic changes in the cochlea render cells relatively insensitive to Atoh1, that Atoh1 is targeted for destruction, that Atoh1 is post-translationally modified ablating its function or that cofactors required for HC-specific transcription factor complexes are down-regulated. Identification of protein co-factors of Atoh1 during development and protein-level repression of Atoh1 postnatally could give an answer. It may also be of value to search for phosphorylation sites conserved in species that do not regenerate HCs but not in species that do. The N terminus domain is well conserved among mammalian species, but not in non-mammals. Furthermore, there are several potential phosphorylation sites in the C terminus outside the serine-rich domain that may only be conserved across species that do not regenerate HCs.

Examination of the regions flanking the bHLH domain and a better understanding of protein-level regulation of Atoh1 could open more avenues of investigation into hearing loss, medulloblastoma, colon cancer, as well as various developmental disorders.

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