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The Role of Atonal Factors in Mechanosensory Cell Specification and Function

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Abstract *Atonal* genes are basic helix-loop-helix transcription factors that were first identified as regulating the formation of mechanoreceptors and photoreceptors in *Drosophila*. Isolation of vertebrate homologs of *atonal* genes has shown these transcription factors to play diverse roles in the development of neurons and their progenitors, gut epithelial cells, and mechanosensory cells in the inner ear and skin. In this article, we review the molecular function and regulation of *atonal* genes and their targets, with particular emphasis on the function of *Atoh1* in the development, survival, and function of hair cells of the inner ear. We discuss cell-extrinsic signals that induce Atoh1 expression and the transcriptional networks that regulate its expression during development. Finally, we discuss recent work showing how identification of *Atoh1* target genes in the cerebellum, spinal cord, and gut can be used to propose candidate *Atoh1* targets in tissues such as the inner ear where cell numbers and biochemical material are limiting.

Keywords Atonal · Atoh1 · Sensory · Cochlea · Hair cells · bHLH · Transcription factors

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Identification of *Atonal* and Its Function in *Drosophila*

Proneural genes play essential roles in the development of the peripheral nervous system (PNS) in *Drosophila* [1]. There are three major sensory organs in *Drosophila* PNS: external sensory organs, chordotonal organs, and multidendritic neurons. Members of the *achaete-scute* complex (*AS-C*) are proneural genes required for the formation of all external sensory organs and some multidendritic neurons [2–5] but not chordotonal organs [6]. In order to identify proneural genes that regulate the formation of chordotonal organs, *atonal* was isolated by polymerase chain reaction (PCR) amplification of *Drosophila* genomic DNA using degenerate primers designed from shared features between members of the *AS-C* complex [7]. *Atonal* is a single-exon gene which encodes a protein of 312 amino acids with a size of 32 kDa. The protein product of *atonal* contains a basic helix-loop-helix (bHLH) motif at its very C-terminus [7]. The bHLH sequence is a ~60-amino acid protein structural motif characterized by two conserved domains: an N-terminal basic domain that binds to DNA consensus sequences called E-boxes (with the core sequence CANNTG) and a C-terminal HLH domain composed of two helices connected by a loop that can form heterodimers with other bHLH proteins [8]. The bHLH motif of atonal shares a high degree of similarity with those of other bHLH proteins: 46 % identity with scute and 30 % with daughterless, although its location within the protein can vary—for example, AS-C proteins do not have the bHLH motif at the C-terminus. Electrophoretic mobility shift assays showed that the atonal protein can form a heterodimer with the ubiquitously expressed bHLH protein daughterless to bind to E-boxes [7].

Chordotonal organs are sensory organs widely distributed throughout the body of adult and developing *Drosophila*, where they function in detecting movements and stretch of the limbs and body wall. In the second segment of the antenna, a large chordotonal organ, termed Johnston's organ, also

serves to detect sound, gravity, and air movements in the environment [9]. In situ hybridization showed that *atonal* transcripts were expressed in the regions of the embryo and developing imaginal discs which give rise to chordotonal organs. In these areas, *atonal* was initially expressed in patches of epidermal cells, followed by a more restricted and stronger expression in the sensory organ precursors (SOPs) of each cluster. Chordotonal organs and some multidendritic neurons are absent from the embryos of *atonal* mutant flies, but external sensory organs are not affected [7]. Gain-of-function experiments in *Drosophila* generated ectopic chordotonal organs observed after global mis-expression of *atonal* [7], suggesting *atonal* is not only necessary but also sufficient for chordotonal organ development in *Drosophila*. In light of the expression of *atonal* in chordotonal organ precursors and its necessity and sufficiency in the development of chordotonal organs, we can conclude *atonal* is a proneural gene specific for chordotonal organ formation [10].

Besides the areas forming the future chordotonal organs, *atonal* expression is also observed in the developing eye in *Drosophila* [7, 11]. In the *Drosophila* eye imaginal disc, *atonal* expression initiates on the anterior edge of the morphogenetic furrow, and then becomes restricted in regularly spaced cells which will differentiate into R8, the first photoreceptor formed in each *Drosophila* ommatidium. Loss- and gain-of-function experiments together suggest *atonal* is both necessary and sufficient for R8 selection during *Drosophila* eye development [11]. Although *atonal* is not directly involved in the development of other photoreceptors (R1–R7), their formation still relies on R8 induction [12]. Therefore, *atonal* also acts as a proneural gene in the formation of *Drosophila* photoreceptors. *Atonal* and *amos* are also necessary for the formation of olfactory and gustatory sensory organ precursors in larval olfactory organs [13].

The Evolution of *Atonal* Homologs

bHLH transcription factors can be found in a wide range of eukaryotes from yeast to humans and play important roles in a large number of developmental processes. Two types of bHLH proteins have been shown to function in neurogenesis. Class I bHLH proteins (also known as E-proteins) are broadly expressed, including E12, E47, HEB, and E2-2 in vertebrates and daughterless in *Drosophila*. They can form homo- or heterodimers to bind E-box sequences and regulate transcription. Class II bHLH proteins are expressed in tissue-specific patterns and usually heterodimerize with class I members to bind E-boxes [8]. In *Drosophila*, both atonal and AS-C members belong to the class II bHLH family.

Atonal homologs show evidence of duplication from an ancestral diploblast *atonal* gene [14]. In *Drosophila*, two *atonal*-related genes were isolated by PCR using degenerate primers based on homology to the atonal bHLH motif. These genes were named *absent multidendritic and olfactory sensilla* (*amos*) [15] and *cousin of atonal* (*cato*) [16]. The bHLH region of *amos* and *cato* are 74 and 64 % identical to that of atonal, respectively. Both *amos* and *cato* are involved in the development of *Drosophila* sensory organs [15, 16]. *Amos* is a proneural gene that regulates the development of two classes of olfactory neurons and a class of multidendritic neurons [15, 17, 18]. *cato*, which is widely expressed in PNS after the selection of neural precursors and before their terminal differentiation, is required for proper sensory neuron morphology [16, 19].

Vertebrate atonal homologs were originally identified as eight members of atonal homolog (Atoh) family. Three of them—Atoh1, Atoh7, and Atoh8—have now been designated as Atoh family members, with the rest of the vertebrate atonal homologs having been re-assigned to a closely related bHLH family, the Neurogenin (Neurog)/Neurogenic differentiation factor (NeuroD) family (Fig. 1). Mouse *Atoh1* (formerly called *Math1*) plays essential roles in the development of different types of cells, including neurons in the brain [22–26] and the dorsal spinal cord [27, 28], intestinal secretory cells [29, 30], Merkel cells [31], and inner ear hair cells [32–34]. The expression of mouse *Atoh7* (formerly called *Math5*) is mostly restricted to the developing retina. *Atoh7* is critical for the formation of retinal ganglion cells and optic nerves [35, 36]. *Atoh8* (formerly called *Math6*) is widely expressed in multiple organs and is involved in the development of brain, kidney, liver, pancreas, retina, and skeletal muscle [37–40].

Unlike the Atoh family, Neurog/NeuroD family genes have been lost in some eukaryote lineages, such as arthropods (including insects such as *Drosophila*) [14]. *Neurog1* (also known as *NeuroD3*) is a key regulator that promotes neurogenesis in the CNS and sensory organs [41–45]. *Neurog2* (also known as *Atoh4*) participates broadly in neurogenesis in the cortex, cerebellum, olfactory system, and cranial ganglia [46–49]. Although *Neurog3* (also known as *Atoh5*) is expressed mainly in the pancreas and plays an important role in the differentiation of endocrine cells [50], it

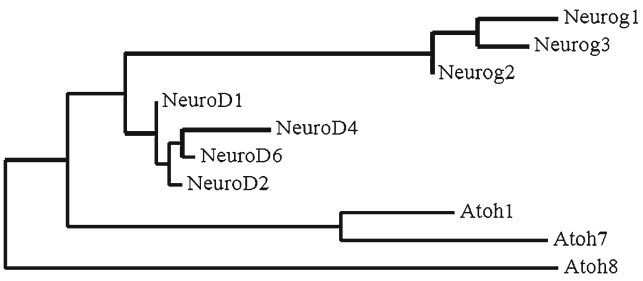

Fig. 1 Phylogenetic tree showing the close relationship between the Atoh and Neurog/NeuroD families. The phylogenetic tree was calculated with full-length amino acid sequences from mouse members of the Atoh and Neurog/NeuroD families and generated by Phylogeny.fr [20, 21]

has also been shown to affect dendrite morphology and the ratio of excitatory/inhibitory synaptic inputs in the hippocampus [51] and is also expressed in the developing hypothalamus [52]. In general, NeuroD subfamily members tend to be expressed in more mature neuronal progenitors or in post-mitotic neurons. NeuroD1 plays critical roles in the development of both the nervous system and the pancreas [53–57]. *NeuroD2* is expressed in the post-mitotic neurons [58] and is involved in synapse development [59–61]. *NeuroD4* (also known as *Atoh3*) is expressed in the brain, retina, and cranial ganglions and is involved in the development of cortical projection neurons, retinal amacrine cells, and branchiomotor neurons [62–64]. *NeuroD6* (also known as *Atoh2*) was shown to be a key regulator of the development of projection neurons of the neocortex and amacrine cells in the retina [65, 66].

The Discovery of *Atoh1* and Its Functions in Cell Type Specification

Mouse *Atoh1* was isolated by PCR using degenerate primers corresponding to the conserved sequence within the bHLH motif of *Drosophila atonal* [67, 68]. *Atoh1* is also a single-exon gene encoding a protein of 351 amino acids with a size of 38 kDa. ATOH1 shares ~70 % identity with atonal in its bHLH motif. However, no significant similarity is found between other regions of the two proteins. Unlike *Drosophila* atonal whose bHLH motif is located at the very end of C-terminus, the bHLH motif of ATOH1 is in the middle of the protein (Fig. 2a). In addition, ATOH1 is rich in prolines which may facilitate protein-protein interactions. The C-terminus of Atoh1 is also rich in serine residues, raising the possibility that phosphorylation may be involved in the regulation of Atoh1 protein function [67]. Comparison of the protein sequences of ATOH1 from different vertebrates showed high degrees of identity in the bHLH motif and C-terminal serine-rich domain. The N-terminus, which is also suggested to be essential for the transcriptional activity, is only strongly conserved among mammals (Fig. 2b) [69]. To determine whether mouse *Atoh1* and *Drosophila atonal* genes were also functional homologs, *Atoh1* was expressed in *atonal* mutant flies and was shown to rescue chordotonal organ and photoreceptor development [70, 71]. In a complementary experiment in which *atonal* was knocked into the *Atoh1* locus, the defects of *Atoh1* null mice, including respiration and development of the cerebellum, spinal cord, inner ear, and gut, were rescued by a single allele of *Drosophila atonal* [71]. Therefore, despite the differences present in the sequences of the *Drosophila* and mammalian homologs, *atonal* and *Atoh1* are functionally equivalent.

In mouse, *Atoh1* is expressed in various organs, including the developing brain, dorsal spinal cord, small intestine, Merkel cells of the skin, and the inner ear [34, 67, 68, 72], but little is known about how its expression is regulated. Some of the first studies of regulation of the *Atoh1* locus identified an enhancer located at the 3′ of *Atoh1* coding region. This enhancer comprised two evolutionarily conserved sequences which contain both E-box and N-box sites (Fig. 3). The enhancer can be recognized and bound by Atoh1 itself and is responsible for the autoregulation of *Atoh1* expression [73]. Transgenic mice carrying a green fluorescent protein (GFP) reporter [72] or Cre/CreEr transgenes [74, 75] driven by this enhancer can recapitulate *Atoh1* expression in most tissues, albeit with some ectopic regions of expression signals [72].

Gain- and loss-of-function studies in mice over the last 15 years have revealed essential roles for *Atoh1* in the organ systems in which it is expressed [22, 24–26, 28–34, 76]. In the following sections, we describe the role of *Atoh1* in the central nervous system (CNS), gut, and skin, and in the following section, we focus in depth on the role of *Atoh1* in the developing inner ear.

The Function of Atoh1 in the Central Nervous System

In the developing mouse CNS, *Atoh1* is first expressed in a restricted region of the dorsal neural tube adjacent to the roof plate, extending posteriorly from the midbrain/hindbrain

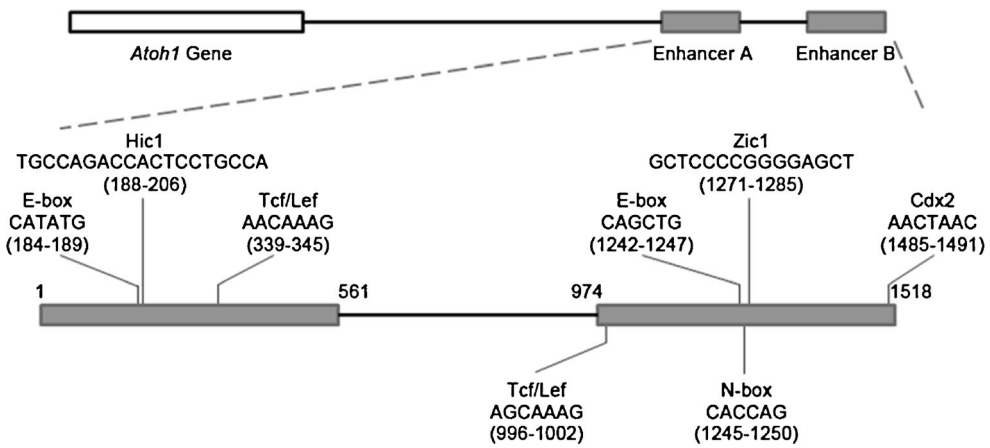

Fig. 2 A diagram of the *Atoh1* autoregulatory enhancer, which is located at the 3′ of *Atoh1* gene and comprised two conserved elements. E-box, N-box, and verified transcription factor-binding sites are marked

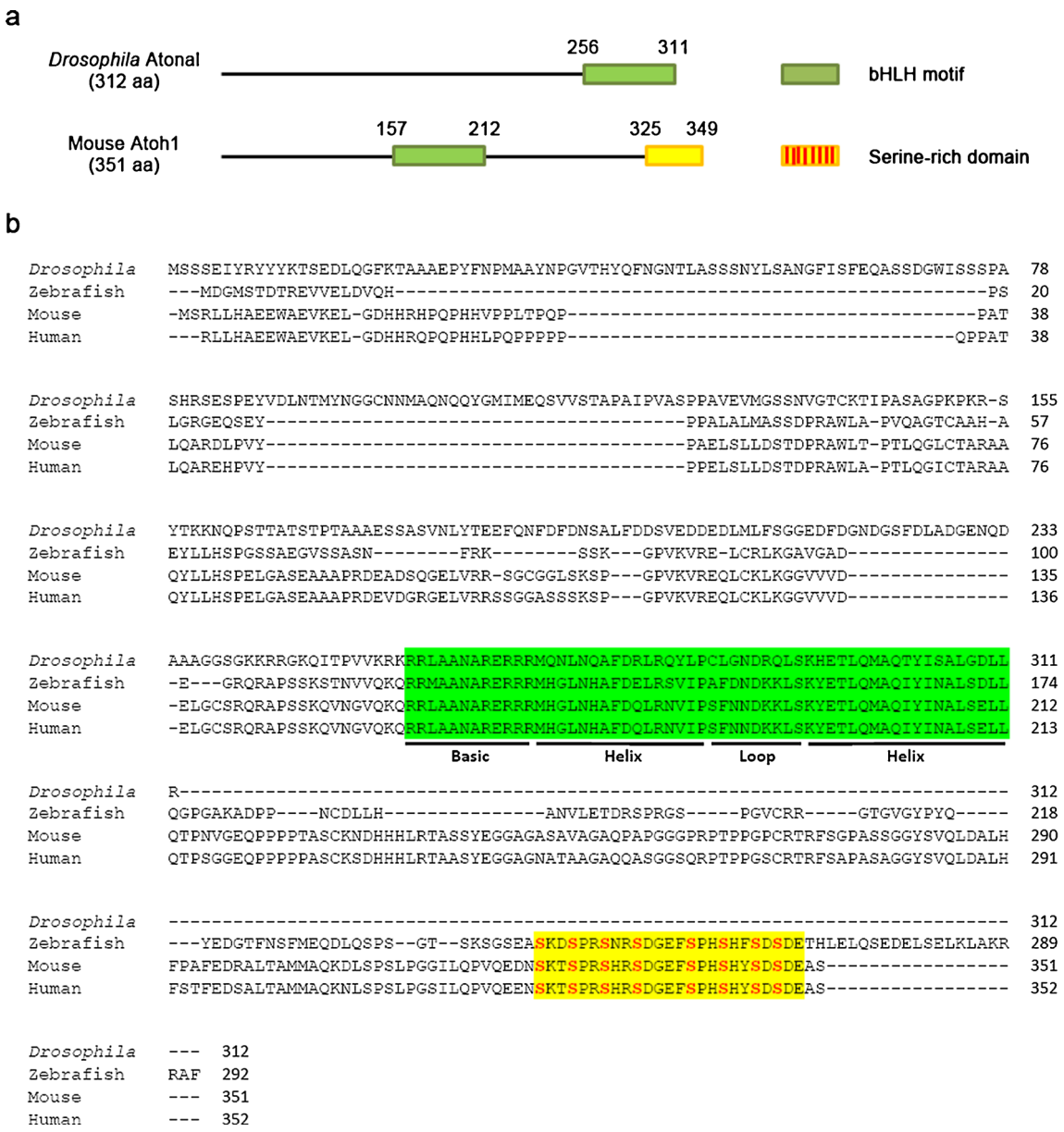

Fig. 3 **a** Schematic diagrams of *Drosophila* atonal and mouse ATOH1 proteins showing the different positions of the bHLH domain and a serine-rich domain that may be a target for post-translational modification. **b** Cross-species comparison of the amino acid sequence of atonal homologs. The bHLH motif is conserved in *Drosophila* and vertebrates (*highlighted in green*). The C-terminal serine-rich domain is only conserved in vertebrates (*highlighted in yellow*). Conserved serine residues are *highlighted in red*. The alignment was created using MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/)

boundary along the whole length of the neural tube. As the embryo develops, *Atoh1* expression in the posterior portion of the developing metencephalon becomes restricted to the rhombic lip [67, 68]. In the rhombic lip, *Atoh1* is expressed in the precursors of cerebellar granule cells. *Atoh1* expression persists in these precursors as they migrate to the external germinal layer (EGL) of the developing cerebellum and is eventually lost from these cells as they differentiate and further migrate inwardly to form the internal granule layer (IGL) [26]. Cerebellar granule cells are the smallest but also the most abundant neurons in the mammalian brain, comprising over half of all brain neurons. As the only excitatory neurons in the cerebellar cortex, they play essential roles in the complex neuronal network of the cerebellum. When *Atoh1* null mice were first generated, it was observed that the cerebellum in these mice was smaller in size compared with their wild-type and heterozygous littermates, and the EGL in these mice was completely missing [25]. Further examination of these mutant mice suggested that the absence of the cerebellar EGL was either due to the loss of granule cell precursors or the failure of granule cell proliferation [26], indicating the crucial role of *Atoh1* in the development of cerebellar granule cells.

Atoh1 null mice die shortly after birth due to the respiratory failure with a slowed central respiratory rhythm. Examination of the hindbrain nuclei involved in the respiratory rhythm generation showed that *Atoh1* is expressed in the retrotrapezoid nucleus (RTN) which regulates neonatal breathing and automatic breathing that maintains a constant level of plasma CO_2 [24]. In the absence of *Atoh1*, RTN neurons fail to properly develop and establish connections with the pre-Bötzinger complex (preBötC), the respiratory rhythm generating center in mammals [24]. A subsequent study using mice with conditional deletion of *Atoh1* specifically in RTN neurons suggests *Atoh1* is not required for the specification or maintenance of RTN neurons but is critical for their migration and projection to the preBötC [22].

In the developing dorsal spinal cord, *Atoh1* is expressed in a specific subset of neurons, the D1 commissural interneurons which give rise to the spinocerebellar tracts. Unlike cerebellar granule cells, in which *Atoh1* is only present in the proliferating precursors, the expression of *Atoh1* in the dorsal spinal cord neurons spans both the proliferating and differentiating phases [27]. Studies of *Atoh1* null mice suggested that *Atoh1* is required for the fate specification and ventral migration of the D1 interneuron precursors. In the absence of *Atoh1*, some D1 precursors acquire roof plate properties [28]. The role of *Atoh1* in the dorsal spinal cord development was further examined and compared with another bHLH factor *Neurog1* expressed in the adjacent precursors of a related class of commissural interneurons, the D3A interneurons. Deletion of *Atoh1* up-regulates *Neurog1* in a subset of D1 interneuron progenitors, the D1A progenitors, while deletion of *Neurog1* up-regulates *Atoh1* in D3A progenitors, suggesting there is a spatial cross-inhibition between the two genes [43].

The Function of Atoh1 in the Intestine

Atoh1 is expressed in the mouse intestinal epithelium from embryonic to adult stages [30, 67]. *Atoh1* function is necessary for the differentiation of the secretory lineages in the intestine, which include the goblet, Paneth, and enteroendocrine cells. In the absence of *Atoh1*, mice fail to form intestinal secretory cells and the cells destined for the secretory lineages undergo a fate switch and become absorptive cells [29, 30]. In complementary experiments, overexpressing *Atoh1* leads to ectopic secretory cells at the expense of absorptive cells [77]. The differentiation of different intestinal cell types is also regulated by an interplay between Atoh1 and Notch signaling and its downstream Hes family effectors. During the fate decision between secretory and absorptive lineages, *Atoh1* and Notch/Hes play opposing roles: *Hes* genes activated by Notch signaling repress *Atoh1* expression and therefore promote the absorptive fate, while cells in which Notch is not activated continue to express *Atoh1* and differentiate into secretory cells [78].

The Function of Atoh1 in Merkel Cells of the Skin

Atoh1 is also required for the development of Merkel cells, the epidermis-derived cells essential for mediating light touch responses [31, 79, 80]. Merkel cell-neurite complexes are sensitive touch receptors which mediate slowly adapting type I (SAI) responses [81]. In the skin, *Atoh1* is observed to be specifically expressed in Merkel cells. Conditional deletion of *Atoh1* using Hoxb1-Cre mice, in which Cre recombinase is expressed throughout the dermis and epidermis of skin in the trunk but not head, results in the absence of Merkel cells all over the body except for the whisker pads [31]. Electrical recordings also showed a loss of SAI responses in the epidermis of these mutant mice [31].

Functions of *Atoh1* in the Inner Ear

Atoh1 is the first transcription factor to be expressed in developing inner ear hair cells [34, 72]. One of the striking phenotypes of *Atoh1* null mice is the complete absence of hair cells in both cochlear and vestibular sensory organs [32, 34, 82]. Since the essential role of *Atoh1* in hair cell development was first discovered, subsequent studies have made great progress in understanding how this gene functions in hair cell development.

Atoh1 Expression in the Inner Ear

In the development of *Drosophila* chordotonal organs and photoreceptors, *atonal* functions as a proneural gene: it is broadly expressed in the proneural clusters before the selection of SOPs and is necessary and sufficient for the development of SOPs [7, 11]. Does vertebrate *Atoh1* also function as a proneural gene? In zebrafish, *atonal* homologs appear to function as proneural genes essential for inner ear development. There are two *atonal* homologs in zebrafish, *atoh1a* and *atoh1b*, which likely arose by large-scale duplication events in the zebrafish genome [83, 84]. The two zebrafish *atoh1* genes have different expression patterns with some overlaps. *atoh1b* is expressed broadly and early in the otic placode and is then confined to the future sensory epithelia before the overt differentiation of hair cells [84]. *atoh1a* expression starts later in the progenitors of the sensory epithelia, with expression up-regulated in the hair cells as they differentiate. Morpholino knockdown of *atoh1a* and *atoh1b* suggests the two *atoh1* genes together regulate zebrafish hair cell development: *atoh1b* is necessary for specifying sensory precursor groups and regulating the development of the earliest hair cells or tether cells, while *atoh1a* is necessary for the formation of later-forming hair cells [84]. It was also shown that *atoh1a* over-expression can induce ectopic hair cells and Notch

signaling negatively regulates both *atoh1* genes [84]. The initial broad expression of *atoh1b* and the necessity and sufficiency of *atoh1a* in regulating hair cell differentiation suggest they act as proneural genes.

At present, there is no definitive consensus on whether *Atoh1* acts as a proneural gene in the mammalian inner ear. Although *Atoh1* has been shown to be both necessary and sufficient for hair cell development [32, 34, 76, 82, 85–88], the major obstacle to determine whether mammalian *Atoh1* is a proneural gene is uncertainty as to whether *Atoh1* is broadly expressed in the progenitors that can give rise to different types of cells, as in *Drosophila* and zebrafish, or is restricted to the progenitors that are already committed to a hair cell fate. Different techniques have been applied to characterize *Atoh1* expression in the cochlea and have yielded inconsistent results. First, examination of mice in which the *Atoh1*-coding region is replaced with a *LacZ* gene encoding β-galactosidase suggested that the *Atoh1* promoter was activated broadly all along the cochlea at E13.5 [33]. However, in situ hybridization for *Atoh1* showed *Atoh1* transcripts could be detected only in the basal turn of the cochlea at around E13, within a narrow band of cells which extend from the basilar membrane to the apical surface thought to be nascent hair cells [89, 90]. Moreover, immunostaining experiments using anti-ATOH1 antibody revealed the first ATOH1-expressing cells in the cochlear duct between E13.5 and E14.5 as a column of cells spanning the cochlear epithelium in a similar pattern to *Atoh1* transcripts [32, 91]. The variability of these results has made it difficult to define what cell populations *Atoh1* is initially expressed in. However, recent studies have made use of a knock-in mouse line, $Atoh1^{A1GFP/A1GFP}$, in which the endogenous *Atoh1* gene is replaced with an $Atoh1^{EGFP}$ fusion gene [24]. Examination of these mice showed that although ATOH1 expression precedes hair cell differentiation, initial ATOH1 expression is restricted to a subset of post-mitotic precursors and is not broadly expressed in the prosensory domain of the cochlea [92].

In two fate-mapping studies, the fate of *Atoh1*-expressing cells in the ear was performed with $Atoh1^{Cre}$ knock-in mice or $Atoh1^{CrePR}$ knock-in mice [90, 91]. When crossed with Cre reporter lines, some labeled supporting cells were observed in both the vestibular and cochlear sensory organs, suggesting *Atoh1* may be initially expressed in progenitors that can turn into both hair cells and supporting cells [90, 91]. However, the number of labeled supporting cells in these studies varied greatly in different sensory organs, suggesting *Atoh1* is not broadly expressed in all precursor cells that will give rise to the future organ of Corti [90, 91]. One interpretation of these data is that *Atoh1* is expressed in hair cell progenitors immediately before they commit to a hair cell fate, but that some of these cells can be diverted to a supporting cell fate, most likely by Notch-mediated lateral inhibition. Based on the restricted *Atoh1* expression observed in both $Atoh1^{GFP}$ and $Atoh1^{Cre}$ knock-in mice, we conclude that in the mammalian inner ear, *Atoh1* may not completely fulfill the strict criteria of a proneural gene. However, it is still possible that *Atoh1* is expressed broadly in the prosensory region of the cochlea at extremely low levels. It is notable that in zebrafish, *atonal* homologs precede the expression of *Sox2* and are both necessary and sufficient for hair cell formation [84, 93], whereas in amniotes, *Sox2* precedes *Atoh1* expression and is necessary and sufficient for hair cell formation [94–99]. This suggests the possibility that the proneural roles of *Sox2* and *Atoh1* may have been transposed in the amniote (bird, reptile, and mammal) and anamniote (fish and amphibian) lineages [100].

Atoh1 expression begins at the mid-basal region of the cochlea. As cochlea grows, *Atoh1* expression progresses to both base and apex [32, 72, 89]. It initially appears in a subset of precursor cells in the prosensory domain, and then is restricted and up-regulated in differentiated hair cells [32, 92]. After birth, *Atoh1* expression in mouse cochlear hair cells starts to decrease. The down-regulation of *Atoh1* also follows a basal-to-apical gradient. By postnatal day 3, *Atoh1* transcripts can barely be detected along the cochlear duct [89]. Interestingly, in $Atoh1^{A1GFP/A1GFP}$ knock-in mice, ATOH-EGFP fusion protein is initially expressed diffusely in the cytoplasm of hair cell precursors; however, in differentiated hair cells expressing Myosin6, ATOH1-EGFP is mostly localized to the nucleus [92]. Immunostaining using a newly described anti-ATOH1 antibody [91] confirmed this observation of a subcellular change of ATOH1 as hair cells undergo differentiation, suggesting that the redistribution is not the consequence of the EGFP tag fused to ATOH1 [92]. It is intriguing that the redistribution of ATOH1 proteins coincides with the expression of hair cell markers and the temporal requirement for ATOH1 in hair cell survival [32, 88, 92]. Many transcription factors undergo cytoplasmic-nuclear shuttling, and the shuttling is often associated with their activities [101–103]. Therefore, the ATOH1 redistribution we observed at the first appearance of hair cell markers may be related to its function in regulating hair cell differentiation and survival. The redistribution of ATOH1 proteins might be a result of clearance of cytosolic ATOH1 or active transport of ATOH1 into the nucleus by chaperones or transcriptional partners of ATOH1. Although fascinating, the exact mechanism and function of this redistribution is not yet clear.

The Function of Atoh1 in Hair Cell Survival and Development

In the *Atoh1* null cochlea, precursor cells in the prosensory domain undergo apoptosis in a basal-to-apical gradient, similar to the hair cell differentiation gradient seen in the wild-

type cochlea. Although *Atoh1* expression in the cochlea starts no later than E13.5, mutant cells do not die until E15.5, the time when early hair cell markers such as Myosin6 start to be expressed in the wild-type cochlea [32]. This suggests precursor cells can survive without *Atoh1* for about 2 days and the trigger of cell death may be their failure to correctly differentiate in the absence of *Atoh1*. It was shown that transient *Atoh1* expression in mice in which *Atoh1* is deleted by $Atoh1^{Cre}$ (a *Cre* transgene driven by *Atoh1* autoregulatory enhancer) is not sufficient to stop the death of most hair cells and cannot support the proper function of the remaining hair cells, suggesting that the level and duration of *Atoh1* expression is critical for the hair cell survival and development [88]. Thus, do hair cells require continuous *Atoh1* expression to maintain their viability? We have recently developed another *Atoh1* conditional knockout (CKO) mouse model in which *Atoh1* can be removed at any time during hair cell development [92]. We observed a critical time window of about 2 days after the initial expression of *Atoh1* in which *Atoh1* is absolutely required for hair cell survival [92]. Within this critical time window, removal of *Atoh1* results in the death of hair cells followed by the death of the surrounding supporting cells. However, if *Atoh1* is deleted 3 days or more after its initial expression, no immediate death of hair cells and supporting cells is observed [92]. However, besides regulating hair cell survival, *Atoh1* also participates in the maturation process of hair cells. Removing *Atoh1* from differentiated hair cells can result in disorganized hair bundles and delayed death of the hair cells [92]. Moreover, re-introduction of *Atoh1* into hair cells with noise-induced damaged hair bundles can repair or regenerate the stereocilia [104]. How *Atoh1* regulates hair bundle development is not clear, but since *Atoh1* has been shown to have targets involved in a wide variety of biological processes in the cerebellum, including regulation of the cytoskeleton [105], it is possible that *Atoh1* might directly or indirectly regulate proteins associated with cytoskeletal networks in hair cell bundles.

The loss of *Atoh1* does not only affect hair cells directly but also disrupts the development of the surrounding supporting cells. In the *Atoh1* null cochlea, the expression of most supporting cell markers is abolished or dramatically decreased [33, 82, 92]. Mis-expression of *Atoh1* can also induce active Notch signaling and supporting cell formation adjacent to the ectopic hair cells [33]. The mechanism of how *Atoh1* affects supporting cell differentiation and survival is still unclear. Since *Atoh1* is not expressed in supporting cells, the regulation that supporting cells receive from *Atoh1* may be cell non-autonomous and may involve Notch signaling-mediated lateral inhibition. Supporting this idea is the observation that conditional deletion of *Atoh1* in hair cells leads to an up-regulation of *Atoh1* promoter activity in supporting cells after hair cell death [92].

The Role of Atoh1 in Hair Cell Regeneration

Hair cell damage or hair cell loss is one of the main causes of hearing loss in humans. Hearing loss can be caused by aging or environmental factors, such as exposure to loud noise and use of ototoxic drugs such as aminoglycoside antibiotics or platinum-containing chemotherapy drugs. It is known that most non-mammalian vertebrates examined, such as fish and birds, can regenerate their hair cells and restore auditory and vestibular function. In these animals, hair cell turnover continually occurs in the vestibular organs and lateral line system and robust hair cell re-generation can also occur after acoustic trauma [106–110]. This re-generation is accomplished by cell proliferation of supporting cells and/or their trans-differentiation into hair cells [111, 112]. In birds, hair cell re-generation starts with direct trans-differentiation of supporting cells, followed by mitotic re-generation [113]. In both zebrafish and birds, re-generation of hair cells is accompanied by a re-expression of *Atoh1* in the nascent hair cells [108, 111, 112, 114]. A small degree of hair cell re-generation can be observed in the cochlea of embryonic and neonatal mice [115–117]. However, mammalian hair cells rapidly lose their regenerative ability as animals get older [118]. For example, spontaneous hair cell re-generation can no longer occur when hair cells are damaged in mice that are 1-week old or older [115]. As a result, deafness in adult mammals caused by hair cell damage or loss is permanent.

Since the first demonstration over 25 years ago that birds and fish can regenerate hair cells, many studies have attempted to induce hair cell re-generation in mammals. One of the most promising strategies is *Atoh1* gene therapy. *Atoh1* alone is sufficient to induce ectopic hair cell formation when mis-expressed in the cochlea of embryonic or neonatal rodents [33, 76, 85, 86, 119, 120]. However, the induction of new hair cells by *Atoh1* is also greatly reduced with age. Two recent studies demonstrated that the efficiency of hair cell re-generation by *Atoh1* over-expression in mice is progressively decreased at postnatal ages and is largely lost when the mice reach 2 weeks of age [86, 87], around the time of the onset of hearing. Although some studies have reported successful hair cell re-generation in adult rodent cochlea by reactivating *Atoh1* expression with viruses [121, 122], in these studies more detailed examination is required to clarify whether the "new" hair cells observed came from hair cell re-generation or the repair of previously damaged hair cells [104], and whether more severe lesions render the cochlear epithelium refractory to Atoh1 activity [123]. Moreover, recent work has shown that a failure to down-regulate *Atoh1* from differentiating hair cells ultimately leads to their death [86, 87, 119]. Moreover, the hair cells generated in these experiments are typically immature, lacking markers such as prestin, oncomodulin, and vGlut3 expression, as well as having electrophysiological properties of immature hair cells. These results suggest that a transient

pulse of *Atoh1* is required to generate viable, mature hair cells, and that additional factors may be required to promote correct maturation of hair cells.

The idea of using *Atoh1* gene therapy for hair cell regeneration in humans is to activate *Atoh1* expression in cochlear supporting cells, so that the supporting cells can trans-differentiate into functional hair cells. However, this strategy will be of little use in clinical applications if adult supporting cells cannot respond to *Atoh1* and become hair cells, as has been shown in mice [86, 87]. Thus, one important question is why *Atoh1* expression in older supporting cells is unable to induce trans-differentiation into hair cells. It is assumed that the reason why older supporting cells lose the competence to trans-differentiate is because Atoh1 fails to correctly regulate some or all of its downstream targets and switch on the hair cell differentiation program in mature supporting cells. Several factors may affect Atoh1's transcriptional activity in cells. First, the epigenetic state of supporting cells may change with age. Many forms of epigenetic regulation have been identified, including DNA methylation and histone post-translational modifications. For individual genes, their epigenetic state can vary depending on cell type, cell age, or even by exposure to different environmental conditions. As supporting cells get older, the epigenetic state of Atoh1 targets that are essential for hair cell differentiation may be converted to a state consistent with transcriptional repression. Alternatively, factors such as Sox2 that may regulate "stem-ness" in supporting cells may also become epigenetically modified with age [124, 125]. In consequence, hair cell target genes may lose their responsiveness to Atoh1 regulation with age and fail to be regulated appropriately in the presence of Atoh1. Comparison of the histone marks in neonatal and adult supporting cells using approaches such as histone chromatin immunoprecipitation-sequencing (ChIP-seq) can provide a global view of epigenetic state change in the whole genome. However, without more information about what genes Atoh1 regulates during hair cell development, it is difficult to interpret such data.

A second reason for the age-dependent decline in the ability of ATOH1 to induce new hair cells is that co-factors which are required for ATOH1 to regulate its downstream targets may be absent in adult supporting cells. As a class II bHLH protein, ATOH1 needs to form a heterodimer with an E-protein for DNA binding and proper function [8]. In *Drosophila*, atonal can form a heterodimer with daughterless and senseless [7, 126]. In mouse, it was also shown that ATOH1 can form dimers with different E-proteins and the activity of these ATOH1/E-protein heterodimers may vary depending on the cellular context [67, 127]. These data suggest different transcriptional co-factors may be involved in assisting individual ATOH1/E-protein heterodimers to differentially regulate downstream targets. However, little is known about the co-factors that are critical for ATOH1 activity in hair cells, with only a few factors, such as TCF3, GATA3, and GFI1 having been shown to co-operate with ATOH1 or atonal [126, 128–130]. ATOH1 immunoprecipitation followed by mass spectrometry may be a way to identify these co-factors.

Mechanisms of *Atoh1* Regulation

Several signaling pathways are known to be involved in regulating *Atoh1* expression, such as Notch [131], Sonic Hedgehog (Shh) [132, 133], Wnts [134–136], and bone morphogenetic proteins (BMPs) [136–139]. Among these signaling pathways, Notch is the best studied, especially in the inner ear. Lateral inhibition mediated by the Notch receptor and its downstream Hes and Hey targets represses *Atoh1* expression in supporting cells and thus stops supporting cells from differentiating into hair cells (Fig. 4) [131]. Notch inhibition by different approaches—such as mutant mice for various Notch components [140–145] or administration of Notch signaling inhibitors [146–149]—was shown to be able to release the lateral inhibition between hair cells and supporting cells and generate supernumerary hair cells at the expense of supporting cells.

Recently, Shh signaling has been implicated to be involved in the regulation of the timing of hair cell differentiation in the cochlea (Fig. 4). *Shh* is expressed in the spiral ganglion which innervates cochlear hair cells [132, 150]. In the spiral ganglion-specific *Shh* knockout mice, the cochlear duct is shortened, prosensory cells exit cell cycle prematurely, and both *Atoh1* expression and hair cell differentiation follow an apical-to-basal gradient which is opposite to the basal-to-apical direction observed in the wild-type cochlea [132]. *Smoothened* (*Smo*) is a G protein-coupled receptor in Shh signaling pathway. Conditional deletion of *Smo* in the prosensory domain results in premature differentiation of cochlear hair cells, a decrease in hair cell number, and hearing impairment. Conversely, conditional activation of *Smo* leads to disrupted differentiation of hair cells and supporting cells [133]. Both studies suggest that Shh signaling is critical for the proper expression of *Atoh1* and proper timing of differentiation of cochlear hair cells.

Using biochemical assays or in silico predictions, several transcription factors have been suggested be able to directly interact with the *Atoh1* enhancers and regulate *Atoh1* transcriptional activity. These studies were mostly done in *Atoh1*-expressing tissues other than hair cells, such as the cerebellum, intestine and spinal cord. Some of these factors enhance *Atoh1* expression (e.g., β-catenin, Cdx2 and Hnf1) [134, 136, 151], while others repress *Atoh1* (e.g., Zic1 and Hic1) [137, 152] (Fig. 3). The *Atoh1*-related bHLH gene *Neurog1* can negatively regulate *Atoh1* expression (Fig. 4). In the inner ear, *Neurog1* and *Atoh1* are required for neural and sensory

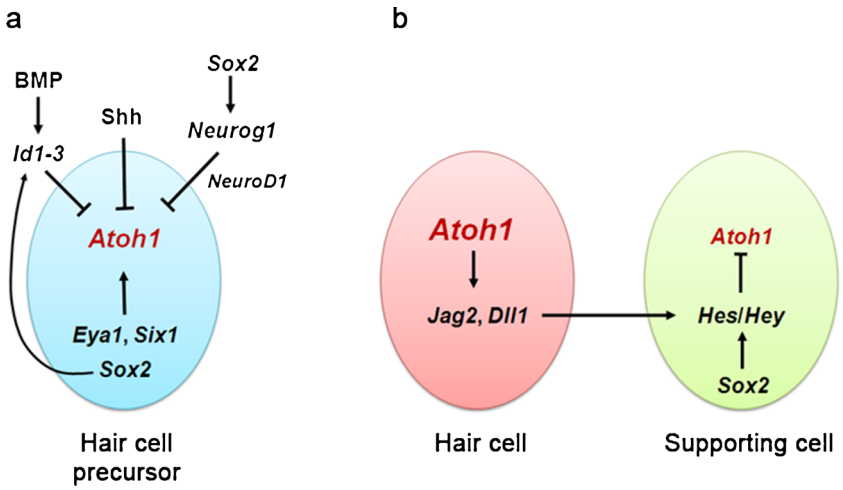

Fig. 4 The regulation of *Atoh1* in cochlear development. **a** In prosensory cell, *Eya1*, *Six1*, and *Sox2* together induce *Atoh1* expression. *Atoh1* expression can be repressed by *Neurog1*, *Id* genes, and Shh signaling. **b** After differentiation, *Atoh1* in hair cells up-regulates the Notch ligands Jag2 and Dll1, which activate Notch signaling in adjacent supporting cells to prevent them from developing as hair cells. Meanwhile, *Sox2* also represses *Atoh1* expression in supporting cells

epithelia development, respectively. *Neurog1* null mice show expanded *Atoh1* expression and over-production of hair cells in the utricle. Conversely, there is a dramatic increase of *Neurog1*+ neural precursors in *Atoh1* null utricle and saccule [44]. Thus, mutual antagonism between *Neurog1* and *Atoh1* plays a role in the neuronal versus hair cell fate decision [100]. Another *Atoh1*-related bHLH factor *NeuroD1* has been suggested as a mediator of the cross-regulation of *Atoh1* expression by *Neurog1* (Fig. 4). Conditional deletion of *NeuroD1* in the inner ear results in the ectopic hair cell formation in the sensory ganglia, suggesting that *NeuroD1* prevents the sensory neurons from developing as hair cells [153]. *NeuroD1* conditional knockouts also show aberrant expression of prosensory markers and a transformation of OHCs into IHCs in apical cochlea, indicating *NeuroD1* also regulates differentiation of subtypes of cochlear hair cells [153]. Finally, *Atoh1* can also be negatively regulated by inhibitor of differentiation genes (*Id1-3*). Id proteins contain a helix-loop-helix (HLH) domain which can dimerize with other bHLH proteins. It has been shown that Id proteins can bind and sequester E-proteins with high affinity, thus inhibiting the formation of functional bHLH heterodimers [154]. In the cochlea, *Id* genes are regulated by BMP signaling (Fig. 4) [139], and ectopic expression of *Ids* in the cochlea reduces *Atoh1* expression and prevents hair cell differentiation [139, 155].

In *Atoh1*^{GFP} transgenic reporter mice, reporter signals in hair cells are abolished when the reporter is bred onto the *Atoh1* null background, suggesting the expression of the GFP reporter is dependent on the continuing presence of *Atoh1* in hair cells [44]. In addition, the onset of this reporter in the developing cochlea is slightly slower than the endogenous Atoh1 transcripts and proteins. The earliest stage that the reporter can be detected in cochlear hair cells is around E14.5 [72], compared with endogenous *Atoh1* expression which can be observed between E13 and E13.5 [89, 90, 92]. Thus, before Atoh1 autoregulation is initiated, what signals turn on the initial expression of *Atoh1*? One recent study suggested that two candidate upstream genes regulating *Atoh1's* initial expression are *Eya1* and *Six1*. In the mouse cochlea, *Eya1* and *Six1* together are sufficient to induce the expression of *Atoh1*, and this induction can be synergistically enhanced by the presence of *Sox2* (Fig. 4). All these three molecules are broadly expressed ahead of *Atoh1* in the prosensory domain and both Six1 and Sox2 are shown to directly bind the *Atoh1* enhancer region [96, 156, 157]. However, it is not clear how the broad expression of *Eya1*, *Six1*, and *Sox2* can lead to the restricted pattern of hair cell-specific expression of *Atoh1*. Intriguingly, *Sox2* plays a dual function in hair cell development. On one hand, *Sox2* promotes sensory formation, in which *Sox2*—together with *Eya1* and *Six1*—up-regulates *Atoh1* expression [96, 156]. On the other hand, *Sox2* prevents hair cell differentiation by inhibiting *Atoh1* expression [158]. Down-regulation of *Sox2* is shown to be necessary for hair cell formation [158]. It has been suggested that *Sox2* may inhibit *Atoh1* expression by activating *Neurog1* and *NeuroD*, *Hes*/*Hey* factors in Notch signaling, and *Id* genes in an incoherent feed-forward loop (Fig. 4) [96, 97, 100]. Besides regulation at the transcriptional level, some studies also showed Atoh1 function may also be controlled through post-transcriptional or post-translational mechanisms. ATOH1 turnover was accelerated with BMP treatment in the granule neuron progenitor (GNP)-like medulloblastoma cells [138]. In vitro cell culture experiments also showed ATOH1 stability was increased or decreased when E47 or Id1/Id2 was co-expressed, respectively [138]. Moreover, in human colon cancer cells, ATOH1 was found to be degraded by glycogen synthase kinase 3β (GSK3β)-mediated proteolysis, which is regulated by Wnt signaling [135]. Since ATOH1 expression is strongly promoted by autoregulation, it is possible that post-translational down-regulation or degradation of ATOH1 protein may be crucial in breaking this autoregulatory loop.

It has been suggested that *Atoh1* in Merkel cells is also directly regulated by the transcription factor *Sox2*. Ablation of *Sox2* in Merkel cells in vivo leads to a decrease of *Atoh1*

expression and loss of Merkel cells. Intriguingly, *Sox2* expression in Merkel cells is controlled by the Polycomb repressor complex, a multi-unit protein complex with histone methyltransferase activity that is involved in transcriptional silencing. Deletion of the key Polycomb subunits *Ezh1* and *Ezh2* from adult skin de-represses *Sox2* and promotes *Atoh1* expression and Merkel cell differentiation [159]. These data provide a novel example of how epigenetic regulation of gene expression plays a role in tissue homeostasis.

Identifying Atoh1 Target Genes

Although *Atoh1* plays essential roles in the development of different cell types and tissues, its molecular function is poorly understood. The reason for this is a lack of knowledge about direct transcriptional targets of ATOH1. Using gene expression profiling combined with alignment of conserved E-boxes, a few genes have been identified as direct targets of ATOH1, such as *BarHl1*, *Pou4f3*, *Hes6*, *Chrna1*, and *Nr2f6* [160–164]. In order to investigate *Atoh1* function in depth, researchers have started to identify ATOH1 direct targets using genome-wide approaches, such as RNA-sequencing (RNA-seq) and ChIP-seq. A recent genome-wide study was performed in the mouse cerebellum to identify the ATOH1 "targetome" in cerebellar granule cells [105]. Three approaches were used in this study, including (1) ATOH1 ChIP-seq to identify Atoh1 binding cites, (2) Histone-seq to identify the global histone H3 lysine 4 (H3K4) methylation status in the cerebellum, and (3) RNA-seq to compare the expression differences between wild-type and *Atoh1* null cerebella. With all three sets of data combined together, a total of 601 genes were identified as potential ATOH1 target genes in mouse cerebellar granule cells [105]. These targets are involved in various biological processes, including cell proliferation and differentiation, cell adhesion and migration, chromosomal organization, and cell metabolism. About one sixth of the target genes are associated with most of the known signaling pathways, such as Shh, Notch, transforming growth factor-beta (TGF-β), retinoic acid (RA), Wnt, and mitogen-activated protein kinase (MAPK) signaling. In this study, an extended E-box sequence composed of ten nucleotides was identified as the unique binding motif of ATOH1 by compiling large numbers of ATOH1 binding sites from ChIP-seq experiments. One or more of such extended Atoh1 E-Box-associated motifs (AtEAMs) are present within 5 kb of the transcriptional start sites of 64 % of the ATOH1 targets identified in the cerebellum, and it is possible that other AtEAMs are located further away from other ATOH1 targets [105]. This extended ATOH1-binding motif identified in mouse cerebellum is close, but not identical, to the one identified for *Drosophila* atonal. This extended ATOH1-binding motif is unlikely to be shared with other closely related bHLH factors, such as NEUROG1. Although ATOH1 and NEUROG1 have high similarity in sequences and bind to core E-boxes that differ by only one nucleotide [165], *Neurog1* cannot fully substitute for *Atoh1*. In knock-in mice in which *Atoh1* coding sequence is replaced by *Neurog1*, the cochlea develops patches of organ of Corti cells with microvillar structures but few hair cells [166]. The less-severe cochlear phenotype of these replacement knock-in mice compared with that in *Atoh1* null mice suggests *Neurog1* may only be able to regulate a subset Atoh1 targets. Therefore, NEUROG1 may possess its own DNA binding motif that is similar to, but distinct from the Atoh1 binding site AtEAM.

To date, only a few ATOH1 direct targets have been identified in inner ear hair cells [162, 163]. Therefore, a systematic study of ATOH1 targets in hair cells will be one of the ways to study the roles *Atoh1* plays during hair cell development and re-generation. However, ChIP-seq experiments using purified hair cells have several technical obstacles. Unlike cerebellar granule cells, in which several million cells are present in each cerebellum and account for over 50 % of the cells in the neonatal cerebellum, cochlear hair cells are present in very limited numbers (only ~3,000 hair cells per mouse cochlea) and only constitute a small proportion of total cells in the whole cochlea. As a result, it is difficult to enrich and collect enough cochlear hair cells to perform a ChIP-seq experiment with current approaches, although recent advances in amplification of small amounts of ChIP-seq material may make these experiments feasible with relatively small numbers of cells. Cross-referencing gene expression sets with the ChIP-seq data obtained from cerebellum, Johnson and colleagues were able to predict the direct targets of ATOH1 in dorsal spinal cord D1 interneurons. Using this strategy, five new genes were identified as the in vivo direct targets of ATOH1 in D1 interneurons [167]. The success of ATOH1 target identification in the dorsal spinal cord suggests this strategy may also be applied to the inner ear for identifying ATOH1 targets in hair cells. That said, a comparison of the transcriptomes of different Atoh1-expressing cell types, such as hair cells, gut epithelial cells, cerebellar granule cells, and Merkel cells is likely to only reveal a minority of genes common to all cell types. This is apparent upon consideration of the different properties of each cell type—for example, *Atoh1*-expressing cerebellar granule progenitors are proliferative, migratory, and need to extend neuronal processes as they mature, whereas *Atoh1*-expressing hair cells are post-mitotic, non-migratory, and do not elaborate axons or dendrites, although they still form synapses with sensory neurons. It will be of great interest to compare direct ATOH1 target genes in these different cell types to understand how higher order chromatin regulation and epigenetic modification are deployed to make different sets of ATOH1 targets available for transcription during the differentiation of different cell lineages.

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