Development/Plasticity/Repair

Characterization of the Transcriptome of Nascent Hair Cells and Identification of Direct Targets of the Atoh1 Transcription Factor

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Hair cells are sensory receptors for the auditory and vestibular system in vertebrates. The transcription factor *Atoh1* is both necessary and sufficient for the differentiation of hair cells, and is strongly upregulated during hair-cell regeneration in nonmammalian vertebrates. To identify genes involved in hair cell development and function, we performed RNA-seq profiling of purified Atoh1-expressing hair cells from the neonatal mouse cochlea. We identified >600 enriched transcripts in cochlear hair cells, of which 90% have not been previously shown to be expressed in hair cells. We identified 233 of these hair cell genes as candidates to be directly regulated by Atoh1 based on the presence of Atoh1 binding sites in their regulatory regions and by analyzing Atoh1 ChIP-seq datasets from the cerebellum and small intestine. We confirmed 10 of these genes as being direct Atoh1 targets in the cochlea by ChIP-PCR. The identification of candidate Atoh1 target genes is a first step in identifying gene regulatory networks for hair-cell development and may inform future studies on the potential role of Atoh1 in mammalian hair cell regeneration.

Key words: **Atoh1; cochlea; hair cells; inner ear**

Introduction

Atoh1 is the first transcription factor to be expressed in hair cells, and is essential for hair-cell development. Previous studies of *Atoh1* mutant mice have revealed multiple functions of *Atoh1* in the genesis, survival, maturation, and function of hair cells (Bermingham et al., 1999; Chen et al., 2002; Woods et al., 2004; Pan et al., 2012; Yang et al., 2012b; Cai et al., 2013). Overexpression of *Atoh1* in immature rodent inner ears can induce ectopic hair cells in both sensory and nonsensory regions of the cochlea (Zheng and Gao, 2000; Woods et al., 2004), suggesting the sufficiency of *Atoh1* for hair-cell formation in parts of the inner ear. However, the ability of *Atoh1* to induce new hair cells in the cochlea declines precipitously with age (Liu et al., 2012; Yang et al., 2012a), although the reasons for this decline are currently not known.

Although *Atoh1* is both necessary and sufficient for hair-cell development, the precise molecular mechanism by which *Atoh1* mediates hair-cell genesis is unknown. A very small number of Atoh1 targets have been identified by expression profiling of tissues or cell lines (Krizhanovsky et al., 2006; Scheffer et al., 2007a,b). Genome-wide studies have also identified Atoh1 targets in the nervous system and intestine (Klisch et al., 2011; Kim et al., 2014). A previous study combined Atoh1 ChIP-seq (to identify Atoh1 binding sites) together with histone-seq (to identify global H3K4 methylation status), and RNA-seq (to compare expression profiles of wild-type and *Atoh1-null* cerebella; Klisch et al., 2011). The resultant Atoh1 "targetome" suggests that *Atoh1* regulates the expression of genes responsible for diverse biological processes, including cell proliferation, differentiation, migration, and metabolism. This study also pinpointed an extended E-box-containing sequence termed AtEAM as a consensus binding site for *Atoh1* (Klisch et al., 2011). A second strategy combining the cerebellar Atoh1 targetome with microarray data from the dorsal spinal cord identified several additional Atoh1 targets specific for dorsal spinal cord interneurons (Lai et al., 2011).

The small number of hair cells in the cochlea has militated against identification of Atoh1 target genes in hair cells by ChIP-seq. However, the success of Atoh1 target identification in the dorsal spinal cord suggests a strategy of hair cell RNA-seq combined with ChIP-seq data from other tissues may allow the identification of some Atoh1 targets in hair cells. We used RNA-sequencing to

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identify transcripts in *Atoh1*-expressing cells from the neonatal mouse cochlea and found 614 genes enriched over tenfold in *Atoh1*-expressing cells. We performed an *in situ* hybridization screen to validate the expression of 60 of these enriched genes, of which 34 showed specific hair cell expression. We searched for the Atoh1-binding sites in 10 of the validated genes and verified direct Atoh1 binding in these gene loci by ChIP-PCR. These Atoh1 targets may be useful tools in the assembly of a hair cell gene regulatory network and may allow us to understand why the ability of Atoh1 to induce hair-cell transdifferentiation declines with age.

Materials and Methods

Experimental animals. *Atoh1* $^{-/-}$ (MGI: *Atoh1*^{tm1Hzo}), *Atoh1*$^{A1GFP/A1GFP}$ (MGI: *Atoh1*$^{tm4.1Hzo}$), and *Atoh1*$^{flox/flox}$ (MGI: *Atoh1*^{tm3Hzo}) mice were generated as previously described (Ben-Arie et al., 1997; Shroyer et al., 2007; Rose et al., 2009). *Atoh1-CreER*^{T2} (MGI: Tg(Atoh1-cre/Esr1*)14Fsh; (Machold and Fishell, 2005) and *R26R-YFP* (MGI: Gt(ROSA)26Sor$^{tm1(EYFP)Cos}$; (Srinivas et al., 2001) transgenic lines were obtained from Jackson Laboratories. Genotyping was performed by PCR using the following primers: for different *Atoh1* alleles, Atoh1-forward (ACG CAC TTC ATC ACT GGC), Atoh1-reverse (GGC ACT GGC TTC TCT TGG), and Neo-forward (GCA TCG CCT TCT ATC GCC) yield a 600 bp wild-type allele band and a 400 bp null allele band. HA-forward (GCG ATG ATG GCA CAG AAG G) and HA-reverse (GAA GGG CAT TTG GTT GTC TCA G) yield a 1 kb *Atoh1* EGFP-tagged allele band and a 350 bp floxed allele band. For *Atoh1-CreER*^{T2}, Cre1F (GCC TGC ATT ACC GGT CGA TGC AAC GA),and Cre1R (GTG GCA GAT GGC GCG GCA ACA CCA TT) yield a 700 bp band. For R26R-YFP, oIMR0316 (GGA GCG GGA GAA ATG GAT ATG), oIMR0883 (AAA GTC GCT CTG AGT TGT TAT), and oIMR4982 (AAG ACC GCG AAG AGT TTG TC) yield a 320 bp YFP+ band. To generate the inducible *Atoh1* conditional knock-out (CKO) mice, *Atoh1-CreER*^{T2}; *Atoh1*$^{\pm}$ males were crossed with *Atoh1*$^{flox/flox}$; *R26R-YFP* homozygous females. One dose of 2 mg tamoxifen and 2 mg progesterone was administered to pregnant females at embryonic day (E)17.5 by oral gavage. Progesterone was co-administered to prevent late fetal abortions (Nakamura et al., 2006). Tamoxifen and progesterone were dissolved together in peanut oil, both at a concentration of 20 mg/ml. The genotypes of embryos or newborn pups from these crosses were determined as above. The Baylor College of Medicine Institutional Animal Care and Use committee approved all animal experiments.

Hair-cell purification. Whole inner ears were dissected from homozygous P0 *Atoh1*$^{A1GFP/A1GFP}$ mice and incubated in Ca^{2+}, Mg^{2+}-free (CMF) PBS. The cochleae were isolated and the spiral ganglia and Reissner's membrane removed to expose the organ of Corti. Isolated cochleae were washed with CMF-PBS and then incubated in 0.1% Trypsin-EDTA (Sigma-Aldrich) diluted in CMF-PBS for 10 min at 37°C. The trypsin solution was removed and the tissue rinsed in DMEM with 5% fetal calf serum (FCS). The tissue was then gently triturated with a 1000 μl pipette tip in CMF-PBS containing 5% FCS for ~100 times to generate a single-cell suspension. Hair cells were purified on a BD FACSAria cell-sorting flow cytometer using a 100 μm nozzle and 488 nm excitation. Gates were set each time using small number of cells from the same sample to identify the viable cells and GFP fluorescence. GFP^+ and GFP^- cells were individually collected in CMF-PBS with 5% FCS. Sorted cells were spun down at 1000 × *g* for 10 min at 4°C. The supernatant was carefully removed and pellets were suspended in cell lysis buffer from an RNeasy Plus Micro kit (Qiagen) and stored at −80°C for future RNA extraction.

qPCR. Total RNA was extracted from fluorescence-activated cell sorting (FACS)-purified cells using an RNeasy Plus Micro kit (Qiagen). cDNA was generated using Superscript III Reverse Transcriptase (Invitrogen). Quantitative PCR (qPCR) was performed with Master SYBR Green Kit (Applied Biosystems) on a Step One Plus real-time PCR system (Applied Biosystems). Each reaction was performed in triplicate. Relative quantification of gene expression was analyzed by the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001) using the ribosomal gene L19 as an internal control. Gene-specific primer sets used for qPCR were as follows: Atoh1-F (ATG CAC GGG CTG AAC CA) and Atoh1-R (TCG TTG TTG AAG GAC GGG ATA); L19-F (GGT CTG GTT GGA TCC CAA TG), and L19-R (CCC GGG AAT GGA CAG TCA).

RNA-sequencing. RNA-seq libraries of FACS purified cells were generated as previously described (Lott et al., 2011). Total RNA was extracted from FACS-purified cells (~100,000 cells for each library) using an RNeasy Plus Micro kit (Qiagen). mRNA purification, RNA fragmentation, first strand and second strand cDNA synthesis, adaptor ligation, and PCR amplification were performed using the Illumina mRNA-Seq Sample Prep Kit. SPRI beads (Ampure XP, Beckman) were used in each purification step after RNA fragmentation for size selection. Duplicate libraries were made for GFP^+ cells and GFP^- cells. All libraries were analyzed for quality and concentration using an Agilent Bioanalyzer. A sample from each library was also cloned into the TOPO-Blunt vector. Ten clones were randomly picked and sequenced to verify that ribosomal RNA was depleted from the libraries. Sequencing was performed at the Genomic and RNA Profiling Core in Baylor College of Medicine using Illumina HiSeq2000 100bp Paired-End Platform. Fastq files of paired-end reads and BigWig files have been deposited in the NCBI GEO database, Accession No. GSE65633.

RNA-seq data analysis. RNA-seq reads were mapped to the mouse reference genome (mm9) using TopHat (Trapnell et al., 2009). Read counts per gene were obtained by counting the number of reads, which overlap the exons of genes defined by RefGene (NCBI). Read counts were normalized to a library size and differentially expressed genes were identified by performing a negative binomial test using the DESeq package (Anders and Huber, 2010). *P* values were adjusted using the Benjamini and Hochberg (1995) multiple testing procedure. Genes with adjusted *p* values $<1 \times 10^{-10}$ were marked as significant.

Prediction of Atoh1 binding sites. To predict Atoh1 binding sites, the MACS package (Zhang et al., 2008) was used to select the top 5000 Atoh1 binding sites from ChIP-Seq data obtained from the neonatal cerebellum (Klisch et al., 2011). MEME-ChIP (Machanick and Bailey, 2011) was used to analyze the flanking sequences of the top 5000 sites, from which 87 motifs were selected that were present >10 times in these 5000 sites. Conserved promoter or enhancer regions within 5 kb of the transcriptional start site of each gene were identified from the UCSC browser conservation track and these regions were searched for the 87 Atoh1 binding motifs.

RNA probe synthesis. Primer sets for each candidate gene were selected to target a 500–700 bp DNA fragment in a single exon of each gene for screen. A T7 RNA polymerase sequence (5′-GGATCCTAATACG ACTCACTATAGGGAG-3′) was added to the 5′ end of each reverse primer. Mouse genomic DNA was used as the template for PCR. The PCR product of the correct size was purified with a PCR Purification Kit (Qiagen). Purified DNA was used as the template for RNA probe synthesis with T7 polymerase (Promega) using standard protocols (Stern, 1998).

In situ *hybridization.* Heads of neonatal mouse pups were fixed in 4% paraformaldehyde in PBS overnight at 4°C, cryoprotected in 30% sucrose in PBS at 4°C, embedded in OCT compound (Sakura Finetek), and cryosectioned at 14 μm. The *in situ* hybridization procedure was modified from previous protocols (Harland, 1991; Birren et al., 1993; Groves et al., 1995). Sections were fixed in 4% paraformaldehyde in PBS, pH 7.2, for 10 min at room temperature, followed by three 5 min washes in DEPC-treated PBS. The sections were treated with 1 μg/ml proteinase K in DEPC-PBS for 5 min at room temperature, followed by three 5 min washes in DEPC-PBS and refixation in 4% paraformaldehyde in PBS, pH 7.2, for 10 min at room temperature. Sections were acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0, for 10 min at room temperature, followed by three 5 min washes in DEPC-PBS. Slides were incubated in hybridization buffer (50% Formamide, 5× SSC, 50 μg/ml Yeast tRNA, 100 μg/ml heparin, 1× Denhardt's Solution, 0.1% Tween 20, 0.1% CHAPS, 5 mM EDTA) for 1–2 h at 65°C. One-hundred microliters of digoxygenin-labeled probe (1 mg/ml) was added to each slide and the slides covered with glass coverslips. The slides were incubated in a chamber humidified with 5× SSC, 50% formamide at 65°C overnight. Coverslips were removed by rinsing in 0.2× SSC and the slides washed in 0.2× SSC at 65°C for 1 h. The slides were then washed in 0.2× SSC for 5

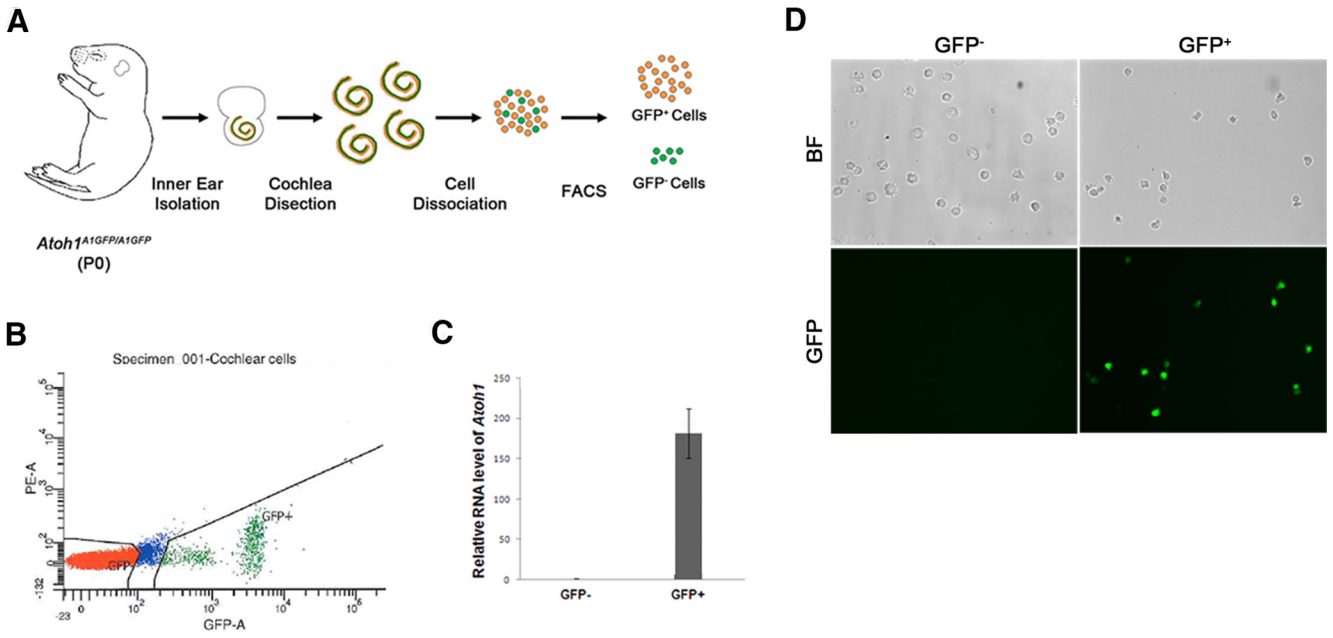

Figure 1. ***A***, Schematic diagram of the purification of GFP cells from the neonatal *Atoh1*$^{A1GFP/A1GFP}$ mouse cochlea. ***B***, Sample FACS profile showing the distribution of GFP-expressing cells (green). ***C***, Q-PCR analysis of GFP-positive and -negative cells sorted from the *Atoh1*$^{A1GFP/A1GFP}$ cochlea shows an almost 180-fold enrichment of *Atoh1* mRNA in the GFP + population. ***D***, Fluorescence and bright field images of the GFP-positive and -negative cell populations.

min at room temperature, followed by another 5 min wash in 0.1% Tween 20 in PBS (PTw). The slides were blocked in 10% lamb serum in PTw at room temperature for 1 h and then stained with anti-digoxygenin-alkaline phosphatase antibody (1:2000) for 1–3 h at room temperature in a humidified chamber. The slides were then washed three times for 5 min each in PTw and equilibrated with freshly made alkaline phosphatase buffer (100 mM Tris, pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20) for 10 min. The slides were developed in alkaline phosphatase buffer containing 0.33 mg/ml NBT and 0.18 mg/ml BCIP in the dark at room temperature until the purple reaction product had developed to a satisfactory degree. The reaction was stopped by washing the slides in PBS three times for 15 min each, followed by fixation in 4% paraformaldehyde in PBS, pH 7.2, for 30 min. The slides were then rinsed and mounted in 80% glycerol in PBS.

Chromatin immunoprecipitation. Cochleae were dissected from P0 *Atoh1*$^{A1GFP/GFP}$ pups and stored in DMEM with 5% FBS. Cochleae were incubated with 500 μM thermolysin in DMEM for 30–45 min at 37°C and washed in DMEM with 5% FBS. The sensory epithelium from each cochlea was dissected out and pooled together in PBS. Eight sensory epithelia were collected for each sample and centrifuged at 470 × *g* for 10 min at 4°C. The pooled sensory epithelia were cross-linked with 500 μl PBS containing 13.5 μl of 36.5% formaldehyde solution for 20 min; the sample was vortexed every 5 min during the incubation. The fixation was then quenched with 57 μl of 1.25 M glycine for 5 min. The cross-linked tissue was centrifuged at 470 × *g* for 10 min at 4°C with soft deceleration. The supernatant was then removed, and the pellet was washed with 500 μl of ice-cold PBS three times. The PBS was removed without disturbing the pellet and the sample snap frozen in liquid nitrogen, and stored at −80°C.

Chromatin immunoprecipitation was performed using the "micro-ChIP" protocol of Dahl and Collas (2009) with some modification. Cross-linked sensory epithelia in the tube were lysed in 120 μl lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS with fresh 1% protease inhibitor and 1 mM PMSF), and incubate for 5 min on ice. The sample was sonicated using a Bioruptor (Diagenode) programmed for 30 s on, 30 s off, for 15 cycles, with vortexing after every five cycles. Four-hundred microliters of RIPA ChIP buffer (10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% SDS, 0.1% Na-deoxycholate, 1% protease inhibitor and 1 mM PMSF) was added to the tube. The sample was then mixed and centrifuged at 12,000 × *g* for 10 min at 4°C. Supernatant (450 μl) was collected into a new tube and the pellet re-extracted with another 400 μl of RIPA ChIP buffer. To precipitate Atoh1^{GFP}-bound DNA, 10 μl of blocking magnetic beads and 10 μl of anti-GFP magnetic beads (Chromotek, GFP-Trap-M) were washed twice with dilution buffer (1.0 M Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA). The ChIP sample solution (400 μl) was added to 400 μl dilution buffer containing the blocking magnetic beads. The sample mixture was then incubated for 30 min at 4°C. After incubation, the tube was placed on a magnetic rack, the beads were captured, and the solution was extracted and mixed with anti-GFP magnetic beads. This mixture was vortexed and incubated for 2 h at 4°C with moderate shaking. The bead-containing solution was placed on the ice-cold magnetic rack to capture the beads and the beads then washed three times with 1 ml ice-cold RIPA buffer at 4°C with moderate shaking for 10 min each. After the final wash, 1 ml TE buffer was added to the beads and incubated for 10 min at 4°C with moderate shaking. After removal of TE, 100 μl of a 10% solution of Chelex-100 beads was added and the sample vortexed for 10 s and boiled for 10 min. One microliter of 25 mg/ml proteinase K was added to the sample, vortexed, and incubated for 40 min at 56°C, with vortexing every 10 min followed by boiling again for 10 min. The sample was cooled to room temperature, centrifuged for 10 s and the supernatant transferred to a chilled clean tube. The sample was then stored at −20°C for up to 1 week or used directly for ChIP-PCR with appropriate primers. As a negative control, GFP ChIP was performed as above from a wild-type mouse.

Results

RNA-seq analysis identifies transcripts enriched in Atoh1-expressing hair cells

We used FACS on cells isolated from *Atoh1*$^{A1GFP/A1GFP}$ knock-in mice (Rose et al., 2009) to purify differentiating hair cells that express Atoh1 (Fig. 1*A*). Cochleae were dissected from newborn (P0) *Atoh1*$^{A1GFP/A1GFP}$ mice, dissociated into single cells, and GFP$^+$ and GFP$^-$ cells were isolated by FACS sorting based on the intensity of GFP fluorescence (Fig. 1*B*). At P0 we saw no observable apical-basal differences in GFP intensity in the intact cochleae of *Atoh1*$^{A1GFP/A1GFP}$ mice (Cai et al., 2013). To confirm Atoh1-GFP expression, both sorted populations were fixed and GFP fluorescence was examined under a microscope. In the

GFP$^+$ population, ~ 90% expressed levels of GFP that were detectable by eye and by costaining with antibodies to Myosin6. No GFP$^+$ cells were detected in the GFP$^-$ population (Fig. 1*C*). To further examine *Atoh1* expression level in the sorted cell populations, we extracted total RNA from both GFP$^+$ and GFP$^-$ populations and performed quantitative reverse-transcriptase PCR to measure the relative mRNA level of *Atoh1*. *Atoh1* transcripts were highly enriched in the GFP$^+$ population by almost 180-fold (Fig. 1*D*).

To identify genes that are enriched in differentiating hair cells, we compared the transcripts in GFP$^+$ and GFP$^-$ cells from P0 *Atoh1*$^{A1GFP/A1GFP}$ mice using RNA-sequencing. We obtained between 98,000,000 and 189,000,000 reads for each sample, with 83–85% of the paired reads mapping correctly to the reference genome. Two biological replicates of each population showed high reproducibility ($r = 0.98$ for the GFP$^+$ populations and 0.96 for the GFP$^-$ populations). By comparing the transcripts between GFP$^+$ and GFP$^-$ populations, we identified 614 transcripts that have over tenfold enrichment in the GFP$^+$ cell population ($p < 1.0E-30$). We also identified 329 transcripts that were downregulated over tenfold in the GFP$^+$ population (data not shown). Of the 614 upregulated genes, 57 have been previously identified as hair cell-specific genes (Table 1), 82 have been identified in adult inner or outer hair cells (Liu et al., 2014), and 74 have also been identified as candidate hair cell genes based on the behavior of chick homologues of these genes during utricle regeneration (Ku et al., 2014). Finally, we also identified 22 enriched transcripts in the GFP$^+$ population that are known deafness genes (*Cabp2*, *Cib2*, *Cldn14*, *Dfnb59*, *Gipc3*, *Gxcr1*, *Gxcr2*, *Ildr1*, *Lhfpl5*, *Msrb3*, *Myo3a*, *Myo6*, *Myo7a*, *Pdzd7*, *Pou4f3*, *Ptprq*, *Smpx*, *Tmc1*, *Tmie*, *Ush1 g*, *Ush2a*).

Genes enriched in Atoh1-expressing hair cells are involved in biological processes associated with sensory organ and neuronal development

To analyze the function of the genes enriched in *Atoh1*-expressing cells, we made use of the gene ontology (GO) database DAVID (Huang et al., 2009a,b). We focused on 313 genes that were enriched by at least tenfold and showed transcript levels at >3000 RPKM. GO analysis suggests several of these genes are involved in biological process associated with sensory organ development (Table 2; 24 genes, $p = 2.11E-10$), as well as molecular functions that are known to be critical for hair cell development, such as cytoskeletal protein binding (20 genes, $p = 2.68E-04$), channel activity (17 genes, $p = 0.001439$), and motor activity (11 genes, $p = 0.001123$). It is known that sensory hair cells share some properties with neurons, such as synapse formation, synaptic vesicle release, and the regulation of membrane potential. Accordingly, we identified a number of genes associated with neuron differentiation in Atoh1-expressing cells (30 genes, $p = 4.92E-11$) and genes known to be synapse components (23 genes, $p = 2.05E-06$).

In situ validation of genes enriched in Atoh1-expressing cochlear cells

We performed an *in situ* hybridization screen in the P0 mouse cochlea to validate the expression of the genes enriched in Atoh1-expressing cochlear cells. We focused on the 313 genes that showed high ranks in both expression and fold-change in our RNA-seq result (expression level >3000 RPKM, fold-change >10). We chose an expression of RPKM>3000 as an arbitrary filter based on our empirical correlation of RPKM values with a positive signal by *in situ* hybridization. We designed RNA probes for 60 genes from this list, and performed *in situ* hybridization on sections of neonatal mouse cochlea to validate their expression. Surprisingly, only 34 of 60 genes showed specific expression in cochlear hair cells (Table 3; Fig. 2*A*,*B*). Although the remaining 26 genes were expressed in hair cells, we observed additional sites of expression such as supporting cells, Kölliker's organ, or the outer sulcus. Some of the validated hair-cell-specific genes were previously shown to be expressed in the inner ear hair cells or associated with hair-cell development and hearing-defect phenotypes, such as *Lhx3* (Hertzano et al., 2007; Rajab et al., 2008), *Lhfpl5* (Kalay et al., 2006), and *Srrm4* (Nakano et al., 2012). However, many of the genes in our screen have never been studied in the inner ear before (Table 3; Fig. 2*B*). These include the phospholipid binding protein *Annexin A4*, the transcription factor and tumor suppressor Castor (*Casz1*; Charpentier et al., 2013), *RBM24*, an RNA-binding protein implicated in Notch-Delta signaling (Maragh et al., 2014), the Notch receptor modifying enzyme *Mfng*, and the lysosome regulatory gene melanoregulin (*Mreg*). Some genes associated with cytoskeletal binding and motor activity are specifically expressed in cochlear hair cells, such as *Eps8l2*, *Kif21b*, and *Pacsin1* (Table 3; Fig. 2*A*,*B*). Several ion channels also show a hair-cell-restricted expression pattern, such as *Chrna9*, *Chrna10*, *Kcnh6*, and *Scn11a* (Table 3; Fig. 2*A*,*B*). Because *Atoh1* is expressed in all differentiating hair cells in both the auditory and vestibular end organs, we also checked the expression of our validated genes in the vestibular organs. All the genes we found to be specifically expressed in cochlear hair cells also show expression in vestibular hair cells at neonatal stages (Table 3; Fig. 2*C*).

Atoh1 expression initiates in the basal region of the cochlea at E13.5 and spreads along the cochlear duct to the apex (Chen et al., 2002; Cai et al., 2013). However, by the day of birth, *Atoh1* expression is beginning to be downregulated in the hair cells in the basal turn of the cochlea, and this downregulation continues for several days along the length of the cochlea to the apex (Groves et al., 2013). Many of the genes in our screen also show a gradient of expression in hair cells along the apical-basal axis of the cochlear duct (Table 3; Fig. 3). Some genes are expressed more strongly in apical hair cells than in the basal ones at P0 in a similar gradient to *Atoh1*, such as *Srrm4* and *Scn11a* (Fig. 3). These genes are likely to be regulated by Atoh1 (although they may not necessarily be direct targets) and like Atoh1 are downregulated as hair cells mature. In contrast, some genes, such as *Chrna10* and *Mreg*, showed an opposite gradient, with higher expression in basal hair cells compared with apical hair cells (Fig. 3). It is likely that these genes reflect the ongoing basal-apical wave of differentiation of hair cells and represent markers of maturing hair cells. Alternatively, it is possible that some of the genes showing differential apical-basal expression maintain this differential expression as the cochlea matures and might, for example, be involved in regulating tonotopic differences in hair cells along the cochlear duct.

Identification of Atoh1 direct target genes in neonatal cochlear hair cells

Although Atoh1 is both necessary and sufficient for hair cell development, little is known about the molecular function of Atoh1 in hair cells and very few genes have been identified as direct targets of Atoh1 in hair cells. To select potential Atoh1 targets, we cross-referenced our validated hair-cell-specific genes from the P0 cochlea with Atoh1 ChIP-seq datasets from mouse cerebellum and intestine (Klisch et al., 2011; Kim et al., 2014). We found that genes for 233 of 313 hair-cell-enriched transcripts contain Atoh1 binding regions within 10 kb of the transcriptional start site in

Table 1. Previously characterized hair cell genes identified in RNA-seq of P1 Atoh1-GFP+ cells

Gene	Expression in GFP+ cells (RPKM)	Expression in GFP− cells (RPKM)	Fold-change (GFP+ vs GFP−)	*p*	References
Atoh1	19042.084	40.151	474.258	6.23E-130	Bermingham et al., 1999; Chen et al., 2002; Cai et al., 2013
Atp7b	1270.502	64.270	19.768	5.07E-43	Ding et al., 2011
Barhl1	561.825	1.982	12.810	2.54E-73	Li et al., 2002
Bdnf	156.249	0.500	283.453	6.73E-44	Ylikoski et al., 1993; Wheeler et al., 1994
Cacnb2	3669.813	288.711	312.498	5.67E-37	Neef et al., 2009
Calb2	9535.022	754.472	12.711	1.41E-35	Dechesne et al., 1991, 1993
Calm1	200561.183	12606.473	12.638	1.84E-42	Walker et al., 1993
Cdh23	45229.825	347.607	15.909	8.06E-27	Müller, 2008
Celsr3	3879.836	63.198	61.391	6.77E-74	Shima et al., 2002
Chrm4	594.397	33.833	17.568	1.21E-34	Maison et al., 2010
Chrna10	25097.049	49.883	503.115	6.37E-127	Elgoyhen et al., 2001
Chrna9	12030.115	17.392	691.694	2.97E-86	Elgoyhen et al., 1994
Cldn14	446.603	5.849	76.361	1.95E-54	Ben-Yosef et al., 2003
Cldn9	23743.783	1413.451	16.798	4.54E-44	Nunes et al., 2006
Ctbp2	2032.242	8.167	70.452	1.72E-31	Schmitz et al., 2000
Dll1	4063.849	57.682	239.864	8.02E-78	Morrison et al., 1999
Dll3	14095.426	58.764	119.103	4.46E-111	Hartman et al., 2007
Foxj1	10620.589	89.172	133.781	3.26E-93	Yu et al., 2011
Fscn2	8200.816	61.300	205.116	3.45E-45	Shin et al., 2010
Gfi1	10651.143	51.927	52.077	5.78E-105	Wallis et al., 2003
Gpr98	17877.609	343.293	505.351	1.37E-37	McGee et al., 2006
Grxcr1	10736.947	21.247	584.267	1.63E-126	Odeh et al., 2010
Grxcr2	8326.395	14.251	19.827	2.45E-127	Imtiaz et al., 2014
Hes6	27504.001	1387.183	75.974	6.40E-47	Qian et al., 2006
Jag2	41818.936	550.437	573.769	2.72E-58	Lanford et al., 1999
Lhfpl5	32615.662	56.845	452.087	5.35E-135	Longo-Guess et al., 2005
Lhx3	14148.409	31.296	21.718	5.58E-128	Hertzano et al., 2007
Lmo1	12269.408	564.951	277.373	2.62E-49	Deng et al., 2006
Loxhd1	6198.799	22.348	22.931	4.21E-81	Grillet et al., 2009
Mcoln3	4954.974	216.084	618.731	1.38E-51	Di Palma et al., 2002
Myo3a	7421.246	11.994	374.468	1.51E-128	Walsh et al., 2002
Myo3b	10718.111	28.622	15.678	2.62E-123	Merritt et al., 2012
Myo6	176638.988	11266.614	49.712	1.76E-43	Avraham et al., 1997
Myo7a	74294.144	1494.476	380.593	1.83E-37	Hasson et al., 1995
Nhlh1	9522.399	25.020	259.873	4.27E-121	Krüger et al., 2006
Pcp4	26486.479	101.921	602.423	1.04E-115	Thomas et al., 2003
Pou4f3	25774.460	42.785	331.022	2.01E-76	Erkman et al., 1996; Xiang et al., 1998
Ptprq	32675.674	98.711	98.435	2.41E-71	Goodyear et al., 2003
Pvalb	23332.031	237.031	13.086	6.02E-88	Pack and Slepecky, 1995
Pvrl1	21596.648	1650.421	17.679	8.31E-38	Togashi et al., 2011
Rab15	32493.128	1837.926	18.510	2.82E-46	Lai et al., 2011
Rassf4	14720.011	795.253	10.701	5.97E-48	Lai et al., 2011
Selm	26344.158	2461.804	275.918	4.66E-32	Lai et al., 2011
Smpx	11162.484	40.456	195.793	2.51E-113	Yoon et al., 2011
Sstr2	480.310	2.453	161.149	5.35E-66	Bodmer et al., 2012
Strc	11791.437	14.699	802.190	3.93E-55	Verpy et al., 2001
Tmc1	1173.027	98.556	11.902	8.21E-33	Kawashima et al., 2011
Tmc2	1621.727	3.260	497.489	5.12E-103	Kawashima et al., 2011
Tmie	4513.184	369.072	12.228	1.03E-35	Mitchem et al., 2002
Tomt	9470.009	143.013	66.218	2.92E-78	Ahmed et al., 2008
Ush1g	815.769	5.931	137.532	3.25E-60	Kikkawa et al., 2003
Ush2a	32496.762	48.641	668.099	2.46E-40	Pearsall et al., 2002

List of known hair cell genes whose transcripts are enriched in neonatal Atoh1-GFP+ cells. The gene name is indicated, together with the expression and fold-change in expression level (RPKM) compared with GFP− cells and the calculated *p* value for difference between the GFP+ and GFP− populations.

either cerebellar or intestinal ChIP-seq experiments, or have AtEAM binding sites within 5 kb of their transcriptional start site (Klisch et al., 2011). We designed specific primers for the binding locus of nine candidate genes (*Anxa4*, *Chrna10*, *Mgat5b*, *Mreg*, *Pcp4*, *Rasd2*, *Rbm24*, *Scn11a*, and *Srrm4*), as well as *Atoh1*, as it is known to regulate its own expression by binding to an autoregulatory enhancer (Helms et al., 2000; Groves et al., 2013; Jarman and Groves, 2013). We isolated sensory epithelia of P0 $Atoh1^{A1GFP/A1GFP}$ cochleae and performed chromatin immunoprecipitation with camelid nanobodies against the Atoh1-GFP fusion protein. By PCR amplification of the immunoprecipitated DNA, we verified all 10 genes to contain upstream or downstream elements that are directly bound by Atoh1 in the neonatal cochlear sensory epithelium. We also analyzed two genes, *Fgf18* and *Cntn1* that have been shown to be direct targets of Atoh1 in the cerebellum (Klisch et al., 2011), but which are not expressed at significant levels in cochlear hair cells. Neither gene region gave a positive result in our ChIP-PCR experiments (Fig. 4). The nine newly identified Atoh1 direct targets include genes that have been previously reported to be essential for cochlear development,

Table 2. Gene ontology analysis of genes identified in RNA-seq of P1 Atoh1-GFP+ cells

GO category	GO term	No. of genes	Genes
Biological process	Neuron differentiation	30	*Myo7a, Uchl1, Jag2, Atoh1, Mcoln3, Lhx3, Pou4f3, Rtn4rl2, Gfi1, Nefl, Kndc1, Ush2a, Cdh23, Tomt, Myo6, Fscn2, Kif5c, Celsr3, Dll1, Gpr98, Sall3, Bbs1, Lhfpl5, 6530402F18Rik, 2610109H07Rik, Chrnb2, Ngfr, Slitrk6, Wnt7a, Grk1*
	Sensory organ development	24	*Tomt, Myo6, Fscn2, Cryab, Myo7a, Tmie, Jag2, Dll1, Pax2, Prph2, Prox1, Gpr98, Ptprq, Lhfpl5, Atoh1, Pvrl1, Chrna9, Mcoln3, Pou4f3, Gfi1, Chrna10, Ush2a, Cdh23, Grk1*
Molecular function	Calcium ion binding	32	*Spock2, Clstn3, Jag2, Cacnb2, Dlk2, Cdh1, Calb2, Necab2, Mmp24, Syp, Pvalb, Pcp4, Pls1, Cib2, S100a1, Cdh23, Dtna, Tesc, Hpcal1, Celsr3, Dll1, Actn3, Anxa4, Gas6, S100a13, Gpr98, Pkd2l1, Chga, Umodl1, Cadps2, Dll4, Calm1*
	Cytoskeletal protein binding	20	*Obscn, Myo6, Baiap2l2, Fscn2, Myo3a, Myo7a, Myo3b, Lmo7, Actn3, Rph3a, Gpr98, Pacsin1, Fhod3, Myo16, Pls1, AI428936, Lmod1, Eps8l2, Ush2a, Tmod1*
	Channel activity	17	*Scn1b, Cacnb2, Pkd2l1, Kcnmb2, Kcna10, Accn4, Chrna9, Mcoln3, P2rx3, Kcnh6, Kcnf1, Scn11a, Chrnb2, Kcnh2, Chrna1, Chrna10, Chrng*
	Motor activity	11	*Myo6, Kif27, Myo3a, Myo7a, Myo3b, Kif5c, Myo16, Kif19a, Dynlrb2, Kif21b, Dnaic2*
Cell component	Cell junction	24	*Gpr156, Ica1, Cldn9, Dlgap3, Rimbp2, Ctnnd2, Lmo7, Cdh1, Rims2, Rph3a, Calb2, Syp, Cbln1, Pvrl1, Cadps2, Pcp4, Snph, Chrnb2, Ssx2ip, Tjp3, Chrna1, Chrna10, Chrng, Dtna*
	Synapse	23	*Gpr156, Rab3b, Ica1, Cplx1, Myo6, Dlgap3, Clstn3, Myo7a, Rimbp2, Rims2, Rph3a, Syp, Cbln1, Chrna9, Cadps2, Snph, Chrnb2, Chrna1, Chrna10, Nefm, Chrng, Cdh23, Dtna*

Gene ontology analysis of the 313 genes whose transcripts showed high ranks in both expression and fold-change in our RNA-seq result (expression level >3000 RPKM, fold-change >10). A representative number of GO categories and terms are shown with >10 genes per category.

Table 3. Summary of hair cell genes validated by *in situ* hybridization

	Expression	Fold-change	P0 vestibule	Gradient (high to low)	Published HC gene	ChIP-seq	Atoh1 direct targets	Expression in Atoh1-CKO
Anxa4	58650.61	74.378	HC	Base to apex	No	Intestine	Yes	Decreased
Atoh1	19042.08	474.258	HC	Apex to base	Yes	Cerebellum, intestine	Yes	Decreased
Calb2	9535.022	12.638	HC	Not observed	Yes	Cerebellum	ND	ND
Casz1	9969.698	85.483	HC	Not observed	No	Cerebellum, intestine	ND	ND
Chrna10	25097.05	503.115	HC	Base to apex	Yes	Intestine	Yes	ND
Chrna9	12030.11	691.694	HC	Not observed	Yes	None	ND	ND
Eps8l2	15530.19	64.052	HC	Base to apex	Yes	Intestine	ND	ND
Grxcr2	8326.395	584.267	HC	Not observed	Yes	None	ND	No change
Kcnh6	12791.54	154.773	HC	Not observed	No	Intestine	ND	ND
Kif21b	8369.936	11.176	HC	Base to apex	No	Cerebellum, intestine	ND	ND
Lhfpl5	32615.66	573.769	HC	Not observed	Yes	Intestine	ND	ND
Lhx3	14148.41	452.087	HC	Not observed	Yes	Intestine	ND	Decreased
Mfng	10493.52	76.080	HC	Not observed	No	Cerebellum, intestine	ND	Decreased
Mgat5b	22037.06	214.706	HC	Not observed	No	Cerebellum, intestine	Yes	No change
Mreg	16063.03	139.686	HC	Base to apex	No	Cerebellum, intestine	Yes	No change
Mycl1	26009.33	41.131	HC	Not observed	Yes	Cerebellum, intestine	ND	ND
Myo3b	10718.11	374.468	HC	Base to apex	Yes	Cerebellum, intestine	ND	ND
Naca	10408.7	24.968	ND	Base to apex	No	Cerebellum, intestine	ND	ND
Pacsin1	4302.44	40.762	HC	Base to apex	No	Cerebellum, intestine	ND	ND
Pcp4	26486.48	259.873	HC	Base to apex	Yes	Cerebellum	Yes	No change
Ptgir	4722.92	133.553	No	Base to apex	No	None	ND	ND
Ptprq	32675.67	331.022	HC	Base to apex	Yes	None	ND	No change
Rab11fip1	3025.383	89.836	HC	Not observed	No	Intestine	ND	ND
Rasd2	127825.8	507.234	HC	Not observed	No	Cerebellum, intestine	Yes	Decreased
Rbm24	16001.61	229.243	HC	Not observed	No	Intestine	Yes	Decreased
Scn11a	11604.04	797.871	HC	Apex to base	Yes	Cerebellum	Yes	Decreased
Sema5b	32913.11	29.366	ND	Base to apex	No	Cerebellum, intestine	ND	ND
Slc6a11	13126.97	309.753	HC	Not observed	No	Cerebellum, intestine	ND	No change
Smpx	11162.48	275.918	HC	Not observed	Yes	Cerebellum	ND	ND
Srrm4	8899.872	164.984	HC	Apex to base	Yes	Cerebellum, intestine	Yes	Decreased
Stard10	30766.48	25.747	HC	Not observed	No	Intestine	ND	ND
Thsd7b	28906.74	298.100	HC	Not observed	No	Intestine	ND	No change
Ttc21a	16745.86	329.525	HC	Not observed	No	Cerebellum, intestine	ND	ND
Umodl1	10437.93	331.222	HC	Not observed	No	Cerebellum, intestine	ND	ND

Summary of genes whose specific expression in hair cells was validated by *in situ* hybridization. Gene name, expression level (RPKM), and fold-change over GFP− cells are indicated, together with expression pattern in the cochlea or vestibular system. If a transcript showed a gradient of expression in the cochlea, this is indicated. The table also lists whether each gene was identified as an Atoh1 target by ChIP-seq in either the cerebellum (Klisch et al., 2011) or small intestine (Kim et al., 2014), as well as whether the gene was shown to be a direct target of Atoh1 in the cochlea by ChIP-PCR (Fig. 5). Finally, the expression of direct Atoh1 target genes was measured *in vivo* 24 h after deletion of *Atoh1* (Fig. 5).

HC, Hair cell; IHC, inner hair cell; ND, not determined.

such as *Chrna10* and *Srrm4* (Simmons and Morley, 2011; Nakano et al., 2012), and genes that have not previously been described in ear development, such as *Anxa4*, *Mgat5b*, *Mreg*, *Pcp4*, *Rasd2*, *Rbm24*, and *Scn11a* (Fig. 4).

We have previously shown that Atoh1 protein is quite unstable in cochlear hair cells, as conditional deletion of *Atoh1* using the Cre-Lox system leads to hair cell death 12 h after the onset of Cre-mediated recombination (Cai et al., 2013). We tested to what

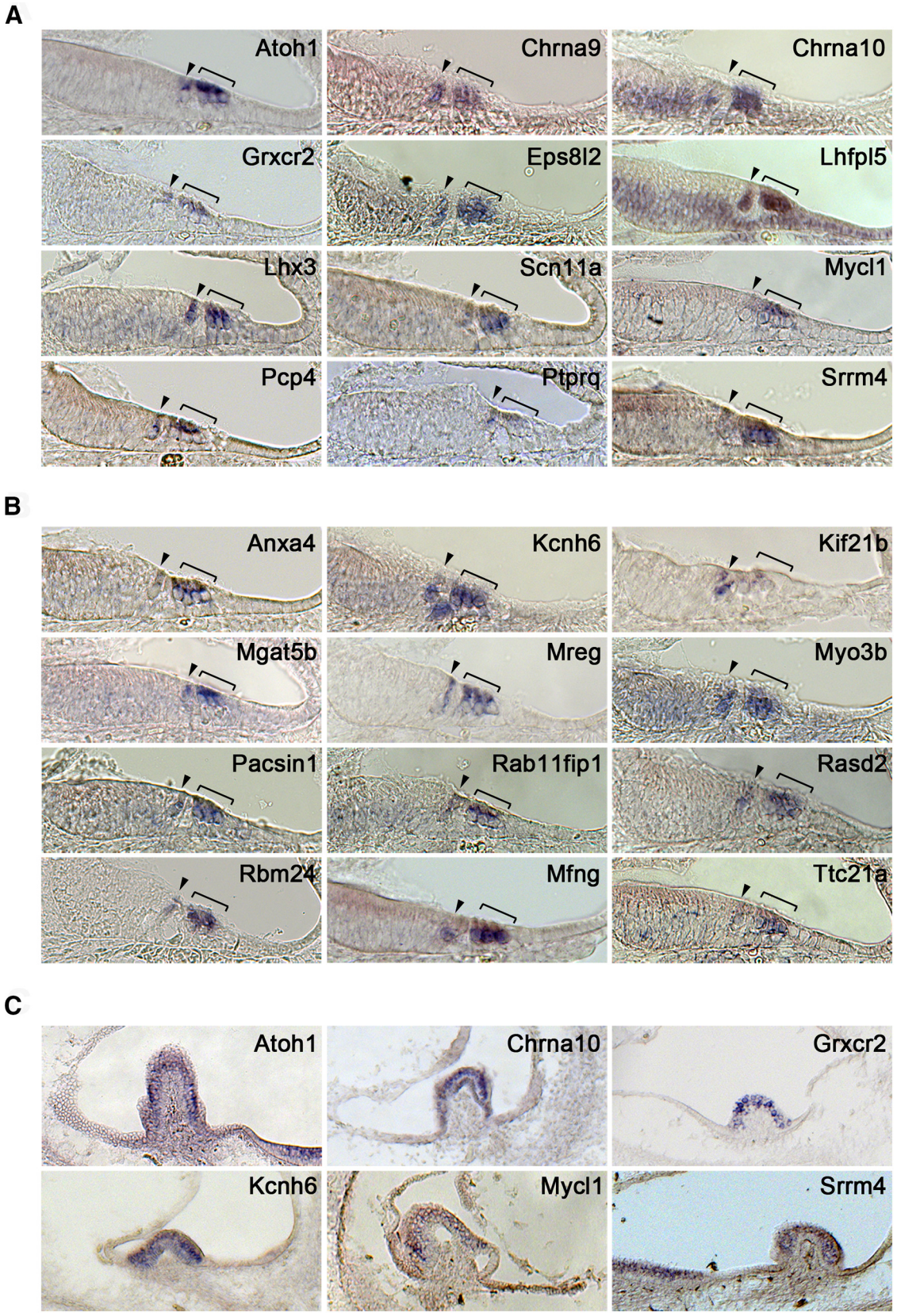

Figure 2. *In situ* hybridization validation of transcripts enriched in Atoh1-GFP+ cells. ***A***, Representative sample of 12 genes whose expression has previously been reported in hair cells. Images are taken from the basal turn of a neonatal mouse cochlea. ***B***, Representative sample of 12 genes whose expression has not previously been reported in hair cells. Images are taken from the basal turn of a neonatal mouse cochlea. Brackets mark the outer hair cell region; arrowhead marks the inner hair cell region. ***C***, Expression of sample of enriched transcripts in the cristae of the neonatal mouse vestibular system.

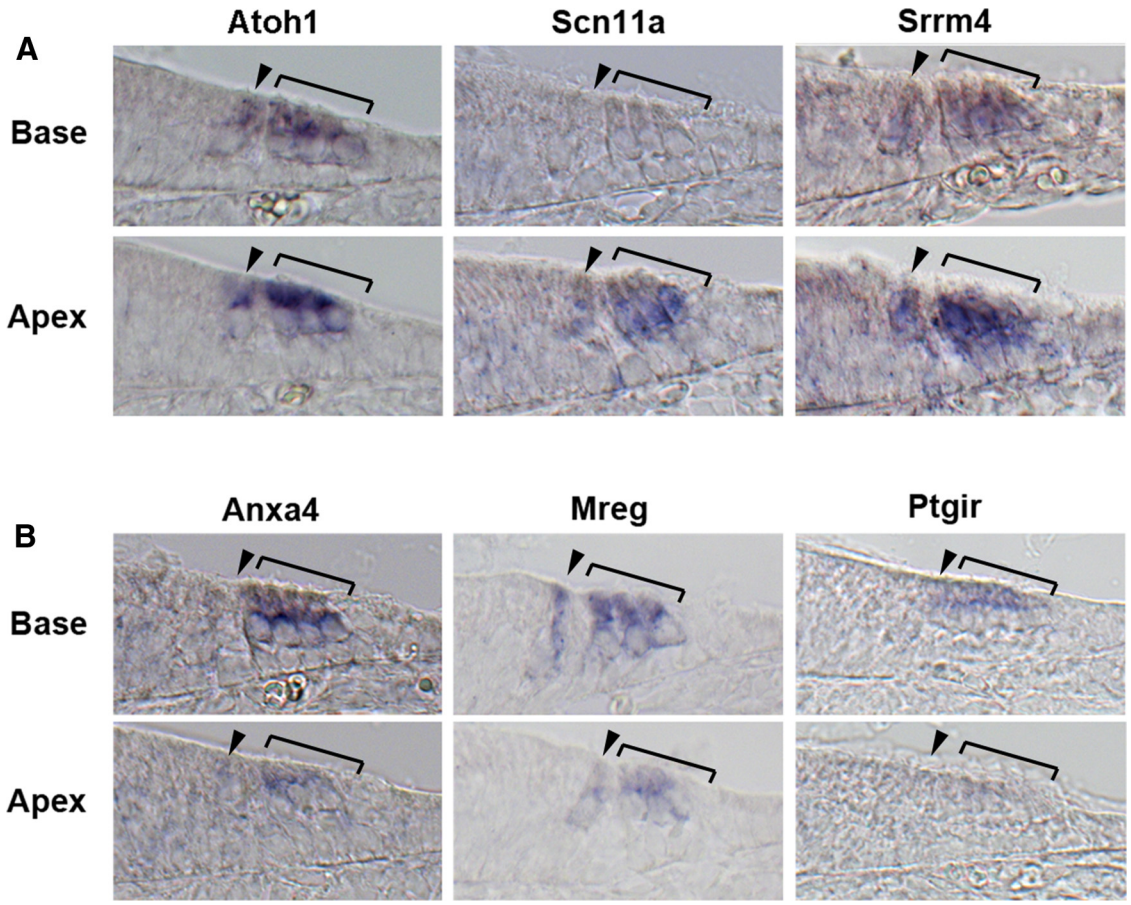

Figure 3. Examples of hair cell transcripts expressed in gradients along the neonatal cochlea. ***A***, *Atoh1*, *Scn11a*, and *Srrm4* are expressed more strongly in the apex than the base of the cochlea, consistent with their being downregulated as hair cells mature. ***B***, *Anxa4*, *Mreg*, and *Ptgir* are expressed more strongly in the base than the apex, consistent with their upregulation and maintenance as hair cells mature. Brackets mark the outer hair cell region; arrowhead marks the inner hair cell region.

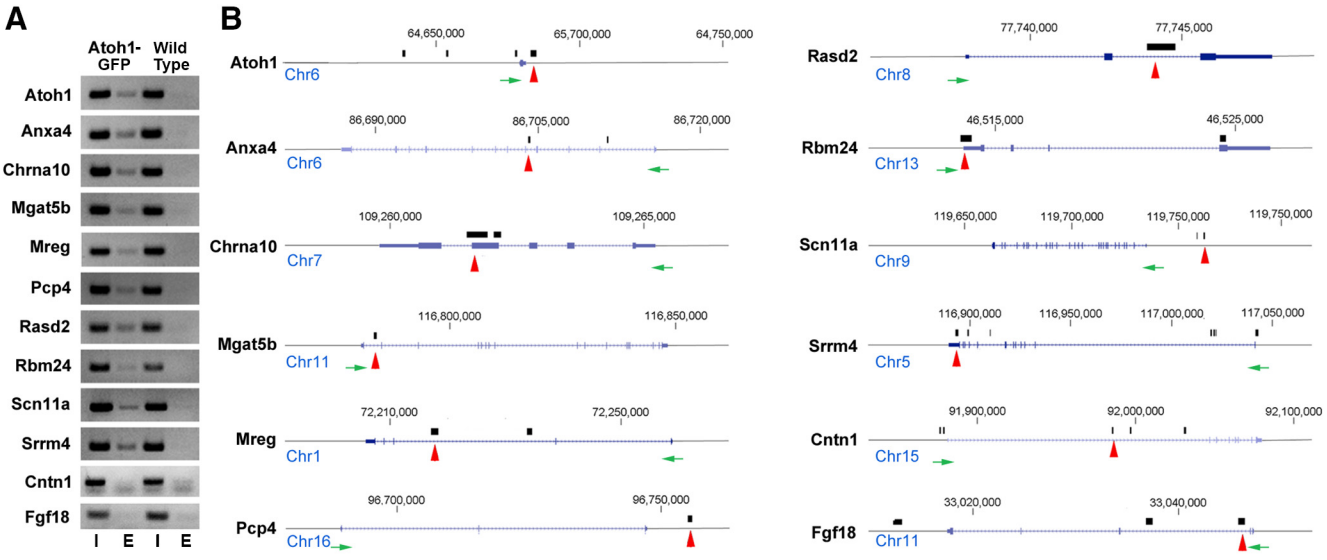

Figure 4. ***A***, Verification of direct targets of Atoh1 by ChIP-PCR. Ten genes that were identified as candidate Atoh1 targets (including *Atoh1* itself as a positive control) were analyzed by chromatin immunoprecipitation from sensory epithelium dissected from neonatal *Atoh1*$^{A1GFP/A1GFP}$ mice. Wild-type mice were used as a negative control. In addition, ChIP-PCR for two genes (*Fgf18* and *Cntn1*) that are directly regulated by Atoh1 in the cerebellum but are not expressed in hair cells were also used as a negative control. PCR amplification of candidate Atoh1 binding regions was performed as described in Materials and Methods. Input DNA and experimental lanes are indicated by I and E, respectively. ***B***, Schematic diagram showing the chromosomal location and structure of each gene, the direction of transcription (green arrows), and the location of Atoh1 binding sites (black bars) identified by ChIP-Seq from cerebellum (*Atoh1*, *Mgat5b*, *Mreg*, *Pcp4*, *Rasd2*, *Scn11a*, *Srrm4*, *Cntn1*, *Fgf18*; Klisch et al., 2011) or intestine (*Anxa4, Chrna10, Rbm24*; Kim et al., 2014). Regions used for ChIP-PCR in ***A*** are shown with red arrows.

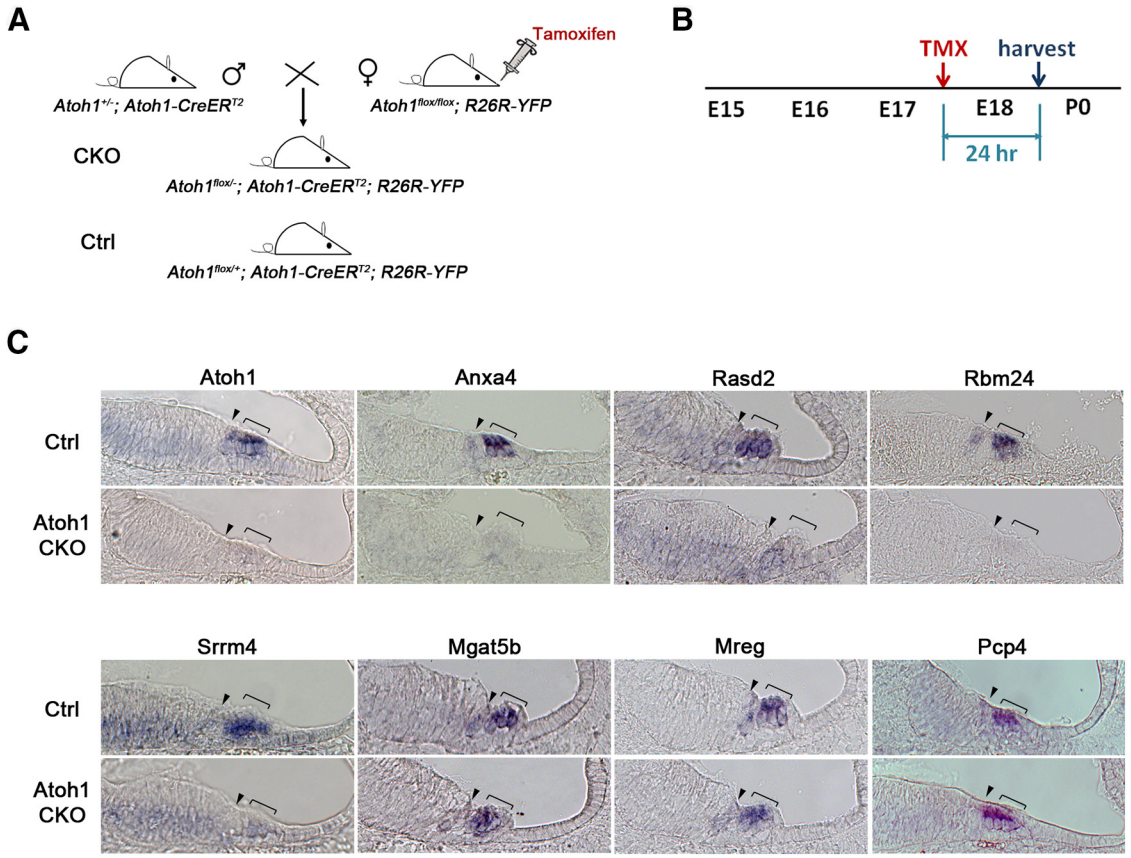

Figure 5. Some, but not all Atoh1 target genes are rapidly downregulated after deletion of *Atoh1*. ***A***, Breeding scheme to generate *Atoh1*-CKO mice. Female *Atoh1*$^{flox/flox}$; *R26R-YFP* mice were mated with *Atoh1*$^{\pm}$; *Atoh1-CreER*^{T2} males to generate mice that carried the *CreER*^{T2} allele, one copy of the *R26R-YFP* Cre reporter and either an *Atoh1*$^{flox/+}$ (50%) or *Atoh1*$^{flox/-}$ (50%) allele. Use of the *R26R-YFP* Cre reporter provides a readout of the leakiness, speed, and efficiency of the system. ***B***, Pregnant dams received one pulse of tamoxifen/progesterone (TMX) at E17.5, followed by kill 24 h later. ***C***, Four of seven genes tested (*Anxa4*, *Rasd2*, *Rbm24*, and *Srrm4*) showed rapid downregulation of transcripts 24 h after tamoxifen administration. However, three other genes (*Mgat5b*, *Mreg*, and *Pcp4*) were still expressed 24 h after tamoxifen administration. Brackets mark the outer hair cell region; arrowhead marks the inner hair cell region.

extent our verified Atoh1 target genes were sensitive to the loss of *Atoh1* by conditionally deleting *Atoh1* from hair cells at E17.5 as previously described (Cai et al., 2013). We crossed *Atoh1*$^{\pm}$; *Atoh1-CreER*^{T2} males with *Atoh1*$^{flox/flox}$; *R26R-YFP* female mice and administered tamoxifen and progesterone to female mice on the 17th day of pregnancy by oral gavage to generate *Atoh1* CKO (*Atoh1*$^{flox/-}$; *Atoh1-CreER*^{T2}; *R26R-YFP*) and control (*Atoh1*$^{flox/+}$; *Atoh1-CreER*^{T2}; *R26R-YFP*) animals. We collected embryos 24 h later, and examined the expression of seven verified Atoh1 target genes, as well as *Atoh1* itself as a positive control. Four genes (*Anxa4*, *Rasd2*, *Rbm24*, and *Srrm4*) were rapidly and completely downregulated in hair cells 24 h after deletion of *Atoh1* (Fig. 5). However, three other genes (*Mgat5b*, *Mreg*, and *Pcp4*) continued to be expressed in *Atoh1* CKO hair cells after 24 h at levels that were indistinguishable from their control counterparts. This persistence may simply reflect the stability of mRNA transcripts for these genes, or may indicate that these genes require Atoh1 for their initiation but not maintenance, due to the action of other transcription factors that can maintain transcription even after Atoh1 is downregulated.

Although Atoh1 is necessary and to some degree sufficient for hair cell development in the inner ear, its function is transient as it is downregulated shortly after birth (Groves et al., 2013). However, some of the genes we have verified to be direct targets of Atoh1, such as *Chrna10*, continue to be expressed in mature outer hair cells after Atoh1 ceases to be expressed (Elgoyhen et al., 2001), and were expressed strongly in basal regions of the cochlea even as *Atoh1* itself was being downregulated (Fig. 3). It is likely that Atoh1 participates in the assembly of transcriptional complexes at these loci, which remain active after Atoh1 expression ceases. For example, the *Pou4f3* gene, which is expressed in hair cells throughout life (Erkman et al., 1996; Xiang et al., 1998), has 5′ regulatory regions that are directly bound and regulated by Atoh1 (Masuda et al., 2011) but these regions also contain sites for other transcription factors such as Etv4, N-Myc, and Ets2 (Ikeda et al., 2015). It is possible that at least three of our verified Atoh1 targets, *Mgat5b*, *Mreg*, and *Pcp4* may be regulated in this way, as they continued to be expressed in the hair cells of *Atoh1* CKO mice (Fig. 3). Alternatively, it is also possible that the transcripts for these genes are quite stable and remain detectable in hair cells at least 24 h after loss of Atoh1 protein.

Discussion

We present one of the first RNA-seq studies to characterize purified cell populations from the mammalian cochlea. We were able to obtain high quality RNA-seq libraries from 100,000 purified Atoh1-GFP^{+} hair cells that gave excellent paired-end mapping of sequenced reads. A number of previous studies have produced valuable gene expression information from microarray profiling of different populations from the inner ear (Sato et al., 2009; Hertzano et al., 2011; Sinkkonen et al., 2011; Son et al., 2012). Our study, together with

other studies such as the recently described SHIELD database (shield.hms.harvard.edu) suggest that RNA-seq analysis of purified cell populations can be successfully accomplished with small numbers of inner ear hair cells; for example, we have recently been able to obtain RNA-seq data of similar quality from as few as 10,000 cells purified from the cochlea using standard RNA-seq library preparation reagents (M. L. Basch and A. K. Groves, unpublished observations).

We found 614 transcripts to be significantly enriched in hair cells, and of these at least 10% have been reported previously to be specifically expressed in hair cells. From our initial list of 614 genes, we selected 313 genes that were enriched >10-fold in Atoh1$^+$ cells, and which gave expression values of >3000 RPKM. Strikingly, when we analyzed 60 genes from this list by *in situ* hybridization, only ~50% (34 genes) showed exclusive and specific expression in hair cells. We note that one of our criteria for selecting genes for validation was the fold-change between Atoh1-GFP$^+$ hair cells and all other cell types in the ear. As such, it follows that a high degree of transcript enrichment in hair cells does not preclude that the transcript in question is present in other cell types in the ear; simply, that it is expressed at lower levels. This emphasizes that validation of RNA-seq data by analysis of mRNA *in situ* expression is a crucial step in the identification and confirmation of truly cell-type specific transcripts, even in cell populations enriched to >90% purity.

Our transcriptomic analysis was performed on hair cells purified from the newborn mouse cochlea. At this age, almost all hair cells have differentiated in the organ of Corti, although they continue to mature over the next 14 d until the onset of hearing at ~2 weeks of age. This maturation is reflected in many ways, for example, the elaboration of the stereociliary hair bundle (Frolenkov et al., 2004), the acquisition of functional mechanotransduction apparatus (Lelli et al., 2009), mature ion currents, and the divergence in morphology and function of inner and outer hair cells (Belyantseva et al., 2000). Some genes are specifically expressed in either inner or outer hair cells from an early age, for example, *Fgf8* is restricted to inner hair cells shortly after the onset of *Atoh1* expression (Jacques et al., 2007), and we observed *Kif21b* expression exclusively in inner hair cells at P1 (Fig. 2*B*). However, other genes, such as *Chrna9*, begin to be expressed in all cochlear hair cells before becoming restricted to outer hair cells (Elgoyhen et al., 1994; Liu et al., 2014; Fig. 2*A*), which may reflect radial (neural–abneural) gradients of differentiation signals. It will be of great interest to determine the transcriptional regulators that establish hair-cell-specific patterns of gene expression, either in development or during hair cell maturation.

At present, very little is known about changes in gene expression that accompany and regulate the maturation of hair cells. Our data suggest that at least some components of mature hair cells are already expressed in neonatal hair cells. First, gene ontology analysis of significantly enriched hair cell genes in our study identified cohorts of genes involved in motor activity, channel activity and calcium binding, synapse formation and function, in addition to genes associated with neural or sensory organ development (Table 2). Second, we were able to detect transcripts for some previously characterized components of the hair bundle (Shin et al., 2013), such as *Apba1*, *Calb2*, *Eps8l2*, *Fscn2*, *Hspa4l*, *Myo3b*, and *Stard10* in addition to the unconventional myosins *Myo6* and *Myo7a* that are expressed early in differentiating hair cells. Third, we compared our data to a recent study that used Affymetrix microarrays to identify the transcriptomes of 2000 individually isolated inner and outer hair cells from 25- to 30-d-old mice (Liu et al., 2014). Comparison of these transcriptomes to our neonatal hair cell data suggest that >80 genes expressed in neonatal hair cells continue to be expressed in mature inner or outer hair cells. Finally, we were also able to identify transcripts for 22 genes identified in syndromic or nonsyndromic forms of deafness.

The challenge and utility of identifying Atoh1 targets in hair cells

Atoh1 plays a central role in the differentiation and survival of hair cells (Jarman and Groves, 2013), and has been proposed as a candidate for gene therapy to restore hair cells lost through injury or aging (Baker et al., 2009; Husseman and Raphael, 2009). Discovering the direct transcriptional targets of Atoh1 is key to understanding its function and possible reasons for its reduced efficacy in promoting hair-cell formation in the aging ear (Kelly et al., 2012; Liu et al., 2012). However, genome-wide analysis of Atoh1 binding sites in hair cells by techniques, such as ChIP-seq, is confounded by the relatively small numbers of hair cells per cochlea and the fact that hair cells represent a small fraction of the total cell types in the cochlear duct. In contrast, it has been possible to obtain ChIP-seq data from Atoh1-expressing cerebellar granule cells (Klisch et al., 2011) and intestinal secretory cells (Kim et al., 2014). In an attempt to identify new Atoh1 targets in hair cells, we interrogated the transcriptome of cochlear hair cells obtained by RNA-sequencing to identify loci that are strongly and specifically expressed in Atoh1$^+$ hair cells and which have enhancers containing Atoh1 consensus binding sites (AtEAMs; Klisch et al., 2011), or which have been shown to be occupied by Atoh1 in the cerebellum or gut by ChIP-seq. This approach allowed us to identify 233 candidate Atoh1 target genes, of which 68 were common to our hair cell transcriptome and to ChIP-seq analyses from the cerebellum and gut. We verified 10 genes, including Atoh1 itself, as strong candidates to be direct targets of Atoh1. Previous studies have also identified several other direct targets of Atoh1 in hair cells, such as *Pou4f3* (Masuda et al., 2011; Ikeda et al., 2015), *Selm*, *Rassf4*, and *Rab15* (Lai et al., 2011).

Atoh1 can regulate a large and diverse array of genes in different cell types with functions including transcriptional control, cell-cycle regulation, cell migration, metabolic control, and even housekeeping functions (Klisch et al., 2011; Lai et al., 2011). These processes are not common to all *Atoh1*-expressing cells, for example, cerebellar granule neuron precursors are both migratory and proliferative, whereas hair cells are not. Clearly then, the cellular context in which Atoh1 functions will determine its selection of targets. Although Atoh1 is expressed in all inner ear hair cells, it is not known whether Atoh1 has distinct targets or overlapping targets in different types of hair cell, for example, auditory versus vestibular hair cells, inner hair cells versus outer hair cells, or type I versus type II vestibular hair cells, or instead simply regulates common, generic aspects of hair-cell development. All 10 of our validated Atoh1 targets showed comparable expression in inner and outer hair cells, and were also expressed in vestibular hair cells (Table 3); although the expression of many more validated Atoh1 targets will need to be tested to determine whether this trend is maintained. It is also possible that Atoh1 establishes a generic pattern of hair-cell gene expression, with later transcription factors modulating these targets in a hair-cell-specific manner after Atoh1 expression is downregulated.

Progress in identifying direct targets of Atoh1 may shed light on the function of this gene in regeneration as well as normal hair-cell differentiation. Ectopic expression of *Atoh1* in the inner

ear, either *in vivo* or *in vitro*, can lead to the formation of ectopic hair cells (Zheng and Gao, 2000; Kawamoto et al., 2003; Izumikawa et al., 2005; Gubbels et al., 2008; Kelly et al., 2012; Liu et al., 2012). Moreover, *Atoh1* is rapidly upregulated in supporting cells of nonmammalian vertebrates as a consequence of hair cell loss (Cafaro et al., 2007; Ma et al., 2008; Ma and Raible, 2009; Lewis et al., 2012). However, recent studies in mice suggest that the ability of inner ear cells to transdifferentiate into hair cells in the presence of ectopic Atoh1 declines with age (Kelly et al., 2012; Liu et al., 2012). There are a number of possible explanations for this decline, for example, the presence or absence of post-translational modifications of Atoh1 itself (Mulvaney and Dabdoub, 2012), an age-dependent upregulation of inhibitors of Atoh1, such as Id HLH factors, or an age-dependent decline in transcription factors or cofactors that cooperate with Atoh1 to activate hair cell genes (Ikeda et al., 2015). Finally, it is possible that direct epigenetic modification of Atoh1 target genes renders them unavailable for transcription, even in the presence of exogenously expressed Atoh1 (Groves et al., 2013). Our identification of direct Atoh1 targets in hair cells in the present study may allow the testing of these hypotheses.

Notes

Supplemental material for this article is available at **https://www.bcm.edu/research/labs/andrew-groves/publications**. Seven data tables and a legend document describe qPCR and PCR primers, a list of all 614 hair cell-enriched genes, 233 candidate *Atoh1* target genes, and comparisons of our hair cell-enriched genes with known deafness genes, genes identified in adult hair cells and genes identified in chick utricle hair cells, and expression levels of 19218 transcripts in Atoh1-GFP$^+$ hair cells at P1. This material has not been peer reviewed.

References

Ahmed ZM, Masmoudi S, Kalay E, Belyantseva IA, Mosrati MA, Collin RW, Riazuddin S, Hmani-Aifa M, Venselaar H, Kawar MN, Tlili A, van der Zwaag B, Khan SY, Ayadi L, Riazuddin SA, Morell RJ, Griffith AJ, Charfedine I, Caylan R, Oostrik J, et al. (2008) Mutations of LRTOMT, a fusion gene with alternative reading frames, cause nonsyndromic deafness in humans. Nat Genet 40:1335–1340. CrossRef Medline

Anders S, Huber W (2010) Differential expression analysis for sequence count data. Genome Biol 11:R106. CrossRef Medline

Avraham KB, Hasson T, Sobe T, Balsara B, Testa JR, Skvorak AB, Morton CC, Copeland NG, Jenkins NA (1997) Characterization of unconventional MYO6, the human homologue of the gene responsible for deafness in Snell's waltzer mice. Hum Mol Genet 6:1225–1231. CrossRef Medline

Baker K, Brough DE, Staecker H (2009) Repair of the vestibular system via adenovector delivery of Atoh1: a potential treatment for balance disorders. Adv Otorhinolaryngol 66:52–63. Medline

Belyantseva IA, Adler HJ, Curi R, Frolenkov GI, Kachar B (2000) Expression and localization of prestin and the sugar transporter GLUT-5 during development of electromotility in cochlear outer hair cells. J Neurosci 20:RC116. Medline

Ben-Arie N, Bellen HJ, Armstrong DL, McCall AE, Gordadze PR, Guo Q, Matzuk MM, Zoghbi HY (1997) Math1 is essential for genesis of cerebellar granule neurons. Nature 390:169–172. CrossRef Medline

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Ser B 57:289–300.

Ben-Yosef T, Belyantseva IA, Saunders TL, Hughes ED, Kawamoto K, Van Itallie CM, Beyer LA, Halsey K, Gardner DJ, Wilcox ER, Rasmussen J, Anderson JM, Dolan DF, Forge A, Raphael Y, Camper SA, Friedman TB (2003) Claudin 14 knockout mice, a model for autosomal recessive deafness DFNB29, are deaf due to cochlear hair cell degeneration. Hum Mol Genet 12:2049–2061. CrossRef Medline

Bermingham NA, Hassan BA, Price SD, Vollrath MA, Ben-Arie N, Eatock RA, Bellen HJ, Lysakowski A, Zoghbi HY (1999) Math1: an essential gene for the generation of inner ear hair cells. Science 284:1837–1841. CrossRef Medline

Birren SJ, Lo L, Anderson DJ (1993) Sympathetic neuroblasts undergo a developmental switch in trophic dependence. Development 119:597–610. Medline

Bodmer D, Brand Y, Radojevic V (2012) Somatostatin receptor types 1 and 2 in the developing mammalian cochlea. Dev Neurosci 34:342–353. CrossRef Medline

Cafaro J, Lee GS, Stone JS (2007) Atoh1 expression defines activated progenitors and differentiating hair cells during avian hair cell regeneration. Dev Dyn 236:156–170. CrossRef Medline

Cai T, Seymour ML, Zhang H, Pereira FA, Groves AK (2013) Conditional deletion of Atoh1 reveals distinct critical periods for survival and function of hair cells in the organ of Corti. J Neurosci 33:10110–10122. CrossRef Medline

Charpentier MS, Taylor JM, Conlon FL (2013) The CASZ1/Egfl7 transcriptional pathway is required for RhoA expression in vascular endothelial cells. Small GTPases 4:231–235. CrossRef Medline

Chen P, Johnson JE, Zoghbi HY, Segil N (2002) The role of Math1 in inner ear development: uncoupling the establishment of the sensory primordium from hair cell fate determination. Development 129:2495–2505. Medline

Dahl JA, Collas P (2009) MicroChIP: chromatin immunoprecipitation for small cell numbers. Methods Mol Biol 567:59–74. CrossRef Medline

Dechesne CJ, Winsky L, Moniot B, Raymond J (1993) Localization of calretinin mRNA in rat and guinea pig inner ear by *in situ* hybridization using radioactive and non-radioactive probes. Hear Res 69:91–97. CrossRef Medline

Dechesne CJ, Winsky L, Kim HN, Goping G, Vu TD, Wenthold RJ, Jacobowitz DM (1991) Identification and ultrastructural localization of a calretinin-like calcium-binding protein (protein 10) in the guinea pig and rat inner ear. Brain Res 560:139–148. CrossRef Medline

Deng M, Pan L, Xie X, Gan L (2006) Differential expression of LIM domain-only (LMO) genes in the developing mouse inner ear. Gene Expr Patterns 6:857–863. CrossRef Medline

Di Palma F, Belyantseva IA, Kim HJ, Vogt TF, Kachar B, Noben-Trauth K (2002) Mutations in Mcoln3 associated with deafness and pigmentation defects in varitint-waddler (Va) mice. Proc Natl Acad Sci U S A 99:14994–14999. CrossRef Medline

Ding D, He J, Allman BL, Yu D, Jiang H, Seigel GM, Salvi RJ (2011) Cisplatin ototoxicity in rat cochlear organotypic cultures. Hear Res 282:196–203. CrossRef Medline

Elgoyhen AB, Johnson DS, Boulter J, Vetter DE, Heinemann S (1994) Alpha 9: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. Cell 79:705–715. CrossRef Medline

Elgoyhen AB, Vetter DE, Katz E, Rothlin CV, Heinemann SF, Boulter J (2001) alpha10: a determinant of nicotinic cholinergic receptor function in mammalian vestibular and cochlear mechanosensory hair cells. Proc Natl Acad Sci U S A 98:3501–3506. CrossRef Medline

Erkman L, McEvilly RJ, Luo L, Ryan AK, Hooshmand F, O'Connell SM, Keithley EM, Rapaport DH, Ryan AF, Rosenfeld MG (1996) Role of transcription factors Brn-3.1 and Brn-3.2 in auditory and visual system development. Nature 381:603–606. CrossRef Medline

Frolenkov GI, Belyantseva IA, Friedman TB, Griffith AJ (2004) Genetic insights into the morphogenesis of inner ear hair cells. Nat Rev Genet 5:489–498. CrossRef Medline

Goodyear RJ, Legan PK, Wright MB, Marcotti W, Oganesian A, Coats SA, Booth CJ, Kros CJ, Seifert RA, Bowen-Pope DF, Richardson GP (2003) A receptor-like inositol lipid phosphatase is required for the maturation of developing cochlear hair bundles. J Neurosci 23:9208–9219. Medline

Grillet N, Schwander M, Hildebrand MS, Sczaniecka A, Kolatkar A, Velasco J, Webster JA, Kahrizi K, Najmabadi H, Kimberling WJ, Stephan D, Bahlo M, Wiltshire T, Tarantino LM, Kuhn P, Smith RJ, Müller U (2009) Mutations in LOXHD1, an evolutionarily conserved stereociliary protein, disrupt hair cell function in mice and cause progressive hearing loss in humans. Am J Hum Genet 85:328–337. CrossRef Medline

Groves AK, George KM, Tissier-Seta JP, Engel JD, Brunet JF, Anderson DJ (1995) Differential regulation of transcription factor gene expression and phenotypic markers in developing sympathetic neurons. Development 121:887–901. Medline

Groves AK, Zhang KD, Fekete DM (2013) The genetics of hair cell development and regeneration. Annu Rev Neurosci 36:361–381. CrossRef Medline

Gubbels SP, Woessner DW, Mitchell JC, Ricci AJ, Brigande JV (2008) Functional auditory hair cells produced in the mammalian cochlea by in utero gene transfer. Nature 455:537–541. CrossRef Medline

Harland RM (1991) *In situ* hybridization: an improved whole-mount

method for *Xenopus* embryos. Methods Cell Biol 36:685–695. CrossRef Medline

Hartman BH, Hayashi T, Nelson BR, Bermingham-McDonogh O, Reh TA (2007) Dll3 is expressed in developing hair cells in the mammalian cochlea. Dev Dyn 236:2875–2883. CrossRef Medline

Hasson T, Heintzelman MB, Santos-Sacchi J, Corey DP, Mooseker MS (1995) Expression in cochlea and retina of myosin VIIa, the gene product defective in Usher syndrome type 1B. Proc Natl Acad Sci U S A 92:9815–9819. CrossRef Medline

Helms AW, Abney AL, Ben-Arie N, Zoghbi HY, Johnson JE (2000) Autoregulation and multiple enhancers control Math1 expression in the developing nervous system. Development 127:1185–1196. Medline

Hertzano R, Dror AA, Montcouquiol M, Ahmed ZM, Ellsworth B, Camper S, Friedman TB, Kelley MW, Avraham KB (2007) Lhx3, a LIM domain transcription factor, is regulated by Pou4f3 in the auditory but not in the vestibular system. Eur J Neurosci 25:999–1005. CrossRef Medline

Hertzano R, Elkon R, Kurima K, Morrisson A, Chan SL, Sallin M, Biedlingmaier A, Darling DS, Griffith AJ, Eisenman DJ, Strome SE (2011) Cell type-specific transcriptome analysis reveals a major role for Zeb1 and miR-200b in mouse inner ear morphogenesis. PLoS Genet 7:e1002309. CrossRef Medline

Huang DW, Sherman BT, Lempicki RA (2009a) Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 37:1–13. CrossRef Medline

Huang DW, Sherman BT, Lempicki RA (2009b) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4:44–57. CrossRef Medline

Husseman J, Raphael Y (2009) Gene therapy in the inner ear using adenovirus vectors. Adv Otorhinolaryngol 66:37–51. CrossRef Medline

Ikeda R, Pak K, Chavez E, Ryan AF (2015) Transcription factors with conserved binding sites near ATOH1 on the POU4F3 gene enhance the induction of cochlear hair cells. Mol Neurobiol 51:672–684. CrossRef Medline

Imtiaz A, Kohrman DC, Naz S (2014) A frameshift mutation in GRXCR2 causes recessively inherited hearing loss. Hum Mutat 35:618–624. CrossRef Medline

Izumikawa M, Minoda R, Kawamoto K, Abrashkin KA, Swiderski DL, Dolan DF, Brough DE, Raphael Y (2005) Auditory hair cell replacement and hearing improvement by Atoh1 gene therapy in deaf mammals. Nat Med 11:271–276. CrossRef Medline

Jacques BE, Montcouquiol ME, Layman EM, Lewandoski M, Kelley MW (2007) Fgf8 induces pillar cell fate and regulates cellular patterning in the mammalian cochlea. Development 134:3021–3029. CrossRef Medline

Jarman AP, Groves AK (2013) The role of atonal transcription factors in the development of mechanosensitive cells. Semin Cell Dev Biol 24:438–447. CrossRef Medline

Kalay E, Li Y, Uzumcu A, Uyguner O, Collin RW, Caylan R, Ulubil-Emiroglu M, Kersten FF, Hafiz G, van Wijk E, Kayserili H, Rohmann E, Wagenstaller J, Hoefsloot LH, Strom TM, Nürnberg G, Baserer N, den Hollander AI, Cremers FP, Cremers CW, et al. (2006) Mutations in the lipoma HMGIC fusion partner-like 5 (LHFPL5) gene cause autosomal recessive nonsyndromic hearing loss. Hum Mutat 27:633–639. CrossRef Medline

Kawamoto K, Ishimoto S, Minoda R, Brough DE, Raphael Y (2003) Math1 gene transfer generates new cochlear hair cells in mature guinea pigs in vivo. J Neurosci 23:4395–4400. Medline

Kawashima Y, Géléoc GS, Kurima K, Labay V, Lelli A, Asai Y, Makishima T, Wu DK, Della Santina CC, Holt JR, Griffith AJ (2011) Mechanotransduction in mouse inner ear hair cells requires transmembrane channel-like genes. J Clin Invest 121:4796–4809. CrossRef Medline

Kelly MC, Chang Q, Pan A, Lin X, Chen P (2012) Atoh1 directs the formation of sensory mosaics and induces cell proliferation in the postnatal mammalian cochlea in vivo. J Neurosci 32:6699–6710. CrossRef Medline

Kikkawa Y, Shitara H, Wakana S, Kohara Y, Takada T, Okamoto M, Taya C, Kamiya K, Yoshikawa Y, Tokano H, Kitamura K, Shimizu K, Wakabayashi Y, Shiroishi T, Kominami R, Yonekawa H (2003) Mutations in a new scaffold protein Sans cause deafness in Jackson shaker mice. Hum Mol Genet 12:453–461. CrossRef Medline

Kim TH, Li F, Ferreiro-Neira I, Ho LL, Luyten A, Nalapareddy K, Long H, Verzi M, Shivdasani RA (2014) Broadly permissive intestinal chromatin underlies lateral inhibition and cell plasticity. Nature 506:511–515. CrossRef Medline

Klisch TJ, Xi Y, Flora A, Wang L, Li W, Zoghbi HY (2011) In vivo Atoh1 targetome reveals how a proneural transcription factor regulates cerebellar development. Proc Natl Acad Sci U S A 108:3288–3293. CrossRef Medline

Krizhanovsky V, Soreq L, Kliminski V, Ben-Arie N (2006) Math1 target genes are enriched with evolutionarily conserved clustered E-box binding sites. J Mol Neurosci 28:211–229. CrossRef Medline

Krüger M, Schmid T, Krüger S, Bober E, Braun T (2006) Functional redundancy of NSCL-1 and NeuroD during development of the petrosal and vestibulocochlear ganglia. Eur J Neurosci 24:1581–1590. CrossRef Medline

Ku YC, Renaud NA, Veile RA, Helms C, Voelker CC, Warchol ME, Lovett M (2014) The transcriptome of utricle hair cell regeneration in the avian inner ear. J Neurosci 34:3523–3535. CrossRef Medline

Lai HC, Klisch TJ, Roberts R, Zoghbi HY, Johnson JE (2011) *In vivo* neuronal subtype-specific targets of Atoh1 (Math1) in dorsal spinal cord. J Neurosci 31:10859–10871. CrossRef Medline

Lanford PJ, Lan Y, Jiang R, Lindsell C, Weinmaster G, Gridley T, Kelley MW (1999) Notch signalling pathway mediates hair cell development in mammalian cochlea. Nat Genet 21:289–292. CrossRef Medline

Lelli A, Asai Y, Forge A, Holt JR, Géléoc GS (2009) Tonotopic gradient in the developmental acquisition of sensory transduction in outer hair cells of the mouse cochlea. J Neurophysiol 101:2961–2973. CrossRef Medline

Lewis RM, Hume CR, Stone JS (2012) Atoh1 expression and function during auditory hair cell regeneration in post-hatch chickens. Hear Res 289:74–85. CrossRef Medline

Li S, Price SM, Cahill H, Ryugo DK, Shen MM, Xiang M (2002) Hearing loss caused by progressive degeneration of cochlear hair cells in mice deficient for the Barhl1 homeobox gene. Development 129:3523–3532. Medline

Liu H, Pecka JL, Zhang Q, Soukup GA, Beisel KW, He DZ (2014) Characterization of transcriptomes of cochlear inner and outer hair cells. J Neurosci 34:11085–11095. CrossRef Medline

Liu Z, Dearman JA, Cox BC, Walters BJ, Zhang L, Ayrault O, Zindy F, Gan L, Roussel MF, Zuo J (2012) Age-dependent in vivo conversion of mouse cochlear pillar and deiters' cells to immature hair cells by atoh1 ectopic expression. J Neurosci 32:6600–6610. CrossRef Medline

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\delta\delta C(T)}$ method. Methods 25:402–408. CrossRef Medline

Longo-Guess CM, Gagnon LH, Cook SA, Wu J, Zheng QY, Johnson KR (2005) A missense mutation in the previously undescribed gene Tmhs underlies deafness in hurry-scurry (hscy) mice. Proc Natl Acad Sci U S A 102:7894–7899. CrossRef Medline

Lott SE, Villalta JE, Schroth GP, Luo S, Tonkin LA, Eisen MB (2011) Noncanonical compensation of zygotic X transcription in early *Drosophila* melanogaster development revealed through single-embryo RNA-seq. PLoS Biol 9:e1000590. CrossRef Medline

Ma EY, Raible DW (2009) Signaling pathways regulating zebrafish lateral line development. Curr Biol 19:R381–R386. CrossRef Medline

Ma EY, Rubel EW, Raible DW (2008) Notch signaling regulates the extent of hair cell regeneration in the zebrafish lateral line. J Neurosci 28:2261–2273. CrossRef Medline

Machanick P, Bailey TL (2011) MEME-ChIP: motif analysis of large DNA datasets. Bioinformatics 27:1696–1697. CrossRef Medline

Machold R, Fishell G (2005) Math1 is expressed in temporally discrete pools of cerebellar rhombic-lip neural progenitors. Neuron 48:17–24. CrossRef Medline

Maison SF, Liu XP, Vetter DE, Eatock RA, Nathanson NM, Wess J, Liberman MC (2010) Muscarinic signaling in the cochlea: presynaptic and postsynaptic effects on efferent feedback and afferent excitability. J Neurosci 30:6751–6762. CrossRef Medline

Maragh S, Miller RA, Bessling SL, Wang G, Hook PW, McCallion AS (2014) Rbm24a and rbm24b are required for normal somitogenesis. PLoS One 9:e105460. CrossRef Medline

Masuda M, Dulon D, Pak K, Mullen LM, Li Y, Erkman L, Ryan AF (2011) Regulation of POU4F3 gene expression in hair cells by 5′ DNA in mice. Neuroscience 197:48–64. CrossRef Medline

McGee J, Goodyear RJ, McMillan DR, Stauffer EA, Holt JR, Locke KG, Birch DG, Legan PK, White PC, Walsh EJ, Richardson GP (2006) The very large G-protein-coupled receptor VLGR1: a component of the ankle link complex required for the normal development of auditory hair bundles. J Neurosci 26:6543–6553. CrossRef Medline

Merritt RC, Manor U, Salles FT, Grati M, Dose AC, Unrath WC, Quintero OA, Yengo CM, Kachar B (2012) Myosin IIIB uses an actin-binding

motif in its espin-1 cargo to reach the tips of actin protrusions. Curr Biol 22:320–325. CrossRef Medline

Mitchem KL, Hibbard E, Beyer LA, Bosom K, Dootz GA, Dolan DF, Johnson KR, Raphael Y, Kohrman DC (2002) Mutation of the novel gene Tmie results in sensory cell defects in the inner ear of spinner, a mouse model of human hearing loss DFNB6. Hum Mol Genet 11:1887–1898. CrossRef Medline

Morrison A, Hodgetts C, Gossler A, Hrabé de Angelis M, Lewis J (1999) Expression of Delta1 and Serrate1 (Jagged1) in the mouse inner ear. Mech Dev 84:169–172. CrossRef Medline

Müller U (2008) Cadherins and mechanotransduction by hair cells. Curr Opin Cell Biol 20:557–566. CrossRef Medline

Mulvaney J, Dabdoub A (2012) Atoh1, an essential transcription factor in neurogenesis and intestinal and inner ear development: function, regulation, and context dependency. J Assoc Res Otolaryngol 13:281–293. CrossRef Medline

Nakamura E, Nguyen MT, Mackem S (2006) Kinetics of tamoxifen-regulated Cre activity in mice using a cartilage-specific CreER^T to assay temporal activity windows along the proximodistal limb skeleton. Dev Dyn 235:2603–2612. CrossRef Medline

Nakano Y, Jahan I, Bonde G, Sun X, Hildebrand MS, Engelhardt JF, Smith RJ, Cornell RA, Fritzsch B, Bánfi B (2012) A mutation in the Srrm4 gene causes alternative splicing defects and deafness in the Bronx Waltzer mouse. PLoS Genet 8:e1002966. CrossRef Medline

Neef J, Gehrt A, Bulankina AV, Meyer AC, Riedel D, Gregg RG, Strenzke N, Moser T (2009) The Ca2+ channel subunit β2 regulates Ca2+ channel abundance and function in inner hair cells and is required for hearing. J Neurosci 29:10730–10740. CrossRef Medline

Nunes FD, Lopez LN, Lin HW, Davies C, Azevedo RB, Gow A, Kachar B (2006) Distinct subdomain organization and molecular composition of a tight junction with adherens junction features. J Cell Sci 119:4819–4827. CrossRef Medline

Odeh H, Hunker KL, Belyantseva IA, Azaiez H, Avenarius MR, Zheng L, Peters LM, Gagnon LH, Hagiwara N, Skynner MJ, Brilliant MH, Allen ND, Riazuddin S, Johnson KR, Raphael Y, Najmabadi H, Friedman TB, Bartles JR, Smith RJ, Kohrman DC (2010) Mutations in Grxcr1 are the basis for inner ear dysfunction in the pirouette mouse. Am J Hum Genet 86:148–160. CrossRef Medline

Pack AK, Slepecky NB (1995) Cytoskeletal and calcium-binding proteins in the mammalian organ of Corti: cell type-specific proteins displaying longitudinal and radial gradients. Hear Res 91:119–135. CrossRef Medline

Pan N, Jahan I, Kersigo J, Duncan JS, Kopecky B, Fritzsch B (2012) A novel Atoh1 "self-terminating" mouse model reveals the necessity of proper Atoh1 level and duration for hair cell differentiation and viability. PLoS One 7:e30358. CrossRef Medline

Pearsall N, Bhattacharya G, Wisecarver J, Adams J, Cosgrove D, Kimberling W (2002) Usherin expression is highly conserved in mouse and human tissues. Hear Res 174:55–63. CrossRef Medline

Qian D, Radde-Gallwitz K, Kelly M, Tyrberg B, Kim J, Gao WQ, Chen P (2006) Basic helix-loop-helix gene Hes6 delineates the sensory hair cell lineage in the inner ear. Dev Dyn 235:1689–1700. CrossRef Medline

Rajab A, Kelberman D, de Castro SC, Biebermann H, Shaikh H, Pearce K, Hall CM, Shaikh G, Gerrelli D, Grueters A, Krude H, Dattani MT (2008) Novel mutations in LHX3 are associated with hypopituitarism and sensorineural hearing loss. Hum Mol Genet 17:2150–2159. CrossRef Medline

Rose MF, Ren J, Ahmad KA, Chao HT, Klisch TJ, Flora A, Greer JJ, Zoghbi HY (2009) Math1 is essential for the development of hindbrain neurons critical for perinatal breathing. Neuron 64:341–354. CrossRef Medline

Sato T, Doi K, Hibino H, Kubo T (2009) Analysis of gene expression profiles along the tonotopic map of mouse cochlea by cDNA microarrays. Acta Otolaryngol Suppl 562:12–17. Medline

Scheffer D, Sage C, Corey DP, Pingault V (2007a) Gene expression profiling identifies Hes6 as a transcriptional target of ATOH1 in cochlear hair cells. FEBS Lett 581:4651–4656. CrossRef Medline

Scheffer D, Sage C, Plazas PV, Huang M, Wedemeyer C, Zhang DS, Chen ZY, Elgoyhen AB, Corey DP, Pingault V (2007b) The α1 subunit of nicotinic acetylcholine receptors in the inner ear: transcriptional regulation by ATOH1 and co-expression with the gamma subunit in hair cells. J Neurochem 103:2651–2664. CrossRef Medline

Schmitz F, Königstorfer A, Südhof TC (2000) RIBEYE, a component of synaptic ribbons: a protein's journey through evolution provides insight into synaptic ribbon function. Neuron 28:857–872. CrossRef Medline

Shima Y, Copeland NG, Gilbert DJ, Jenkins NA, Chisaka O, Takeichi M, Uemura T (2002) Differential expression of the seven-pass transmembrane cadherin genes Celsr1–3 and distribution of the Celsr2 protein during mouse development. Dev Dyn 223:321–332. CrossRef Medline

Shin JB, Longo-Guess CM, Gagnon LH, Saylor KW, Dumont RA, Spinelli KJ, Pagana JM, Wilmarth PA, David LL, Gillespie PG, Johnson KR (2010) The R109H variant of fascin-2, a developmentally regulated actin cross-linker in hair-cell stereocilia, underlies early-onset hearing loss of DBA/2J mice. J Neurosci 30:9683–9694. CrossRef Medline

Shin JB, Krey JF, Hassan A, Metlagel Z, Tauscher AN, Pagana JM, Sherman NE, Jeffery ED, Spinelli KJ, Zhao H, Wilmarth PA, Choi D, David LL, Auer M, Barr-Gillespie PG (2013) Molecular architecture of the chick vestibular hair bundle. Nat Neurosci 16:365–374. CrossRef Medline

Shroyer NF, Helmrath MA, Wang VY, Antalffy B, Henning SJ, Zoghbi HY (2007) Intestine-specific ablation of mouse atonal homolog 1 (Math1) reveals a role in cellular homeostasis. Gastroenterology 132:2478–2488. CrossRef Medline

Simmons DD, Morley BJ (2011) Spatial and temporal expression patterns of nicotinic acetylcholine alpha9 and alpha10 subunits in the embryonic and early postnatal inner ear. Neuroscience 194:326–336. CrossRef Medline

Sinkkonen ST, Chai R, Jan TA, Hartman BH, Laske RD, Gahlen F, Sinkkonen W, Cheng AG, Oshima K, Heller S (2011) Intrinsic regenerative potential of murine cochlear supporting cells. Sci Rep 1:26. CrossRef Medline

Son EJ, Wu L, Yoon H, Kim S, Choi JY, Bok J (2012) Developmental gene expression profiling along the tonotopic axis of the mouse cochlea. PLoS One 7:e40735. CrossRef Medline

Srinivas S, Watanabe T, Lin CS, William CM, Tanabe Y, Jessell TM, Costantini F (2001) Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev Biol 1:4. CrossRef Medline

Stern CD (1998) Detection of multiple gene products simultaneously by in situ hybridization and immunohistochemistry in whole mounts of avian embryos. Curr Top Dev Biol 36:223–243. Medline

Thomas S, Thiery E, Aflalo R, Vayssettes C, Verney C, Berthuy I, Créau N (2003) PCP4 is highly expressed in ectoderm and particularly in neuroectoderm derivatives during mouse embryogenesis. Gene Expr Patterns 3:93–97. CrossRef Medline

Togashi H, Kominami K, Waseda M, Komura H, Miyoshi J, Takeichi M, Takai Y (2011) Nectins establish a checkerboard-like cellular pattern in the auditory epithelium. Science 333:1144–1147. CrossRef Medline

Trapnell C, Pachter L, Salzberg SL (2009) TopHat: discovering splice junctions with RNA-Seq. Bioinformatics 25:1105–1111. CrossRef Medline

Verpy E, Masmoudi S, Zwaenepoel I, Leibovici M, Hutchin TP, Del Castillo I, Nouaille S, Blanchard S, Lainé S, Popot JL, Moreno F, Mueller RF, Petit C (2001) Mutations in a new gene encoding a protein of the hair bundle cause non-syndromic deafness at the DFNB16 locus. Nat Genet 29:345–349. CrossRef Medline

Walker RG, Hudspeth AJ, Gillespie PG (1993) Calmodulin and calmodulin-binding proteins in hair bundles. Proc Natl Acad Sci U S A 90:2807–2811. CrossRef Medline

Wallis D, Hamblen M, Zhou Y, Venken KJ, Schumacher A, Grimes HL, Zoghbi HY, Orkin SH, Bellen HJ (2003) The zinc finger transcription factor Gfi1, implicated in lymphomagenesis, is required for inner ear hair cell differentiation and survival. Development 130:221–232. CrossRef Medline

Walsh T, Walsh V, Vreugde S, Hertzano R, Shahin H, Haika S, Lee MK, Kanaan M, King MC, Avraham KB (2002) From flies' eyes to our ears: mutations in a human class III myosin cause progressive nonsyndromic hearing loss DFNB30. Proc Natl Acad Sci U S A 99:7518–7523. CrossRef Medline

Wheeler EF, Bothwell M, Schecterson LC, von Bartheld CS (1994) Expression of BDNF and NT-3 mRNA in hair cells of the organ of Corti: quantitative analysis in developing rats. Hear Res 73:46–56. CrossRef Medline

Woods C, Montcouquiol M, Kelley MW (2004) Math1 regulates development of the sensory epithelium in the mammalian cochlea. Nat Neurosci 7:1310–1318. CrossRef Medline

Xiang M, Gao WQ, Hasson T, Shin JJ (1998) Requirement for Brn-3c in maturation and survival, but not in fate determination of inner ear hair cells. Development 125:3935–3946. Medline

Yang J, Bouvron S, Lv P, Chi F, Yamoah EN (2012a) Functional features of trans-differentiated hair cells mediated by Atoh1 reveals a primordial mechanism. J Neurosci 32:3712–3725. CrossRef Medline

Yang SM, Chen W, Guo WW, Jia S, Sun JH, Liu HZ, Young WY, He DZ (2012b) Regeneration of stereocilia of hair cells by forced Atoh1 expression in the adult mammalian cochlea. PLoS One 7:e46355. CrossRef Medline

Ylikoski J, Pirvola U, Moshnyakov M, Palgi J, Arumäe U, Saarma M (1993) Expression patterns of neurotrophin and their receptor mRNAs in the rat inner ear. Hear Res 65:69–78. CrossRef Medline

Yoon H, Lee DJ, Kim MH, Bok J (2011) Identification of genes concordantly expressed with Atoh1 during inner ear development. Anat Cell Biol 44: 69–78. CrossRef Medline

Yu X, Lau D, Ng CP, Roy S (2011) Cilia-driven fluid flow as an epigenetic cue for otolith biomineralization on sensory hair cells of the inner ear. Development 138:487–494. CrossRef Medline

Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, Nusbaum C, Myers RM, Brown M, Li W, Liu XS (2008) Model-based analysis of ChIP-Seq (MACS). Genome Biol 9:R137. CrossRef Medline

Zheng JL, Gao WQ (2000) Overexpression of Math1 induces robust production of extra hair cells in postnatal rat inner ears. Nat Neurosci 3:580–586. CrossRef Medline