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The goal of the project is to test experimental therapies for hearing loss in transgenic mouse models. We have identified a list of targets of the Atoh1 transcription factor which is a key to regenerating hair cells. We have shown that another candidate pathway, the Notch signaling pathway, is no longer active in the mature animal, and thus is not a viable therapeutic target for hearing loss. We have also generated the means to activate Atoh1 and a supporting factor, Gfi1, in deafened mice to promote hair cell regeneration.
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1. INTRODUCTION:

Hearing and balance dysfunction are frequently seen in military personnel exposed to blast injuries. The most common cause of these disorders is the death of inner ear sensory hair cells. Once hair cells are killed, they are not replaced and combat-induced hearing and balance disorders are therefore permanent. At present, there is no treatment that allows the replacement of these sensory cells. The long term goal of this research is to develop gene or drug-based therapies that allow the regeneration of sensory hair cells and the restoration of hearing and balance in combat personnel. The transcription factor **Atoh1** is one of the first genes to be switched on when hair cells form. Atoh1 has been shown to induce new hair cells when activated in embryonic or neonatal inner ears. We have attempted to re-activate Atoh1 by genetic or pharmacological methods in an animal model to test its ability to promote regeneration of sensory hair cells.

2. KEYWORDS

Hearing Loss
Hair Cells
Cochlea
Atoh1
Gfi1
Mice
Regeneration
Supporting Cells
Adenovirus
3. OVERALL PROJECT SUMMARY:

Throughout this Final report, we have summarized the main findings discussed in previous Annual Reports and have provided a list of references to publications and abstracts that were generated by this work.

Aim 1: To determine the genetic targets of Atoh1

The transcription factor Atoh1 is both necessary and sufficient for the differentiation of hair cells, and is strongly up-regulated during hair cell regeneration in non-mammalian vertebrates. In order to understand more about the mechanism by which Atoh1 regulates the production of hair cells, we proposed the following goals for this Aim:

- to obtain comprehensive transcriptomic data sets from hair cells purified from wild type and Atoh1 mutant mice (which are unable to make hair cells)
- to use an epitope-tagged version of Atoh1 to identify direct targets of Atoh1 in cochlear hair cells by chromatin immunoprecipitation and sequencing (ChIP-Seq).

We initially began these studies by using microarray technology to identify transcripts present in purified hair cells, and we obtained microarray data from wild type hair cells purified by fluorescence-activated cell sorting. However, within the first six months of the project, the advent of RNA sequencing (RNA-seq), in particular the technology to obtain RNA-sequencing data from relatively small numbers of cells, allowed us to obtain much more representative and complete transcriptomic data from purified hair cells. As a result, we switched to RNA-seq for the remainder of the project and obtained high quality, reproducible, well-mapped RNA-seq data sets from purified neonatal cochlear hair cells (Cai et al., 2015).

Our original goal of generating RNA-seq data from Atoh1 mutant mouse cochleas was confounded by our discovery that in these mutants, the progenitors of hair cells in the cochlea die shortly after the time at which they would normally differentiate. Thus, Atoh1 is critically required for the survival of newly-generated hair cells. This result meant that it was not technically possible to purify the progenitors of hair cells from Atoh1 mutant mice, as they die almost as soon as they receive the signals to differentiate. However, we were able to use a conditional knockout mutant of Atoh1 to demonstrate that the requirement of Atoh1 for hair cell survival is only present for the first few days after hair cells differentiate. We were also able to publish the results of this study (Cai et al., 2013).

We carried out several attempts to perform ChIP-sequencing using transgenic mice that carried an epitope-tagged version of Atoh1. We did this both on whole cochleas and also on 300,000 purified hair cells carrying the tagged version of Atoh1. However, in both cases the signal-noise ratio we observed when analyzing the sequencing data made it impossible to accurately predict binding sites of Atoh1 in the hair cell genome. As a result, we adopted an alternative, bioinformatic-driven comparative strategy to identify potential targets of Atoh1 in hair cells. We cross-referenced our hair cell RNA-seq data with Atoh1 ChIP-seq data sets from two other populations that express Atoh1- cerebellar granule cells and intestinal epithelial cells. Our criteria for a candidate Atoh1 target gene was one of the following:

- It was significantly expressed and enriched in hair cells (fold change>10, RPKM>3000) and contained at least one predicted Atoh1 binding site within 5kb of the gene’s transcriptional start site, OR
- It was shown to an Atoh1 binding region within 10kb of the transcriptional start site that was revealed through ChIP-seq in either cerebellum or intestinal epithelium.

Using these parameters, we identified 233 candidate Atoh1 target genes in cochlear hair cells, and demonstrated that 10 of them were indeed direct Atoh1 targets by ChIP-PCR. We also showed that some of these genes were down-regulated when Atoh1 was conditionally deleted from cochlear hair cells. This data was published earlier this year (Cai et al., 2015).
Aim 2: To activate Atoh1 in damaged cochlear organ cultures to promote hair cell regeneration

Aim 3: To activate Atoh1 in deafened mice to promote hair cell regeneration

These two aims used two different strategies to activate Atoh1, and we consider each of them in turn together with a discussion of how they were applied to organ culture and intact mouse model systems.

A: Inhibition of the Notch signaling pathway

It is well-established that hair cells use the Notch signaling pathway to inhibit surrounding supporting cells from adopting a hair cell fate. Accordingly, in animals that can regenerate their hair cells (such as birds), newly-regenerated hair cells that up-regulate Atoh1 re-express Notch ligands on their cell surface. Similarly, inhibition of the Notch signaling pathway in neonatal mammals leads to supporting cells switching on the Atoh1 transcription factor and differentiating into hair cells. Thus, in these two aims, we proposed to inhibit Notch signaling in organ cultures and in living, deafened mice using a variety of means.

We quickly found that although inhibition of the Notch signaling pathway works very well in neonatal animals, the ability of Notch inhibition to generate new hair cells declines precipitously after birth, such that very little effect is seen in three day old mouse cochleas and no effect is seen by the time the mice are six days old. We should stress that this decline simply reflects the aging or maturation of mouse supporting cells, as no supporting cells are either lost or gained during this time period. We obtained a similar result in a different way by using transgenic over-expression of Notch inhibitory molecule (a dominant negative version of the Mastermind co-activator protein), but again saw the same result. We characterized this effect at the level of organ cultures and the single cell level, and these results were recently published (Maass et al., 2015).

We then attempted to determine the transcriptional changes that occurred in supporting cells in the first week after birth in mice. We purified supporting cells from new born or 6 day old transgenic mice used by our lab to label supporting cells with GFP. We performed RNA-seq on relatively small numbers of cells from these mice and were able to identify hundreds of transcripts that were either significantly up- or down-regulated between these two ages. This work was presented in abstract form (Maass et al., 2014); we are currently preparing the work for publication.

To understand the reason for why a blockade of Notch signaling is effective in neonatal but not older animals, we considered a number of possibilities. It was formally possible that a large number of transcriptional changes were occurring in DAPT-treated cultures in six day old animals, but that these changes were simply insufficient to induce a hair cell differentiation program. To test this, we performed RNA-seq experiments on supporting cells purified from neonatal and six day old supporting cells that were treated with Notch inhibitors for 24 hours. In P1 supporting cells that were exposed to DAPT for 24 hours, over 2000 genes were significantly up- or down-regulated. However, in P6 supporting cells that were exposed to DAPT for 24 hours, only 2 genes were significantly up-regulated and 18 were down-regulated. Our results therefore demonstrate that supporting cells become essentially refractory to Notch signaling between P1 and P6, and that Notch inhibition by itself is unlikely to be a viable therapy to restore hair cells.

B: Genetic activation of Atoh1

Prior to our proposal, a number of studies had suggested that ectopic expression of Atoh1 in the inner ear was able to generate ectopic hair cells. The majority of these studies had been performed in neonatal animals, but a number of studies also used viruses to infect supporting cells with Atoh1 in older animals. The second strategy we proposed to activate Atoh1 in the cochlea was to use transgenic mice in which Atoh1 was expressed using the Cre-LoxP system – we proposed to mate an inner ear- or supporting cell-specific Cre driver line with mice in which Atoh1 could be expressed in a Cre-dependent fashion. Our first attempt to make these mice, using a transgenic chick beta-actin promoter and a LoxP-flanked stop cassette, gave very low expression of Atoh1. We therefore switched to targeting the
ubiquitously-expressed ROSA locus using previously published targeting cassettes incorporating a GFP reporter and a LoxP-flanked stop cassette. Once again, this line gave a very low induction of Atoh1, for reasons which are still unclear to us.

At the same time that we were testing these mice, two independent studies were published that also used two different Cre-inducible systems to activate expression of Atoh1 in the inner ear. Similar to our Notch studies reported above, these two papers showed that although transgenic activation of Atoh1 can generate large numbers of new hair cells in neonatal animals, the same transgenic approach does not produce new hair cells in animals older than about 7 days. We therefore proposed to test the hypothesis that additional transcription factors may be required to co-operate with Atoh1 in older animals. We chose to pursue the zinc finger transcription factor Gfi1, as its homologue in Drosophila, senseless, has been shown to co-operate with and enhance the function of the Drosophila Atoh1 homologue, atonal. We therefore generated a second set of transgenic mice in which Atoh1 and Gfi1 were co-expressed from the same transgenic construct in a Cre-inducible fashion. As discussed above, our first attempt to co-express these two genes, using transgenic mice made from a previously published targeting cassettes incorporating a GFP reporter and a LoxP-flanked stop cassette, proved unsuccessful. We therefore re-engineered our mice using an alternative Cre-inducible expression construct developed and published by the Allen Brain Institute. We also generated adenoviral constructs that allowed us to infect the cochlea with either Atoh1 and a reporter gene, or Atoh1 and Gfi1 together with a reporter gene.

In the course of generating these new mice, we became concerned that the strategy we used for co-expression of Atoh1 and Gfi1 might be compromised by the picornavirus 2A sequence used to join the two gene coding regions together. After ribosomal cleavage of the 2A peptide to release Atoh1 and Gfi1 separately, a single proline residue was left attached to the N-terminus of Gfi1. Since the SNAG repressor domain of Gfi1 occurs immediately at the N-terminus of the protein, it is possible that the additional proline may interfere with this domain. We directly compared the repressive function of a Gfi1-2A-TdTomato construct (in which Gfi1 has no N-terminal proline), with a TdTomato-2A-Gfi1 construct, and found that the latter construct had a significantly (75%) lower repressive activity. At this point, we do not know if the repressive function of Gfi1 is necessary for hair cell formation, as there is some evidence that Gfi1 can directly interact with Atoh1 without DNA binding, but in the light of these results, it will be prudent to re-engineer our constructs with Gfi1 as the first sequence in the construct. However, the grant terminated before we could complete this work.

As we were completing the project, we were able to demonstrate that unlike the adult cochlea, adenoviral transduction of Atoh1 into the adult utricle was able to generate large numbers of extra hair cells. Based on our candidate list of Atoh1 targets generated in Aim 1, we are now in an excellent position to interrogate these targets in the adult utricle and to find how these targets are differentially regulated in the cochlea and utricle, such that regeneration is possible in the adult utricle but not the cochlea.
4. KEY RESEARCH ACCOMPLISHMENTS:

• Developed techniques to purify supporting cells from cultured organ of Corti
• Generated a validated list of genes expressed in hair cells using RNA-seq
• Generated a list of Atoh1 target genes in cochlear hair cells
• Generated high-quality RNA-Seq library from neonatal and six day old supporting cells.
• Generated high-quality RNA-Seq libraries to compare the effects of DAPT on neonatal and six day old supporting cells.
• Validated RNA-Seq library construction on small numbers of cells
• Confirmed that the new line of Sox2-CreER mice is able to efficiently cause gene activation or deletion in cochlear supporting cells.
• Generated a second set of targeting constructs to conditionally activate Atoh1 and Gfi1 in any cell or tissue type in the mouse.
• Generated adenoviral constructs to over-express Atoh1 and Gfi1 in any cell or tissue type in the mouse.
5. CONCLUSION:

The long-term goal of this project is to use activation of the Atoh1 gene by pharmacological or genetic means to promote hair cell production in the damaged cochlea as a means of hearing restoration.

The three goals of the proposal were to identify the genetic targets of Atoh1 and to demonstrate as a proof of principle that activation of Atoh1 can generate hair cells in organ culture and transgenic mouse models.

Aim 1 – to identify targets of Atoh1 itself – has now been completed and two manuscripts have been published. Mouse cochlear supporting cells execute their final division at late embryonic stages. One explanation for the failure of hair cell regeneration in mature animals is that direct transcriptional targets of Atoh1 become epigenetically modified in supporting cells with increasing age and are rendered unavailable for transcription, even in the presence of ectopically expressed Atoh1. It is now accepted that genome-wide changes in epigenetic marks on promoters and enhancers accompany cell differentiation and maturation. In addition to DNA methylation of CpG sequences associated with transcriptional silencing, post-translational modification of histones can also render genes active or silent. In addition, many genes can be held in a transcriptionally “poised” state during development: they contain both repressive and activating histone marks and are transcriptionally silent, but can be rapidly activated by removal of the repressive epigenetic modifications. It is possible that Atoh1 targets are transcriptionally poised in neonatal supporting cells, but these targets become completely silent and associated with heterochromatin as supporting cells mature. Now that we have a panel of validated Atoh1 targets, these questions are now experimentally tractable.

Aims 2 and 3 – to activate Atoh1 in organ culture and in the intact animal by transgenic over-expression or by inhibition of Notch signaling – were been impeded for two reasons. First, we established in the course of years 1 and 2 that the Notch pathway is no longer active in the mature cochlea. We have now characterized this at the cellular and molecular level using organ cultures, gene expression analysis and RNA-seq of normal and Notch-inhibited supporting cells of different ages. We have shown beyond reasonable doubt that inhibiting the Notch signaling pathway in supporting cells from six day old mice causes almost no significant transcriptional changes at all. This suggests that any future therapies involving Notch inhibition in the inner ear are unlikely to work by themselves. Future work must now concentrate on how hair cell-specific genes are prevented from being re-activated in mature supporting cells, since they are clearly not being repressed by Notch signaling.

The second impediment to our in vivo experiments has been to generate suitable transgenic mice to over-express Atoh1 and Gfi1. We have re-targeted mouse ES cells with new targeting constructs, but our final conclusion is that another modification of the construct will be necessary to ensure that the Gfi1 protein expressed in these mice is maximally functional. Interestingly, a recent study showed that over-expression of Atoh1, Gfi1 and Pou4f3 in ES cells was sufficient to convert them into cells with some properties of hair cells. This study used a similar 2A fusion strategy to the one we have used, but in that study, Gfi1 was placed at the 5’ end of the expression construct, a practice that we will adopt in the future.
6. BIBLIOGRAPHY OF PUBLICATIONS, ABSTRACTS AND PRESENTATIONS:


LIST OF PERSONNEL WHO RECEIVED PAY FROM THE RESEARCH EFFORT, 2010-2015

Andrew K. Groves, Ph.D., Principal Investigator

Juan C. Maass, M.D., Ph.D., Postdoctoral Fellow
Eduardo Martin Lopez, Ph.D., Postdoctoral Fellow
Joanna Asprer, Ph.D., Postdoctoral Fellow

David Chung, Graduate Student

Rende Gu, M.D., Staff Scientist

Hongyuan Zhang, Research Technician
8. REPORTABLE OUTCOMES:

Informatics: We have compiled two databases of genes whose expression is enriched in hair cells by both microarray and RNA-seq. These data have been uploaded to a publicly accessible database (NCBI GEO, accession number GSE65633). We have cross-referenced these databases to extract genes in these lists that contain Atoh1-binding sites within 5kb upstream or downstream by bioinformatic interrogation with a consensus AtEAM site. We have established that the two different methods give distinct but overlapping results. We have identified over 200 Atoh1 targets and have manually validated 10 of these by ChIP-PCR. We have published two manuscripts on these findings.

ES cell production: We have generated successfully targeted ES cell lines to generate Cre-inducible forms of Atoh1 and both Atoh1 and Gfi1 simultaneously. We have also generated adenoviral expression constructs to express these genes in the organ of Corti if required.

Notch regulation in the organ of Corti: We have published one manuscript describing the cessation of Notch signaling in the organ of Corti. We have also generated RNA-seq data for genes expressed in P1 and P6 supporting cells and for supporting cell treated with DAPT; these data sets will be uploaded to NCBI GEO when a final paper describing them is published.
Conditional Deletion of Atoh1 Reveals Distinct Critical Periods for Survival and Function of Hair Cells in the Organ of Corti

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Atohal homolog 1 (Atoh1) encodes a basic helix–loop–helix protein that is the first transcription factor to be expressed in differentiating hair cells. Previous work suggests that expression of Atoh1 in prosensory precursors is necessary for the differentiation and survival of hair cells, but it is not clear whether Atoh1 is required exclusively for these processes, or whether it regulates other functions later during hair cell maturation. We used EGFP-tagged Atoh1 knock-in mice to demonstrate for the first time that Atoh1 protein is expressed in hair cell precursors several days before the appearance of differentiated markers, but not in the broad pattern expected of a proneural gene. We conditionally deleted Atoh1 at different points in hair cell development and observe a rapid onset of hair cell defects, suggesting that the Atoh1 protein is unstable in differentiating hair cells and is necessary through an extended phase of their differentiation. Conditional deletion of Atoh1 reveals multiple functions in hair cell survival, maturation of stereociliary bundles, and auditory function. We show the presence of distinct critical periods for Atoh1 in each of these functions, suggesting that Atoh1 may be directly regulating many aspects of hair cell function. Finally, we show that the supporting cell death that accompanies loss of Atoh1 is likely caused by the abortive trans-differentiation of supporting cells into hair cells. Together our data suggest that Atoh1 regulates multiple aspects of hair cell development and function.

Introduction

The organ of Corti, the auditory sensory organ in mammals, comprises a precise and invariant pattern of mechanosensory hair cells and nonsensory supporting cells in the cochlea. Cochlear hair cells and supporting cells arise from a common post-mitotic precursor population, the prosensory domain (Kelley, 2006). This differentiation follows a gradient, starting near the basal region of the cochlea and progressing toward the apex over a period of several days (Li and Ruben, 1979; Lim and Anniko, 1985; Chen et al., 2002; Lumpkin et al., 2003; Montcouquiol and Kelley, 2003; Kelley, 2006). Atoh1, the mouse homolog of the Drosophila proneural gene atonal, is a basic helix–loop–helix (bHLH) transcription factor that is the earliest known gene expressed in differentiating hair cells (Bermingham et al., 1999; Chen et al., 2002; Woods et al., 2004). In the cochlea, Atoh1 is both necessary and sufficient for hair cell development: the absence of Atoh1 results in a complete loss of hair cells (Bermingham et al., 1999), while ectopic expression of Atoh1 is sufficient to direct ectopic hair cell formation in the greater epithelial ridge, the nonsensory epithelium next to the organ of Corti (Zheng and Gao, 2000). In Atoh1-null mice, massive cell death is observed within the presumptive sensory epithelia at the base of the cochlea at embryonic day (E) 15.5. Later, the apoptosis progresses to the whole cochlear duct (Chen et al., 2002). Transient expression of Atoh1 in mice in which Atoh1 is conditionally deleted by an Atoh1-Cre transgene is not sufficient to prevent the majority of hair cells from dying and cannot support the proper function of the remaining hair cells (Pan et al., 2012), indicating the level and duration of Atoh1 expression is critical for maintaining the viability and differentiation of hair cells. However, it remains unclear whether there is a critical period for Atoh1 to keep hair cells alive and whether Atoh1 functions differently at later stages of hair cell development. A recent genome-wide survey of Atoh1 target genes in the cerebellum suggested that Atoh1 regulates a broad range of biological processes, including cell proliferation, differentiation, migration, and metabolism (Klisch et al., 2011). The variety of pathways Atoh1 might regulate during cerebellar
development suggests that Atoh1 might be involved in multiple developmental processes in hair cells as well.

To dissect the function of Atoh1 during hair cell development, we established a conditional knockout (CKO) system to delete Atoh1 at specific developmental stages. By exposing pregnant or neonatal mice to tamoxifen to activate Cre-mediated recombination driven by an Atoh1 autoregulatory enhancer (Atoh1-CreER<sup>22</sup>), we deleted Atoh1 from hair cells at different embryonic and neonatal stages. We found a critical time window, ~2 d after initiation of Atoh1 expression, in which Atoh1 is absolutely required for hair cell survival. Atoh1 deletion within this time window also led to the loss of the surrounding supporting cells, and we used a Cre reporter allele to show that some of these supporting cells attempt to upregulate Atoh1 in response to hair cell loss. Significantly, later deletion of Atoh1 also revealed a time-dependent requirement for its activity in hair bundle maturation and auditory function. Together, our data suggest Atoh1 has multiple functions in the survival, differentiation, and maturation of hair cells during cochlear development.

**Materials and Methods**

**Experimental animals.** Atoh1<sup>+/−</sup> [Mouse Genome Informatics (MGI): Atoh1<sup>H11001/H11002</sup>, Atoh1<sup>CreERT2</sup>/Atoh1<sup>CreERT2</sup> (MGI: Atoh1<sup>CreERT2/CreERT2</sup>), and Atoh1<sup>CreERT2</sup> (MGI: Atoh1<sup>CreERT2/CreERT2</sup>) mice were generated as previously described (Bari-Erie et al., 1997; Shroyer et al., 2007; Rose et al., 2009). Atoh1<sup>CreERT2</sup> [MGI: Tg[Atoh1-cre/ES1]<sup>Fsh</sup>] (Machold and Fishell, 2005) and R26R-YFP [MGI: Gt(Rosa)26Sortm1(EYFP)Cos]<sup>*)</sup> (Srivinas 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 (GCC ACT GGC TTC TCT AGC GCC) and Neo-forward (GCA TCG CCT ACA TCC TTC) yield a 600 bp wild-type allele band and a 400 bp null allele band. HA-forward (GGC ACT GGC TTC TCT AGC GCC) 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-CreERT2<sup>+</sup>, Cre1F (GCC TGC ATT ACC GGT CGA TGC AAG CA) and Cre1R (GTG GCA GAT GGC GCA GCA ACA CTA C) yield a 700 bp band. For R26R-YFP, olMR0316 (GGA GGC GGA GAA ATG GAT ATG), olMR0883 (AAA ATG GCT GCT AGT GTG TAT T) and olMR4982 (AAG ACC GCG AAG ATG TGG TC) yield a 320 bp yellow fluorescent protein-positive (YFP +) band. To generate the inducible Atoh1 CKO mice, Atoh1-CreERT2<sup>+</sup>, Atoh1<sup>−/−</sup> males were crossed with Atoh1<sup>CreERT2</sup>, R26R-YFP homozygous females. One dose of 2 mg tamoxifen and 2 mg progesterone was administered to pregnant females at E13.5, E16.5, or E17.5 by oral gavage. Tamoxifen and progesterone were dissolved to get in peanut oil, both at a concentration of 20 mg/ml. For delivering tamoxifen into newborn animals, tamoxifen was dissolved in peanut oil at a concentration of 10 mg/ml and subcutaneously injected into animals at a dose of 75–100 µg/g body weight. 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.

**Cochlea isolation.** Cochleas from E13.5 to E16.5 embryos were dissected in PBS and then incubated in calcium-magnesium-free PBS containing dispase (1 mg/ml; Invitrogen) and collagenase (1 mg/ml; Worthington) for 8 min at room temperature. The enzyme solution was then replaced by DMEM containing 10% FBS and the mesenchymal tissue was removed by fine syringes to free the cochlear duct. For neonatal pups, cochleas were dissected in PBS, with the spiral ganglia and Reissner’s membrane removed to expose the organ of Corti. To obtain adult cochlear whole-mount preparations, temporal bones were dissected and the cochleas were slowly perfused through the oval window with 4% paraformaldehyde (PFA) in PBS, postfixed for 2 h at room temperature, and decalcified in 150 mM EDTA for 5–7 d at 4°C before further dissection.

**Immunohistochemistry.** Primary antibodies used in this study were anti-active Caspase 3 (1:500, rabbit; R&D Systems), anti-GFP (1:1000, chicken; Abcam; this detects GFP, EGFP, and YFP), anti-Hey2 (1:500, rabbit; Doetzlhofer et al., 2009), anti-Jag1 (1:200, rabbit; Santa Cruz Biotechnology), anti-Myo1m (1:500, rabbit; Proteus), anti-Myo5m (1:100, mouse; Developmental Studies Hybridoma Bank), anti-p27<sup>kip1</sup> (1:250, mouse; NeoMarker), anti-Prox1 (1:500, rabbit; Millipore Bioscience Research Reagents), anti-Sox2 (1:500, rabbit; Millipore), anti-Sox10 (1:250, goat; Santa Cruz Biotechnology), anti-ß tubulin (TuJ1, 1:1000, mouse; Covance), anti-TMHS (tetraspan transmembrane protein of hair cell stereocilia) (1:1000, rabbit; a gift from Kenneth Johnson; Longo-Guess et al., 2005), anti-espin (1:1000, rabbit; gift from Jim Bartles; Sekerkova et al., 2004), and anti-Atoh1 (1:10,000, chick; a gift from Matthew Kelley and Thomas Coate; Driver et al., 2013). Secondary antibodies were Alexa 488, Alexa 594, or Alexa 647 (1:2000; Invitrogen). Actin in stereociliary bundles was labeled with Alexa 594-conjugated phallolidin (1:1000, Invitrogen). Cell nuclei were labeled by DAPI. For sections of ear tissue, animal heads were fixed 1–2 h in 4% PFA at room temperature, cryoprotected in 30% sucrose in PBS at 4°C, embedded in OCT compound, and cryosectioned at 12–14 µm. The immunohistochemistry procedure followed standard protocols with some minor modifications. For anti-Atoh1 staining, tissue was fixed in 4% paraformaldehyde for 30 min before sectioning, and sections were blocked in PBS containing 0.5% Triton X-100, 10% goat serum, and 10% Hen-blocker (Aves Labs). For anti-p27<sup>kip1</sup>, Jag1, and Sox2 staining, sections were boiled for 10 min in 10 µM citric acid, pH 6.0.

**Uptake of FM1-43.** FM1-43 (Invitrogen) was prepared in water at a stock concentration of 5 mM. Cochleas were dissected from postnatal day (P) 5 mouse pups and briefly rinsed in HBSS (Invitrogen). Cochleas were exposed to a 5 µM solution of FM1-43 in HBSS for 15 s before being washed several times, mounted, and viewed with fluorescence microscopy.

**Auditory brainstem response recording.** Before testing, mice were anesthetized by intraperitoneal injection of a ketamine–xylazine mix at a dose of 100 mg/kg ketamine and 10 mg/kg xylazine. Testing was performed in a soundproof booth. Normal body temperature was maintained throughout the procedure by placing the mice on a heating pad. Pure tone bursts (0.1 ms rise/fall, 2 ms duration, 21 presentations/s) from 4 to 48 kHz were generated using System 3 digital signal processing hardware and software (Tucker Davis Technologies). The intensity of the tone stimuli was calibrated using a type 4938 one-quarter inch pressure-field calibration microphone (Bruel & Kjær). EC1 ultrasonic, low-distortion electrostic speakers were coupled to the ear canal to deliver stimuli within 3 mm of the tympanic membrane. Response signals were recorded with subcutaneous needle electrodes inserted at the vertex of the scalp (channel 1), the postauricular bulla region (reference), and the back leg (ground), and averaged over 500 presentations of the tone bursts. Electrode-recorded activity was filtered (high pass, 300 Hz; low pass, 3kHz; notch, 60Hz) before averaging to minimize background noise. Auditory thresholds were determined by decreasing the sound intensity of each stimulus to 10 dB from 90 dB in 5 dB steps until the lowest sound intensity with reproducible and recognizable waveforms was detected. Thresholds were determined to within 5 dB for each frequency by two raters to ensure reliability. Mean hearing thresholds ± SD (dB SPL) were plotted as a function of stimulus frequency (kilohertz) and analyzed for group differences at individual frequencies using two-tailed t-tests accompanied by a one-way ANOVA to reveal overall trends. R (version 2.13) was used for all statistical analyses.

**Results**

**Atoh1 protein marks hair cell precursors during mouse cochlear development.** The organ of Corti differentiates from a band of prosenycial cells that runs along the length of the cochlear duct (Kelley, 2007; Kelly and Chen, 2009). This prosenycial domain begins to exit the cell cycle in mice at E12.5, starting at the apex of the cochlea. Over the next 2–3 d, cell cycle exit proceeds along the cochlea from the apex to the base (Ruben, 1967; Lee et al., 2006). The differentiation of this postmitotic precursor domain into hair cells and supporting cells begins between E13 and E14, starting close to the
base of the cochlea and spreading along the length of the cochlea to the apex over the next 4 d (Li and Ruben, 1979; Lim and Anniko, 1985; Chen et al., 2002; Montcouquiol and Kelley, 2003). At intermediate times, different regions of the cochlea are therefore at different stages of differentiation. For example, at E16, the basal region of cochlea contains a full complement of one row of inner hair cells and three rows of outer hair cells as revealed by the hair cell marker Myosin6, while at the apex of the cochlea, no Myosin6-expressing hair cells can be detected (Fig. 1D). In the middle regions of the cochlea, the first inner hair cells can be observed differentiating, but their neighboring outer hair cells and associated supporting cells have yet to differentiate. Thus, different regions of the cochlea contain hair cells or their precursors at different stages of differentiation.

**Atoh1** is one of the first genes expressed in differentiating hair cells and is both necessary and in some circumstances sufficient for their differentiation (Bermingham et al., 1999; Zheng and Gao, 2000). However, the onset of **Atoh1** expression in the cochlea has been hard to visualize accurately, with different techniques giving different results. For example, using a β-gal reporter knocked in to the **Atoh1** locus, Woods et al. showed that **Atoh1** promoter activity can be observed in a wide patch of cells along most of the cochlear duct at E13 (Woods et al., 2004), suggesting **Atoh1** is a marker of hair cell and supporting cell pre-

**Figure 1.** Atoh1 protein expression in the mouse cochlea. **A**, Schematic diagram of the Atoh1
targeted to the Atoh1 genomic locus. **B**, Whole-mount Atoh1
tissues from E13.5 to P0. The right two rows show higher magnification of the base and apex regions marked by box at the left. Hey2 (red) was used to mark the prosensory domain at E13.5 and E14.5. Scale bars, 200 μm. **C**, Sections through regions of E16.5 Atoh1
tissue. Hair cells were labeled by Myosin6 (red). Hair cell differentiation begins near the base of the cochlea and spreads down to the apex over a period of days. Sections through the base, middle turn, and apex of the cochlea at E16.5 therefore reveal different stages in the differentiation of hair cell precursors. In the base, most Atoh1-EGFP protein is localized in the nucleus of Myosin6-expressing hair cells. However, in the apex, much cytoplasmic Atoh1-EGFP protein can be observed in precursors that have not yet expressed Myosin6. **E**, An Atoh1 antibody also reveals nuclear staining in hair cells in the base of the cochlea, but no detectable staining in the apex. Arrowhead, inner hair cells; bracket, outer hair cells.
The earliest stage at which we could detect Atoh1-EGFP expression in the Atoh1A1GFP/A1GFP cochlea was E13.5 (Fig. 1 B, C). In whole-mount preparations of the E13.5 cochlea, we observed expression of EGFP in a diffuse, salt-and-pepper patch of cells in the midbasal region, localized within the prosensory domain marked by the transcription factor Hey2 (Fig. 1 B) (Hayashi et al., 2008; Doetzthofer et al., 2009). We did not observe broad domains of Atoh1 protein expression in sections of the E13.5 cochlea. Instead, cells expressing Atoh1-EGFP frequently lined up in columns in the sensory epithelium or existed as single cells (Fig. 1 C). Costaining with p27kip1 and Hey2 indicated that these columns or isolated EGFP-labeled cells localized within the prosensory domain on its neural side (Fig. 1 C). This arrangement of the earliest Atoh1-expressing cells in the Atoh1A1GFP/A1GFP cochlea was consistent with previous immunohistochemical studies using anti-Atoh1 antibodies (Chen et al., 2002; Driver et al., 2013). However, the higher sensitivity of the anti-EGFP antibody in our study suggests that the onset of Atoh1 protein expression in the mouse cochlea begins as early as E13.5.

At E16.5 in the basal cochlea, Atoh1-EGFP protein was restricted to hair cells that express the early hair cell differentiation marker Myosin6, forming one row of inner hair cells and three rows of outer hair cells (Fig. 1 D). However, in the middle turn region, where Myosin6 is just beginning to be expressed in outer hair cells, Atoh1-EGFP was expressed in columns of cells spanning the sensory epithelium. This pattern of EGFP-expressing cells was also seen in the apex of E16.5 cochlea, where there were no signs of Myosin6 expression (Fig. 1 D). This suggests that Atoh1 is expressed in a subset of postmitotic precursors within the cochlear sensory primordium, before the differentiation of hair cells. As differentiation proceeds, Atoh1 becomes restricted to Myosin6-labeled hair cells (Fig. 1 D). Intriguingly, we observed that Atoh1-EGFP expression in hair cells in the basal third of the cochlea was largely restricted to the nucleus. However, in the middle turn and apical regions that contain Myosin6-negative hair cell precursors at earlier stages of differentiation, EGFP was diffusely localized throughout the cell cytoplasm (Fig. 1 D). These changes in subcellular distribution of Atoh1 protein between precursors and differentiating hair cells suggests there is a redistribution of cytosolic Atoh1 or an active transport of Atoh1 protein from the cytoplasm into the nucleus during hair cell differentiation.

Figure 2. Precursor cells in the sensory epithelia start to undergo apoptosis at E15.5 in the Atoh1-null (−/−) mice. A–C, E–G, Whole-mount cochleas from E15.5 Atoh1-null (A–C) and Atoh1 heterozygous (E–G) littermates. p27kip1 (green) marks the prosensory epithelial region. ActCasp3 (red) labels apoptotic cells in the prosensory epithelium of the Atoh1-null cochlea. C', G', Higher-power magnifications of the midbasal region of the cochlea marked by box in C and G, respectively. The dotted lines highlight the prosensory epithelium. D, H, Sections of the basal cochlea at E15.5. ActCasp3 (red) staining was only observed in the Atoh1 mutants (D) but not their heterozygous littermates (H).

Cell death in the prosensory domain of Atoh1 mutant mice

Genetic inactivation of Atoh1 in mice results in the death of a subpopulation of cells in the prosensory domain and a consequent failure of hair cell formation (Chen et al., 2002; Pan et al., 2011, 2012). To examine the time of onset of the apoptosis in detail, we performed immunohistochemistry on whole-mount Atoh1-null (Atoh1−/−; Bermingham et al., 1999) cochleas at different stages, using antibody against the active form of Caspase3 (ActCasp3). No apoptotic cells were observed in the prosensory domain of the Atoh1-null cochlea at E14.5 (data not shown), but we observed many ActCasp3-labeling cells at the base of E15.5 cochleas (Fig. 2A–C'). Staining of the cochlear sections showed the dying cells were located within the sensory epithelial region labeled by p27kip1 (Fig. 2D). These data suggest there is an ~48 h delay between the time at which Atoh1 protein expression would...
normally initiate in the cochlea and the death of hair cell precursors in the absence of Atoh1.

A transgenic mouse system to conditionally delete Atoh1

Our immunohistochemistry data from Atoh1^{A1GFP/A1GFP} cochleas suggest Atoh1 expression is sustained in cochlear hair cells for at least a week after hair cell differentiation. We next asked whether Atoh1 is continuously required for the survival of hair cells after differentiation has begun. We established a CKO system to remove Atoh1 from hair cells at different stages using the inducible Cre-lox system. We crossed Atoh1^{+/H11001}/H11002; Atoh1-CreERT2; R26R-YFP female mice. By oral gavage of the pregnant females or injecting mouse pups subcutaneously with tamoxifen, we were able to generate Atoh1 CKO (Atoh1^{flox/-}; Atoh1-CreERT2; R26R-YFP) and control (Atoh1^{flox/+}; Atoh1-CreERT2; R26R-YFP) animals (Fig. 3A). One advantage of this system is that the R26R-YFP reporter provides a clear visualization of the Cre recombination efficiency in the cochlea and reveals cells in which Atoh1 has been deleted. The system displayed almost no recombination in the absence of tamoxifen. Figure 3B shows regions from a neonatal Atoh1^{flox/+}; Atoh1-CreERT2; R26R-YFP mouse that had not been exposed to tamoxifen. No YFP can be seen in either apical or basal regions of the cochlea; we typically saw one YFP-labeled cell in every five cochleas from untreated control mice. Moreover, we saw no evidence of YFP-labeled supporting cells in control animals treated with tamoxifen at any age between E15.5 and P2 (data not shown).

Tamoxifen-mediated recombination occurred rapidly in our CKO system. Figure 3C shows examples of embryos in which tamoxifen was administered at E17.5 and the animals killed 8, 24, or 48 h later. We could readily detect YFP expression in the embryonic cochleas 8 h after tamoxifen treatment. After 24 h, >80% of hair cells in the base of the cochlea were labeled with YFP. At the apex, which contains less mature hair cells, fewer (50%) hair cells were labeled by Cre recombination, but by 48 h, similar numbers (80–85%) of YFP-labeled cells could be seen in both apical and basal regions.

Atoh1 expression is gradually downregulated in cochlear hair cells in the first few days after birth, commencing at the base of the cochlea and finally being extinguished at the apex several days later. For example, at P0, Atoh1 is being downregu-
lated in the base of the cochlea and is strongly expressed in the apex. Accordingly, when tamoxifen was injected into P0 pups, we saw few YFP-labeled cells in the base of the cochlea but many more (50%) in the apex (Fig. 3D). We continued to see ~50% labeled cells in the apical third of the cochlea when tamoxifen was administered at P2 (Fig. 3D), suggesting that the *Atoh1-CreER<sup>22</sup>* allele is still active at this time.

Identification of a critical period for *Atoh1* function in hair cell survival

To test whether there is a critical time window for *Atoh1* to maintain the survival of hair cells, we delivered one dose of tamoxifen to pregnant females at three stages, E15.5, E16.5, or E17.5 (Fig. 4A). Animals were allowed to develop to the day of birth (P0). B–D, Different regions of whole-mount cochleas from P0 *Atoh1* CKO and control littermates in which one dose of tamoxifen was administered at E15.5 (B), E16.5 (C), and E17.5 (D). YFP (green) was used as a reporter to show Cre-mediated recombination. Red, Myosin6-labeled hair cells. The number of hair cells in both CKO and control cochleas is quantified and shown in the graphs at the right (n = 3, error bars show SD).

**Figure 4.** *Atoh1* is required for the survival of hair cells in a time-dependent manner. A, Diagram showing the experimental design for the analysis of *Atoh1* CKO cochleas in B–D. Female *Atoh1<sup>flox/flox</sup>*;R26R-YFP mice were mated with *Atoh1<sup>−/−</sup>*; *Atoh1-CreER<sup>22</sup>* males and received a single dose of tamoxifen (TMX) at E15.5, E16.5, or E17.5. Mouse pups were analyzed on the day of birth (P0). B–D, Different regions of whole-mount cochleas from P0 *Atoh1* CKO and control littermates in which one dose of tamoxifen was administered at E15.5 (B), E16.5 (C), and E17.5 (D). YFP (green) was used as a reporter to show Cre-mediated recombination. Red, Myosin6-labeled hair cells. The number of hair cells in both CKO and control cochleas is quantified and shown in the graphs at the right (n = 3, error bars show SD).
cochleas, we first observed apoptotic cells 20 h after tamoxifen treatment, with large numbers of dying cells observed by 24 h (see Fig. 8A). Since we first detect recombination in our mice 8 h after tamoxifen administration (Fig. 3C), this suggests that Atoh1 protein is unstable and that loss of Atoh1 from differentiating hair cells causes rapid cell death. Moreover, since hair cells mature in a basal–apical gradient along the cochlea starting at E13.5, our data suggest that Atoh1 is required for the survival of hair cell precursors in the base of the cochlea for 72 h after the first appearance of Atoh1 protein, but that deletion of Atoh1 after this critical period (for example, at E16.5 or later in the base of the cochlea) does not compromise hair cell survival.

Identification of a second critical period for Atoh1 in hair cell function

We next addressed whether surviving hair cells in the Atoh1 CKO cochlea retain any aspects of normal structure or function. We tested the effect of Atoh1 deletion on hair bundle structure by staining the actin-rich stereocilia with fluorescently labeled phalloidin. Administration of tamoxifen at E17.5 causes hair cell loss in the apex, but not in the midregion or basal region (Fig. 4D). In these animals, we observed disorganized hair bundles in both in the middle turn (where hair cell numbers were normal) and in the apical regions of the cochlea (which show significant hair cell loss; Fig. 5A). We did not observe abnormal hair bundle structure in the basal, more mature hair cells of CKO cochleas (Fig. 5A). We saw similar results with antibodies to the hair bundle proteins Espin (Sekerkov á et al., 2004; Fig. 5B) and TMHS (Longo-Guess et al., 2005; data not shown). We also examined cochleas in which we delivered tamoxifen to neonatal pups at P0, and only observed very subtle defects at the apex of the CKO organs (Fig. 5A). These data suggest that Atoh1 plays an additional role in hair bundle development as well as in regulating hair cell survival. Since deletion of Atoh1 at E17.5 can cause hair cell defects without hair cell death, this suggests that there is a second delayed critical period for Atoh1 in the regulation of hair bundle morphology independent of cell survival.

To determine whether Atoh1 can influence assembly of the mechanotransduction machinery in hair cell stereociliary bundles, we labeled living cochleas with FM1-43, a fluorescent styryl dye that can rapidly enter hair cells through open mechanotransduction channels (Meyers et al., 2003; Lelli et al., 2009). We administered tamoxifen at E17.5 and let the mice develop for 5 d after birth. Dissected cochleas were then bath-exposed to FM1-43 for 15 s and then washed and examined for FM1-43 fluorescence. We detected dye uptake in hair cells all along the CKO cochleas regardless of bundle morphology, even at the apex, where significant hair cell loss had occurred (Fig. 5C). Similar results were observed by injecting mouse pups with the fixable dye AM1-43 (data not shown). This suggests that deletion of Atoh1 does not affect the correct expression of mechanotransduction channels in hair cells over the time period examined, and that the assembly of mechanotransduction apparatus does not require morphologically normal hair bundles.

Figure 5. Atoh1 is required for stereociliary bundle development in hair cells. A, Phalloidin staining of different regions of whole-mount cochlea preparations from P2 Atoh1-CKO and control littermates. One dose of tamoxifen administration at E17.5 resulted in disorganized hair bundles in the middle and apical turns of CKO cochleas, but no defects in the more mature hair cells in the basal region. However, only very subtle defects in hair bundles were observed in the apical cochlea of CKO animals that received one dose of tamoxifen at P0. This suggests that Atoh1 is initially required for hair cell survival, but later for other functions, including hair bundle morphology. B, Whole-mount cochleas from Atoh1-CKO and control animals receiving one dose of tamoxifen at E17.5 and stained with anti-espin antibodies at P2. C, Whole-mount cochleas from Atoh1-CKO and control animals receiving one dose of tamoxifen at E17.5 and then dissected at P5 and exposed to 5 μM of the styryl dye FM1-43 for 15 s to detect dye uptake through mechanotransduction channels. Surviving hair cells from apical regions of the cochlea displaying abnormal bundle morphology (A, B) still take up FM1-43. Scale bars, 20 μM. TMX, tamoxifen.
To determine the degree of hearing loss caused by deletion of Atoh1, we performed auditory brainstem response (ABR) measurements on 6-week-old Atoh1-CKO animals in which tamoxifen was administered at E17.5. We were surprised to see that these mice had a severe hearing loss at all frequencies tested (Fig. 6A), despite the fact that mice given tamoxifen at E17.5 only display apical hair cell loss at birth (Fig. 4D). This suggested that the loss of Atoh1 might lead to further hair cell loss or dysfunction as the animals age. We therefore treated mice with tamoxifen at E17.5 and isolated cochleas from these CKO at different postnatal stages and performed immunohistochemistry for Myosin6 to label hair cells and the TuJ1 anti-tubulin antibody to label neuronal fibers. At P7, no hair cells loss was observed in basal or middle turn regions of the CKO cochleas (Fig. 6C), although significant hair cell loss was seen in the apical regions, as we had observed at P0 (Fig. 4D). However, by P15, we started to observe loss of outer hair cells in the middle turn region of the cochlea (Fig. 6C). In 6-week-old adult mice, most outer hair cells were missing along the entire length of the cochlea (Fig. 6C) and efferent innervation of the outer hair cell region was severely disrupted. These data suggest that although outer hair cells in the basal and middle turns of the cochlea initially survive following loss of Atoh1, it is required for their integrity and survival in the week before the onset of hearing.

To determine whether this delayed effect of Atoh1 loss on hair cell survival also occurred within a critical time window, we administered tamoxifen to CKO pups at 2 d after birth and again measured hearing and cochlear morphology at 6 weeks of age. At P2, Atoh1 is still expressed in the apex of the cochlea, and Atoh1-CKO mice still show recombination in 50% of hair cells (Fig. 3D). In contrast to mice in which Atoh1 was deleted at E17.5, P2 CKO mice had ABR thresholds only marginally higher than those of controls (Fig. 6B). Moreover, the morphology and innervation of the cochlea of 6-week-old CKO was similar to controls along the length of the cochlea, and only very few missing outer hair cells were observed at the apex (Fig. 6D). These data suggest that although deletion of Atoh1 hair cells at E17.5 can cause delayed, severe hair cell death, hair cells in P2 mice no longer require Atoh1 for their short-term or long-term survival.

**Atoh1 is indirectly required for differentiation and survival of cochlear supporting cells**

Previous studies have suggested Atoh1 expression in hair cells might play an indirect role in the development and maturation of...
the neighboring supporting cells (Woods et al., 2004). Consistent with this idea, immunostaining of cochleas from Atoh1-null and heterozygous embryos at E19. The expression of supporting markers Prox1, Sox2, and Jag1 was abolished in the knock-out mutants. Similar, albeit slightly milder phenotypes were also seen in Atoh1-CKO mice that received three daily doses of tamoxifen between E13.5 and E15.5 and were killed at E19. B–D, Whole-mount cochleas from P0 Atoh1-CKO and control littermates receiving one dose of tamoxifen at E15.5 (B), E16.5 (C), and E17.5 (D). This is the same experimental regimen shown in Figure 4A. Supporting cells were visualized by staining with a Prox1 antibody (red). The number of Prox1-labeled cells in both CKO and control cochleas on each injection day is quantified and shown in the graph.

Figure 7. Atoh1 is required for the survival of the surrounding supporting cells in a time-dependent manner. A, Sections of middle turn regions of the cochlea from Atoh1-null and heterozygous embryos at E19. The expression of supporting markers Prox1, Sox2, and Jag1 was abolished in the knock-out mutants. Similar, albeit slightly milder phenotypes were also seen in Atoh1-CKO mice that received three daily doses of tamoxifen between E13.5 and E15.5 and were killed at E19. B–D, Whole-mount cochleas from P0 Atoh1-CKO and control littermates receiving one dose of tamoxifen at E15.5 (B), E16.5 (C), and E17.5 (D). This is the same experimental regimen shown in Figure 4A. Supporting cells were visualized by staining with a Prox1 antibody (red). The number of Prox1-labeled cells in both CKO and control cochleas on each injection day is quantified and shown in the graph.
ing cells from basal to middle turn regions of the CKO cochleas (Fig. 7B). However, when tamoxifen was administered at E17.5, supporting cell numbers were comparable between CKO and control organs at the base. Significant decrease of supporting cells was only observed in the middle turn to apical regions (Fig. 7D).

The loss of supporting cells in the Atoh1-CKO cochlea closely followed the loss of hair cells (Fig. 4B–D). These data suggest that the critical period of Atoh1 requirement for the survival of hair cells is approximately the same as that which indirectly regulates the survival of surrounding supporting cells.

To gain more insight into the mechanism of supporting cell death, we used anti-ActCasp3 antibodies to identify cells undergoing apoptosis in the cochlear sections of CKO mice that we harvested 24 h after tamoxifen treatment at E17.5. YFP shows cells that underwent recombination after tamoxifen administration. ActCasp3 (red) labels apoptotic cells in the apex of CKO organ (A). Myosin6 labels differentiated hair cells (B). Prox1 (C) and Sox10 (D) were used to examine supporting cell development. YFP + cells (arrow) in the supporting cell layer were only observed in the apical region of CKO cochlea. This suggested that cells in the supporting cell layer of the CKO, but not wild-type cochlea, were activating the Atoh1-CreERT2 transgene. In the apex of control cochlea, we often saw inner hair cells labeled by Sox10 (D, arrowhead).

E, Quantification of YFP + cells in the hair cell and supporting cell layers of Atoh1-CKO and control mice harvested 24 h after tamoxifen treatment at E17.5. The total number of YFP + cells was quantified in regions approximating to the basal and apical thirds of the cochlea from three different specimens of each genotype. Cells were assigned based on their position in the hair cell or supporting cell layers. Significant numbers of YFP-labeled cells (79) were observed in the supporting cell layer from the apical regions of three Atoh1-CKO cochleas. In contrast, only two YFP-labeled supporting cells from three cochleas could be observed in the apical turn of control cochleas.

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Figure 8. Atoh1 is indirectly required for differentiation and survival of cochlear supporting cells. A–D, Sections of apical regions of the cochlea from Atoh1-CKO and control animals harvested 24 h after tamoxifen treatment at E17.5. YFP shows cells that underwent recombination after tamoxifen administration. ActCasp3 (red) labels apoptotic cells in the apex of CKO organ (A). Myosin6 labels differentiated hair cells (B). Prox1 (C) and Sox10 (D) were used to examine supporting cell development. YFP + cells (arrow) in the supporting cell layer were only observed in the apical region of CKO cochlea. This suggested that cells in the supporting cell layer of the CKO, but not wild-type cochlea, were activating the Atoh1-CreERT2 transgene. In the apex of control cochlea, we often saw inner hair cells labeled by Sox10 (D, arrowhead). E, Quantification of YFP + cells in the hair cell and supporting cell layers of Atoh1-CKO and control mice harvested 24 h after tamoxifen treatment at E17.5. The total number of YFP + cells was quantified in regions approximating to the basal and apical thirds of the cochlea from three different specimens of each genotype. Cells were assigned based on their position in the hair cell or supporting cell layers. Significant numbers of YFP-labeled cells (79) were observed in the supporting cell layer from the apical regions of three Atoh1-CKO cochleas. In contrast, only two YFP-labeled supporting cells from three cochleas could be observed in the apical turn of control cochleas.
chleas, about half of the YFP+ cells were localized in the supporting cell layer (79 cells of a total of 170 YFP+ cells counted from 3 different cochleas; Fig. 8E). In contrast, we observed only two YFP+ cells in total within the supporting cell layer in the apical regions of three control cochleas (Fig. 8E). Since the Atoh1 auto-regulatory enhancer used to drive CreER\textsuperscript{T2} expression requires Atoh1 expression (Helms et al., 2000; Machold and Fishell, 2005; Raft et al., 2007), the presence of YFP in the supporting cell layer suggests that loss of hair cells induces the upregulation of Atoh1 in the surrounding supporting cells, followed by Cre-mediated deletion of Atoh1 in these cells and their rapid death.

**Discussion**

**Atoh1 is first expressed in a subset of precursors before hair cell differentiation**

In *Drosophila*, atonal acts as a proneural gene: it is expressed in proneural clusters and the sensory precursors that give rise to the chordotonal organs, and is both necessary and sufficient for the differentiation of sensory organ precursors (Jarman et al., 1993). However, different techniques to reveal Ator1 mRNA or protein expression in the mouse cochlea have yielded inconsistent results (Chen et al., 2002; Woods et al., 2004). We attempted to resolve this issue by using an Atoh1-EGFP fusion construct knocked into the Atoh1 locus. The Atoh1-EGFP allele is fully functional (Rose et al., 2009) and displays no observable phenotypes, being able to breed normally and survive to adulthood, unlike Atoh1-null mice. Our data show that Atoh1 protein is expressed before the expression of hair cell and supporting cell markers, but its expression is restricted to a subset of precursors in a salt-and-pepper pattern close to the border of the prosensory domain with the greater epithelial ridge (Fig. 1B, C). This region will give rise to inner hair cells, the first hair cell type to differentiate in the organ of Corti (Lim and Amnoko, 1985; Chen et al., 2002; Lumpkin et al., 2003; Jacques et al., 2007). The restricted expression of Atoh1 suggests that, unlike atonal in *Drosophila*, Atoh1 is not expressed in a manner consistent with a proneural gene in the mammalian cochlea.

We observed a striking change in the subcellular localization of Atoh1-EGFP protein as hair cell precursors differentiated. Before hair cell differentiation, Atoh1-EGFP is ubiquitously expressed in the nucleus and cytoplasm of hair cell precursors (Fig. 1D). However, as cells started expressing the hair cell differentiation marker Myosin6, Atoh1-EGFP becomes restricted to the nucleus. We observed similar results with a recently described polyclonal antibody to Atoh1 (Driver et al., 2013), although the antibody appeared to be less sensitive than the Atoh1-EGFP fusion protein (compare Fig. 1D and 1E). The change in Atoh1 subcellular localization might be caused by the nuclear transport of Atoh1 when precursors differentiate into hair cells. Although nuclear-cytoplasmic shuttling has been reported for many transcription factors, there are few examples of this process regulating cell differentiation and function of hair cells. We observed disorganized hair bundles in Atoh1-CKO cochleas when Atoh1 was deleted at E17.5, but not when Atoh1 was removed at P0, suggesting Atoh1 is required for the formation and orientation of stereocilia in a time-dependent manner. The fact that disorganized hair bundles can be observed in regions of Atoh1-CKO cochleas that have a normal complement of hair cells and supporting cells (Fig. 5A, B) suggests that this effect is a direct result of Atoh1 loss, rather than morphological rearrangements as an indirect consequence of cell death. Our FM1-43 labeling data suggests that mechanotransduction channels are still present in surviving Atoh1-CKO hair cells bearing disorganized stereociliary bundles (Fig. 5C). However, our ABR measurements of Atoh1-CKO mice suggest that animals in which Atoh1 is deleted at E17.5 have severe hearing loss at 6 weeks. Our whole-mount data suggests the hearing loss results from the degeneration of hair cells, particularly outer hair cells, and that this degeneration is already apparent by the onset of hearing at 2 weeks. We suggest that one factor contributing to
the delayed loss of hair cells in the CKO animals seen at adult stages might be disrupted formation of stereocilia. It has been previously shown that loss of genes involved in stereocilia development, such as *Whirlin*, *Cadherin23* and *Pcdh15*, can cause hair cell degeneration with age (Agramam et al., 2001; Holme et al., 2002; Mustapha et al., 2007; Kane et al., 2012), suggesting healthy stereociliary bundles are essential for the long-term survival of hair cells. Once again, we observe another critical period in which *Atoh1* is required for hair bundle formation and long-term hair cell survival, as we observed far less severe hearing loss and hair cell loss in CKO mice treated with tamoxifen at P2. Our histology data show that despite significant (>50%) recombination in the apex of the P2 cochlea, these mice lack only a few outer hair cells in this region, with no obvious signs of hair cell degeneration in basal and middle turn regions. The slight loss of hair cells in the apex may explain why these CKO mice show some degree of hearing loss at lower frequencies (<16 kHz).

Hair cell loss leads to trans-differentiation and death of surrounding supporting cells

Previous studies have shown that *Atoh1*-null mice lack both hair cells and supporting cells (Woods et al., 2004; Pan et al., 2012). It has been proposed that nascent hair cells may regulate the differentiation of supporting cells, but the ongoing role of hair cells in supporting cell survival is more controversial (Woods et al., 2004). We show here that conditional deletion of *Atoh1* in cochlear hair cells leads to supporting cell death, but only in regions in which hair cells are also missing. This suggests that hair cells are indirectly regulating the survival of supporting cells. A possible mechanism for this death is suggested by our observation of Cre-mediated recombination in some supporting cells after tamoxifen treatment. We see strong expression of EGFP in cells in the lower layer of the CKO cochlea, colocalizing with both Prox1 and Sox10. We have never observed this expression in control co-chick cilia. This suggests that nascent hair cells normally send inhibitory signals (likely through the Notch pathway) to neighboring supporting cells, preventing their differentiation into hair cells (Lanford et al., 1999; Kiernan et al., 2005). Moreover, a number of previous studies in embryonic or neonatal mammals have shown that loss of hair cells following trauma can cause supporting cells to trans-differentiate into hair cells (Kelley et al., 1995; Burns et al., 2012). Atoh1 upregulation in the supporting cells of CKO cochleas might therefore be the consequence of loss of lateral inhibition following hair cell death. This would suggest that supporting cells might undergo an ill-fated attempt to trans-differentiate into hair cells, which then undergo Atoh1 deletion due to the continuing presence of circulating tamoxifen (Reinert et al., 2012).

In conclusion, our data suggest that *Atoh1* fulfills several separate functions during the development and maturation of hair cells. In addition to being required for the initial differentiation of hair cells, conditional deletion of *Atoh1* has also revealed the existence of distinct critical periods for hair cell survival, stereociliary bundle maturation, and the long-term viability of hair cells. By analogy to the recent finding that *Atoh1* can directly regulate a wide variety of cellular functions in the developing cerebellum (Klisch et al., 2011), we suggest that *Atoh1* may have a similarly diverse set of functions during the maturation of hair cells. A systematic survey of *Atoh1* targets in hair cells, similar to that performed in the cerebellum, is likely to reveal further candidates and pathways that promote hair cell survival and function.

References


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

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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 cell targets 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 target genes in hair cells. We used RNA-sequencing to...
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: Atoh1tm1Hzo), Atoh1 

\( ^{1GFP/1GFP} \), Atoh1 

\( ^{1GFPA/1GFPA} \) (MGI: Atoh1 

\( ^{1GFP/1GFP} \), and Atoh1 

\( ^{1GFP/1GFP} \) (MGI: Atoh1 

\( ^{1GFP/1GFP} \) expressing mice were generated as previously described (Ben-Arie et al., 1997; Shroyer et al., 2007; Rose et al., 2009). Atoh1-CreERT2 (MGI: Tg(Atoh1-cre/ Esr1)14Flo; (Machold and Fishell, 2005) and R26R-YFP (MGI: Gt(Rosa)26Sortm1(EYFP)Cgn; (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 ACT ACG GCT), and Atoh1-reverse (GGC ACT GGC TTC TCT TGG), and Neo-forward (GCA TCG CCT ACT TAC GCC) yield a 600 bp wild-type allele band and a 400 bp null allele band. HA-forward (GGG ATG AGA CAG AAG G) and HA-reverse (GAG GGG CAT TGG GTT GTC TCA G) yield a 1 kb Atoh1 EGF-tagged allele band and a 350 bp floxed allele band. For Atoh1-CreERT2, CreF (GGC TGC ATT ACC GGT CAG TAC GCC GA), and CreR (GGC TGC ATT ACC GGT CAG TAC GCC GA) yield a 700 bp band. For R26R-YFP, oligo71866 (GGA GCG GGA GAA ATG GAT ATG), oIMR0883 (AAA GTC GCT GGC GCA AGA ATG TC) yield a 320 bp YFP band, and R26R-YFP, oligo71877 (GGA GGG GGA AAT GAT ATG), oligoMR0883 (AAG GCA GCC AAG ATG TT C) yield a 320 bp YFP + band. To generate the inducible Atoh1 conditional knock-out (CKO) mice, Atoh1-CreERT2; Atoh1 

\( ^{1GFP/1GFP} \) males were crossed with Atoh1 

\( ^{1GFP/1GFP} \) females to target a 500 –700 bp DNA fragment in a single exon of each gene for direct Atoh1 binding in these gene loci by ChIP-PCR. These binding motifs.

Conserved promoter or enhancer regions within 5 kb of the transcription 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 RetGene (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 \( \geq 10 \) 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 selected 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‘-GGATCCTAATACGACTCACTATA-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. 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 fixation 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, 5X SSC, 50 μg/ml Yeast tRNA, 100 μg/ml heparin, 1X Denhardt’s Solution, 0.1% Tween 20, 0.1% CHAPS, 5 mg/EDTA) for 1–2 h at 65°C. One-hundred microliters of digoxigenin-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 0.2X SSC and the slides washed in 0.2X SSC at 65°C for 1 h. The slides were then washed in 0.2X SSC for 5
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-digoxigenin-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 MgCl₂, 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/A1GFP} pups and stored in DMEM with 5% FBS. Cochleae were incubated with 500 μl thermoslysin 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 supernatant was then removed, and the pellet was washed with 500 μl of 1.25M glycine for 5 min. The cross-linked tissue was centrifuged at 470 × g for 5 min. The cross-linked tissue was then centrifuged at 470 × g for 10 min at 4°C with soft deceleration. The supernatant was 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 epithelium 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 then 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 were mixed twice with dilution buffer (1.0 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA). 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 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. 1A). 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. 1B). At P0 we saw no observable apical-basal differences in GFP intensity in the intact cochlea 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 staining with antibodies to Myosin6. No GFP+ cells were detected in the GFP− population (Fig. 1C). 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. 1D).

To identify genes that are enriched in differentiating hair cells, we compared the transcripts in GFP+ and GFP− cells from P0 Atoh1H11011/GFP/H11011 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+ cell population (data not shown). Of the 614 upregulated genes, 57% have previously been 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 (Tu et al., 2014). Finally, we also identified 22 enriched transcripts in the GFP+ population that are known deafness genes (Caldp2, Cib2, Cldn14, Dnbbn59, Gip3, Gxc1, Gxc2, Ildr1, Lhfp5, Msrb3, Myo3a, Myo6, Myo7a, Pdxd7, Poud43, Ptprg, Snpm, Tmcl1, Tmie, Ush1g, 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, $p = 0.00123$). 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. 2A,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), Lhfp5 (Kalay et al., 2006), and Srrm4 (Nakanou et al., 2012). However, many of the genes in our screen have never been studied in the inner ear before (Table 3; Fig. 2B). These include the phospholipid binding protein Annexin A4, the transcription factor and tumor suppressor Castor (Cas1; 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 melanoreulin (Mreg). Some genes associated with cytoskeletal binding and motor activity are specifically expressed in cochlear hair cells, such as Eps8l2, Kif21b, and Pascin1 (Table 3; Fig. 2A,B). Several ion channels also show a hair-cell-restricted expression pattern, such as Chrna9, Chrna10, Kenh6, and Scn11a (Table 3; Fig. 2A,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. 2C).

**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 (Klisich 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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression in GFP+ cells (RPKM)</th>
<th>Expression in GFP− cells (RPKM)</th>
<th>Fold-change (GFP+ vs GFP−)</th>
<th>p</th>
<th>References</th>
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<td>Atoh1</td>
<td>19042.084</td>
<td>40.151</td>
<td>474.258</td>
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<td>Atf7b</td>
<td>1270.502</td>
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<td>5.07E-43</td>
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<td>Bdnf</td>
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<td>8.06E-27</td>
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<td>Cd93</td>
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<td>Clcn1</td>
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<td>Srrm4</td>
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<td>2.46E-40</td>
<td>Pearse et al., 2002</td>
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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 AtoEAM binding sites within 5 kb of their transcriptional start site (Klisch et al., 2011). We specified design primers for the binding locus of nine candidate genes (Arxαd, Chm4a10, Mgat5b, Mreg, Pcp4, Rasd2, Rbm24, Scl11a, 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/GFP+ 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,
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, Mga5b, Mreg, Pcp4, Rasd2, Rhm24, 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<sup>+/GFP</sup>/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. **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, Cntnt1, Fgf18; Klisch et al., 2011*) or intestine (*Anxa4, Chna10, Rbm24; Kim et al., 2014*). Regions used for ChIP-PCR in **A** are shown with red arrows.
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<sup>+/−</sup>; Atoh1-CreER<sup>T2</sup> males with Atoh1<sup>fl<sub>x</sub>/fl<sub>x</sub></sup>; Atoh1-CreERT2<sup>−</sup>; R26R-YFP female mice and administered tamoxifen and progesterone to female mice on the 17<sup>th</sup> day of pregnancy by oral gavage to generate Atoh1 CKO (Atoh1<sup>fl<sub>x</sub>/fl<sub>x</sub></sup>; Atoh1-CreERT2<sup>−</sup>; R26R-YFP) and control (Atoh1<sup>fl<sub>x</sub>/fl<sub>x</sub></sup>; Atoh1-CreERT2<sup>−</sup>; 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<sup>+</sup> 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, Fgfr8 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. 2B). However, other genes, such as Chrm9, 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. 2A), 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; Hussenet 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, Rasfl4, 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 chick utricle hair cells, and expression levels of 19218 transcripts in Atoh1-GFP+ hair cells at P1. This material has not been peer reviewed.

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Changes in the regulation of the Notch signaling pathway are temporally correlated with regenerative failure in the mouse cochlea

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Sensorineural hearing loss is most commonly caused by the death of hair cells in the organ of Corti, and once lost, mammalian hair cells do not regenerate. In contrast, other vertebrates such as birds can regenerate hair cells by stimulating division and differentiation of neighboring supporting cells. We currently know little of the genetic networks which become active in supporting cells when hair cells die and that are activated in experimental models of hair cell regeneration. Several studies have shown that neonatal mammalian cochlear supporting cells are able to transdifferentiate into hair cells when cultured in conditions in which the Notch signaling pathway is blocked. We now show that the ability of cochlear supporting cells to transdifferentiate declines precipitously after birth, such that supporting cells from six-day-old mouse cochlea are entirely unresponsive to a blockade of the Notch pathway. We show that this trend is seen regardless of whether the Notch pathway is blocked with gamma secretase inhibitors, or by antibodies against the Notch1 receptor, suggesting that the action of gamma secretase inhibitors on neonatal supporting cells are likely to be inhibiting Notch receptor cleavage. The loss of responsiveness to inhibition of the Notch pathway in the first postnatal week is due in part to a down-regulation of Notch receptors and ligands, and we show that this down-regulation persists in the adult animal, even under conditions of noise damage. Our data suggest that the Notch pathway is used to establish the repeating pattern of hair cells and supporting cells in the organ of Corti, but is not required to maintain this cellular mosaic once the production of hair cells and supporting cells is completed. Our results have implications for the proposed used of Notch pathway inhibitors in hearing restoration therapies.

Keywords: notch, hair cell, supporting cell, cochlea, regeneration
Introduction

The Notch signaling pathway is an evolutionarily ancient form of cell-cell communication. During Notch signaling, the binding of membrane-bound ligands of the Delta and Jagged/Serrate families to Notch receptors causes the cleavage of the receptor and release of an intracellular domain which travels to the nucleus and participates in transcriptional activation (Artavanis-Tsakonas et al., 1999; Ilagan and Kopan, 2007; Artavanis-Tsakonas and Muskavitch, 2010; Mori et al., 2013). Notch signaling is deployed in the development of many tissues, and can influence cell fate through lateral inhibition with feedback (Chitnis, 1995; Formosa-Jordan et al., 2013). Inductive signaling (frequently to establish boundaries of different cell types) or by the asymmetrical inheritance of fate determinants that affect Notch signaling (Bray, 2006).

Notch signaling regulates many aspects of inner ear development (Kelley, 2003, 2006, 2007; Murata et al., 2012). During the induction of the otic placode, the anlagen of the inner ear, Jagged1 activation of Notch signaling acts to strengthen otic placode fate in response to FGF and Wnt signals (Jayasena et al., 2008; Groves and Fekete, 2012). As the first neuroblasts begin to differentiate and delaminate from the otocyst, Notch-Delta signaling regulates the proportion of progenitor cells that differentiate as neurons by lateral inhibition (Adam et al., 1998; Abelló et al., 2007; Daudet et al., 2007; Kiernan, 2013; Neves et al., 2013a). As the first sensory regions of the inner ear begin to develop, Notch-Jagged1 signaling helps maintain and promote the fate of vestibular sensory regions of the ear through lateral induction (Eddison et al., 2000; Daudet and Lewis, 2005; Kiernan et al., 2005a; Brooker et al., 2006; Daudet et al., 2007; Hartman et al., 2010; Pan et al., 2010, 2013; Neves et al., 2011, 2013a,b), although it is less clear if this mode of sensory induction also occurs in the cochlea (Bashch et al., 2011; Yamamoto et al., 2011). Finally, as hair cell and supporting cells begin to differentiate from sensory progenitor cells in prosensory patches, hair cells begin to express the Notch ligands Delta1 and Jagged2 (Dll1, Jag2) on their cell surface, and signaling by these ligands through the Notch1 receptor on nascent supporting cells induces and maintains supporting cell fate though lateral inhibition. Accordingly, pharmacological or genetic disruption of Notch1, Dll1 or Jag2, singly or in combination, leads to a failure of Notch signaling and an increase in the number of hair cells at the expense of supporting cells, likely through loss of lateral inhibition (Kiernan et al., 2005a; Brooker et al., 2006). Mutation or knock-down of downstream transcriptional effectors of Notch signaling, such as members of the Hes and Hey gene families, also leads to an increase in hair cell numbers at the expense of supporting cells (Zheng et al., 2000; Zine et al., 2001; Hayashi et al., 2008; Li et al., 2008; Doetzlhofer et al., 2009; Tateya et al., 2011; Benito-Gonzalez and Doetzlhofer, 2014).

A number of studies suggest that Notch signaling between hair cells and supporting cells continues in the sensory end organs of the mammalian inner ear after birth (Zine et al., 2000; Murata et al., 2006; Yamamoto et al., 2006; Hartman et al., 2007, 2009; Hori et al., 2007; Batts et al., 2009; Doetzlhofer et al., 2009; Lin et al., 2011; Liu et al., 2012a,b). Downstream effectors of Notch signaling can be detected in the cristae and maculae of the vestibular system (Hartman et al., 2009; Wang et al., 2010; Lin et al., 2011; Slowik and Bermingham-McDonogh, 2013), and blockade of Notch signaling in the adult vestibular system can induce the formation of ectopic hair cells at the expense of supporting cells (Lin et al., 2011; Slowik and Bermingham-McDonogh, 2013). Similarly, inhibition of Notch signaling in the neonatal organ of Corti also down-regulates some downstream effectors of Notch signaling in supporting cells and leads to the rapid formation of extra hair cells (Doetzlhofer et al., 2009; Korrapati et al., 2013; Mizutari et al., 2013; Bramhall et al., 2014). In both the neonatal cochlea and adult vestibular system, the generation of hair cells has been proposed to occur through a direct trans-differentiation of supporting cells without cell division (Doetzlhofer et al., 2009; Lin et al., 2011; Bramhall et al., 2014), a mode of differentiation that has also been observed during hair cell regeneration in birds (Stone and Cotanche, 2007).

Recently, application of gamma secretase inhibitors that attenuate the Notch pathway to the noise-damaged cochlea has been shown to generate small numbers of new hair cells and a partial restoration of hearing (Mizutari et al., 2013), suggesting that the Notch pathway may still be active in the mature cochlea. However, two issues remain unaddressed by this study. First, it is not clear to what extent components of the Notch signaling pathway—Notch receptors, Notch ligands and their downstream effectors—are expressed in the maturing cochlea (Batts et al., 2009). Indeed, two studies examining the presence of the active cleaved intracellular portion of the Notch1 receptor found very little evidence for Notch activation in the cochlea 1 week after birth (Murata et al., 2006; Liu et al., 2013). Moreover, although gamma secretase inhibitors are known to inhibit cleavage and activation of Notch receptors, they also cleave many other membrane proteins, and so it is possible that their effects in the cochlea may not be specific to the Notch pathway (Kopan and Ilagan, 2004).

In the present study, we compared the effects of gamma secretase inhibitors or blocking antibodies to the Notch1 receptor on the patterning of hair cells and supporting cells in organ cultures of the neonatal cochlea. In each case, we found that inhibitor treatment causes an increase in hair cell numbers at the expense of supporting cells, suggesting that both inhibitors are likely causing supporting cell trans-differentiation through inhibition of the Notch pathway. However, we find a precipitous age-dependent decline in the ability of these inhibitors to cause supporting cell trans-differentiation into hair cells. This decline in response to Notch inhibition progresses in a basal-apical gradient along the organ of Corti, consistent with the gradient of cellular maturation in the cochlea, and by 6 days after birth, the organ of Corti is essentially unresponsive to Notch inhibition in culture. We combined in situ hybridization, Q-PCR quantitation and single cell Fluidigm analysis of Notch pathway components and showed that Notch receptors,
ligands and effectors are down-regulated from the organ of Corti in basal-apical gradient during the first postnatal week, and are at least an order of magnitude lower in mature animals, even after noise damage. Our results suggest that the Notch signaling pathway is deployed to establish the pattern of hair cells and supporting cells in the cochlea, but is not required to maintain this pattern in the mature organ of Corti.

Materials and Methods

Mice

Atoh1<sup>Al1GFP/A1GFP</sup> (MGI: Atoh1<sup>tm4.1Hzo</sup>) knock-in mice and Atoh1<sup>GFP</sup> transgenic reporter mice were generated as previously described (Lumpkin et al., 2003; Rose et al., 2009). ICR mice were used for Notch Intracellular Domain (NICD) immunostaining. Noise damage experiments were performed on wild type CBA/CaJ mice. Pillar cells and Deiters’ cells were purified from Fgfr3<sup>-/-CreER<sup>2</sup></sup> (Young et al., 2010) mice mated with Ai14:Rosa<sup>tdTom</sup> reporter mice (Jackson, #007908). All animal experiments were approved by the Baylor College of Medicine or Stanford University Institutional Animal Care and Use committees.

Cochlear Organ Culture

Cochleas were dissected in ice cold HBSS immediately after euthanasia. Briefly, the heads were bisected, the temporal bone was removed from the skull base and the otic capsule was removed with forceps until the intact membranous cochlea was separated from the bony structures. For P0 and P3 animals, the cochlear duct was peeled out from the modiolus and the medial structures (Kölliker and Corti’s organs) were separated from the lateral wall, Reissner’s membrane and the stria vascularis. For P6 mice, in order to preserve the structures in the organ of Corti, the cochlear duct was gently separated from the modiolus by cutting between them with forceps and the lateral wall, stria vascularis and Reissnér’s membrane were partially removed after cutting with 27 gauge needle. Immediately after dissection, the explants were placed on top of filter membranes with 1 µm pores (SPI-pore or Whatman) floating in DMEM/F12 (Hepes) supplemented with B27 supplements (Life Technologies), 1mM N-acetylcysteine (Sigma), 5 ng/ml EGF and 2.5 ng/ml FGF2 and 67 µg/ml penicillin. In some cultures DMEM/F12 medium was supplemented with N2 supplements (Life Technologies), N-acetylcysteine and penicillin. For Notch inhibition experiments, cultures were supplemented with 0.75–10 µM DAPT (Gamma secretase inhibitor IX, Calbiochem EMD) or DMSO 0.04% v/v (Life Technologies). Anti Notch1-specific antibodies and control IgD antibodies (Wu et al., 2010) were provided by Genentech and used at 2 µg/ml. The cultures were maintained for 1 h, 1, 2 or 3 days in vitro (DIV) at 37°C in 5% CO₂.

Immunostaining, Microscopy and Quantification

Whole cochlear explants were fixed in 4% paraformaldehyde, then permeabilized and blocked in 0.2% Triton X-100 and 10% donkey serum in PBS. The explants were incubated with primary antibodies overnight at 4°C, washed in PBST (0.1% Triton X-100 in PBS) and incubated with secondary antibodies for 2 h at room temperature, followed by 3 further washes in PBST and then incubated in 10 µg/ml DAPI for 10 min. Primary antibodies used were rabbit polyclonal anti-Mycosin VIIa (1:500; Proteus Biosciences) and mouse monoclonal anti-GFP (1:200; Invitrogen). Secondary antibodies were Alexa Fluor 594 and 488 (Invitrogen). Images were obtained on an Axio Observer Zeiss microscope with an Apotome2 structured illumination attachment and analyzed in Axiovision 4.8 (Zeiss) and Image J (NIH) using Bioformat and the Cell Counter plugins. The cochlea was divided in five pieces: the tip, the apex, the middle, the base and the hook. Cell counts across different areas of the cochlea were normalized to cells per 100 µm and were expressed as a percentage increase with respect to control conditions. Significant differences were analyzed using a Mann-Whitney test for pairwise comparisons.

For NICD immunostaining in sections the protocol was modified from Morimoto et al. (2010). Briefly the heads of P0, P3 and P6 ICR mice were mounted in paraffin blocks and sectioned at 10 µm. The sections were rehydrated, bleached in H₂O₂ and boiled in a pressure cooker in antigen unmasking solution (Vectorlabs) for 20 min. The sections were then permeabilized, blocked and incubated with a cleaved Notch1 antibody (val1744; 1:100; Cell signaling) and anti- Mycosin VIIa (1:500; Proteus Biosciences). The signal was amplified with Vectastain ABC Kit (rabbit IgG) (Vectorlabs) as indicated by manufacturer. The color was developed for 5–10 min with TSA Tyramide Cy3 Reagent (diluted 1:100 after reconstitution; Perkin Elmer) and then stained with DAPI. Images were obtained on a Zeiss Axio Observer microscope with an Apotome2 structured illumination attachment.

In Situ Hybridization

Digoxigenin-labeled in situ probe synthesis was performed on linearized plasmid DNA using standard protocols (Stern, 1998).The following mouse cDNA probes were used in the study and kindly provided by the investigators listed: Notch1, Jag1, Dll1 (Gerry Weinmaster), Notch3 (Urban Lendahl), Hes5 (Ryoichirō Kageyama), Hey1, Hey2, HeyL. (Manfred Gessler), and Atoh1 (Huda Zoghbi). A cDNA clone for Jag2 (BC010982) was purchased from open Biosystems. The in situ hybridization procedure for frozen sections was modified from previous protocols (Harland, 1991; Birren et al., 1993; Groves et al., 1995). Heads of perinatal 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. 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 re-fixation in 4% paraformaldehyde in PBS, pH 7.2 for 10 min at room temperature.
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. 100 µl of digoxigenin-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 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-digoxigenin-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, followed by fixation in 4% paraformaldehyde in PBS, pH 7.2 by washing the slides in PBS three times for 15 min each, at room temperature until the purple reaction product had developed. 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. Whole mount in situ hybridization was carried out as recently described in detail (Khatri and Groves, 2013).

RNA Isolation and Q-PCR

For each experimental condition, total RNA was extracted from 4 uncultured whole cochlear explants or 3 cultured cochlear explants using the RNeasy mini kit (Qiagen). RNA yield ranged from 480 to 1200 ng and was used for cDNA preparation using random primers and SuperScript III First-Strand Synthesis System (Invitrogen). qPCR reaction was performed with SYBRGreen PCR Master Mix in StepOnePlus RealTime PCR System (Applied Biosystems), using the reaction cDNA at 0.3–0.6 ng/µl and primers at 50 nM (excluding 100 nM for the Hes5 reaction). Primers sequences are provided in Table 1. GAPDH and L19 primers were used as reference genes. Significant differences were analyzed using a Mann-Whitney test for pairwise comparisons. Multiple comparisons or pairwise correction for multiple comparisons were not performed.

Table 1 | Q-PCR primers for Figures 1, 3, 5, 7

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<td>L19 5′-GGTCTGGTGGGATCCAC-3′</td>
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Noise Damage

Noise damage of six-week-old CBA/CaJ mice was performed as previously described (Liu et al., 2011). Briefly, a custom-built box contained six piezo horns (TW-125, Pyramid Car Audio, Brooklyn, NY, USA) inserted through the cover. Band-passed white noise (4–22 kHz) was generated digitally with RPvds software (Version 6.6, Tucker-Davis Technologies, Alachua, FL, USA), converted to analog by a digital-to-analog converter, and then transferred to the power amplifier (Servo 550, Sampson, Hauppauge, NY, USA) to drive the speakers. A cage containing the mice was placed inside the box and the mice were exposed to noise at 98 dB ± 2 dB for 4 h.

RNA Isolation and Q-PCR

For each experimental condition, total RNA was extracted from 4 uncultured whole cochlear explants or 3 cultured cochlear explants using the RNeasy mini kit (Qiagen). RNA yield ranged from 480 to 1200 ng and was used for cDNA preparation using random primers and SuperScript III First-Strand Synthesis System (Invitrogen). qPCR reaction was performed with SYBRGreen PCR Master Mix in StepOnePlus RealTime PCR System (Applied Biosystems), using the reaction cDNA at 0.3–0.6 ng/µl and primers at 50 nM (excepting 100 nM for the Hes5 reaction). Primers sequences are provided in Table 1. GAPDH and L19 primers were used as reference genes. Significant differences were analyzed using a Mann-Whitney test for pairwise comparisons. Multiple comparisons or pairwise correction for multiple comparisons were not performed.

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Results

The Neonatal Cochlea Exhibits a Position-Dependent Variation of Supporting Cell Trans-Differentiation in Response to Gamma Secretase Inhibitors

A number of studies have demonstrated conversion or trans-differentiation of cochlear supporting cells into hair cells after treatment with gamma secretase inhibitors (Takebayashi et al., 2007; Hayashi et al., 2008; Doetzlofer et al., 2009; Korrapati et al., 2013; Mizutari et al., 2013; Bramhall et al., 2014), although results in different studies have often been obtained with different gamma secretase inhibitors or at different concentrations of a given inhibitor. We confirmed these results in cochlear explant cultures from postnatal day 0 (P0) Atoh1+A1GFP/A1GFP and Atoh1-GFP mice, using the gamma secretase inhibitor DAPT at a concentration of 2 µM. We quantified the number of supernumerary hair cells in the apical, middle, and basal regions of the cochlea (Figures 2A,B; the approximate positions of the three regions are indicated in Figure 2C). We excluded from our counts the most basal hook region as it was more susceptible to variable damage during dissection and the most apical tip region because of its more variable behavior. We observed a clear effect of position on the number of supernumerary hair cells generated in the cultures over the 3 day culture period, with the apex producing 20% more hair cells after 3 days, whereas the base generated only 32% more hair cells in the same time period (Figures 2A,B). These results suggested that more mature supporting cells at the base of the cochlea were far less likely to trans-differentiate into hair cells in response to DAPT than their younger counterparts at the apex.

The Response of Supporting Cells to Gamma Secretase Inhibition or Notch Inhibition Declines Rapidly with Age

To test whether the response of cochlear supporting cells to DAPT was indeed age-dependent, we established cochlear cultures from newborn (P0), 3 and 6 day old mice and cultured them for 2 days in 5 µM DAPT or DMSO vehicle for 1–3 days and counted the numbers of Atoh1-GFP or Myosin VIIA-expressing hair cells in the apical, middle, and basal regions of the cochlea. We saw significant numbers of supernumerary hair cells in DAPT-treated P0 cochlear cultures compared to DMSO vehicle, but observed no significant increase in hair cell numbers when either P3 or P6 cultures were treated with DAPT (Figures 3A,B). Most experiments in which gamma secretase inhibitors have been used to promote supporting cell trans-differentiation into hair cells are interpreted on the assumption that the inhibitors are targeting the cleavage of Notch receptors in cochlear supporting cells. However, since gamma secretases cleave many other membrane proteins (Kopan and Ilagan, 2004), it is possible that some of the effects of gamma secretase inhibitors may be due to the inhibition of cleavage of other membrane proteins. To test this, we used specific blocking antibodies to the Notch1 receptor (Wu et al., 2010) at a concentration of 2 µg/ml, previously shown to effectively inhibit Notch1 signaling in vitro (Wu et al., 2010). Cochlear cultures from P0 animals showed similar age-dependent responses to Notch1 blocking antibodies (48%
increased in Myosin VIIa+ hair cells) as cultures incubated for 2 days in 5 µM DAPT (41% increase) compared to control cultures containing DMSO or a control IgD (Figures 3A,B). However, we saw no significant response when P3 or P6
cultures were treated with blocking antibodies as we previously saw with 5 μM DAPT (Figures 3A,B). We also observed a comparable down-regulation of supporting cell-specific Hes and Hey genes and the supporting cell marker Jag1 in P0 cultures treated with DAPT or Notch1 blocking antibodies, together with a comparable up-regulation of Atoh1 (Figure 3C). These results suggest that the majority of the effects of the gamma secretase inhibitor DAPT on supporting cell transdifferentiation in neonatal cultures are likely specific to the Notch pathway.

**Notch Pathway Genes are Down-Regulated in the Cochlea During the First Postnatal Week**

The preceding results suggest that the Notch pathway is deployed to stabilize supporting cell fate of neonatal cochlear supporting cells, but that inhibition of the Notch pathway has no effect on supporting cell fate even a few days later. To determine out if this change in the response of supporting cells to Notch inhibition was related to changes in the endogenous activity of the Notch pathway, we examined the expression of mRNA for Notch receptors (Notch1 and Notch3), ligands (Dll1, Jag1,

---

**FIGURE 3** | Age-dependent decline in the effect of Notch inhibition on supporting cells in the presence of gamma secretase inhibitors or Notch1 blocking antibodies. (A) Immunostaining of apical portions of cochlear explants obtained at 0, 3 and 6 postnatal days (P0, P3 and P6) from Atoh1<sup>GFP</sup> transgenic reporter mice treated with 5 μM DAPT, 2 μg/ml Notch1 antibodies (Anti Notch1), 2 μg/ml control IgD and DMSO cultured 2 days in vitro (DIV), Myo7a: red. Scale 50 μm. (B) The increase in numbers of Myosin VIIa-labeled cells in the apical portion of the cochleas shown in (A). N = 6, 6 and 3 for P0, P3 and P6 respectively. *p = 0.0044 and 0.0045 for DMSO/DAPT and Anti Notch1/Control IgD comparisons respectively (Mann Whitney). Error bars: SEM. (C) mRNA amount of Notch pathway genes obtained by qPCR in whole cochlear explants of Atoh1<sup>GFP</sup> transgenic reporter P0 newborn mice cultured in the presence of DAPT or DMSO and in Notch1 antibodies or control IgD antibodies. Blue columns (top): level of expression after DAPT treatment relative to DMSO. Red columns (bottom): level of expression after anti Notch1 antibodies (aN1) relative to control IgD antibodies. N = 3. Error bars: SEM. Note that error bars are present for each condition but are very small in some cases.
FIGURE 4 | Notch pathway components are down-regulated during the first postnatal week. In situ hybridization of Notch pathway genes in the cochlea at 0, 3 and 6 postnatal days (P0, P3 and P6). Left panels: Whole mount in situ of cochlear explants, with the samples curved clockwise from apex to base. Scale = 200 µm. Right panels: In situ hybridization of frozen sections; a: apex region, b: basal region. Scale 50 µm.

and Jag2) and downstream effectors of Notch signaling (Hey1, Hey2, HeyL and Hes5) in the cochlea from P0 to P6 by in situ hybridization on whole mount cochleas, sectioned cochleas and by Q-PCR of cochlear tissue (Figures 4, 5A). In general, all components of the Notch pathway evinced a down-regulation between P0 and P6 starting at the base and proceeding down to the apex. Specifically, Notch1 and Notch3 were expressed throughout the supporting cell layer and into Kölliker’s organ.
and the outer sulcus, and both receptors showed a basal-apical down-regulation between P0 and P6. Jag2 and Dll1 were both down-regulated in hair cells between P0 and P6, along with the hair cell marker Atoh1. Hey2 and Hes5 were down-regulated from pillar cells and Deiters’ cells respectively in a basal-apical gradient, whereas Hey1, HeyL and Jag1 were expressed in all supporting cells and cells of Kölliker’s organ, and down-regulated again in a basal-apical direction. The speed of down-regulation varied considerably from gene to gene—for example, Dll1 was down-regulated in hair cells more quickly than Jag2, and Hes5 was down-regulated much more quickly in supporting cells than Hey1. We also saw a general trend towards down-regulation of each gene by Q-PCR (Figure 5A), although the degree of down-regulation measured by this method was somewhat blunted as a result of including the entire basal-apical extent of the cochlear duct in each sample. To confirm that activation of the Notch receptor was also decreasing between P0 and P6, we immunostained cochlear sections with antibodies to the Notch1 intracellular domain (Notch1-ICD) which is released following damage. We examined the expression of Atoh1 and Hes5 in cochleas isolated from cohorts of mice which received noise damage at P42 and were analyzed at one, three, or seven days later. Our controls were non-noise exposed cohorts of P0 and P49 mice. The level of noise we applied has been shown to be adequate to damage the cochlear epithelium and elicit changes in gene expression. In particular, it produces large temporary threshold
FIGURE 6 | Comparison of Notch pathway genes in P1 and P21 cochlea at the single cell level. Pillar and Deiters’ cells were purified from P2 and P21 FGFR3-CreER;ROSA-TdTomato mice and RNA extracted from 162 (P2) and 123 (P21) single cells and subjected to qPCR analysis using the Fluidigm system (Durruthy-Durruthy et al., 2014) with primers for housekeeping genes and Notch pathway genes. (A) Graph showing the percentage of cells at each age that expressed detectable levels of each gene under analysis. (B) Violin plot showing the distribution of expression levels for each gene in all cells including the cells with no expression (Log2x = 0) presented in a combination of box plots and kernel density plots. White Crosses indicate the mean, white boxes the median expression levels. (C) Violin plot similar to (B), excluding cells with undetectable levels of expression for each gene.

Elevations, and mild permanent threshold shifts, 17% OHC loss and 3% IHC loss, and increases in prestin gene expression in residual OHCs (Wang et al., 2010; Xia et al., 2013). We found that levels of the hair cell-specific transcription factor Atoh1 in 7 week old animals were less than 10% of their neonatal counterparts (Figure 7) and that these levels did not change significantly over a 7 day period after noise damage. Similar results were observed for Hes5 (Figure 7). These data suggest that the Notch pathway remains down-regulated in the mature cochlea and that it is not significantly perturbed by noise damage.

Discussion

The Notch signaling pathway is deployed during the differentiation of hair cells and supporting cells and has been proposed to regulate the proportion of each cell type...
Many studies have used gamma secretase inhibitors as a reagent to inhibit Notch signaling, despite the fact that gamma secretases are known to cleave scores of other transmembrane receptors, ligands and downstream effectors in the first postnatal week (Takebayashi et al., 2013) or in neonatal cochlear cultures to Notch inhibitors (Figure 2)—are also supported by a careful analysis of basal-apical differences in the response of neonatal cochlear cultures to Notch inhibitors (Figure 2). Since hair cells and supporting cells in the mid-base of the cochlea differentiate at least 3 days before cells at the apex (Chen et al., 2002; Cai et al., 2013), analysis of whole cochlear explants allows us to directly compare different states of supporting cell differentiation in the same tissue. In P0 mice, we saw a higher proportion of ectopic outer hair cells vs. inner hair cells in the apex of the cochlea, and an even smaller proportion of ectopic inner hair cells at the base. Since inner hair cells begin to differentiate before outer hair cells in any given region of the cochlea (Chen et al., 2002; Cai et al., 2013), it is possible that these differences reflect a neural-abneural gradient of response to Notch inhibition as well as an apical-basal response. Alternatively, since different supporting cell types express different combinations of Hes and Hey genes (Zheng et al., 2000; Zine et al., 2001; Hayashi et al., 2008; Li et al., 2008; Doetzlhofer et al., 2009; Murata et al., 2009; Tateya et al., 2011), it is possible that these differences reflect the different sensitivities of these Notch target genes to Notch inhibition (Ong et al., 2006).

We observe a down-regulation of mRNA levels of Notch receptors, ligands and downstream effectors in the first postnatal week. The degree and rate of down-regulation varies, but analysis of Notch1 signaling in cochlear supporting cells over this time
period (Figure 5; Murata et al., 2006; Basch et al., 2011) suggests that very little cleavage of the Notch1 receptor is occurring by the end of the first postnatal week. The mechanism of this down-regulation is currently not known, although given the absence of significant Notch pathway expression in the adult cochlea, it is possible that the loci of Notch pathway genes are becoming epigenetically modified and placed beyond use. It is also possible that this epigenetic silencing is accompanied by silencing of the direct targets of Notch effectors such as the Hes and Hey genes. However, it should be noted that the Notch pathway appears to be down-regulated in mature supporting cells in the chicken basilar papilla, as this sensory organ also fails to respond to gamma secretase inhibitors in the undamaged state (Daudet et al., 2009). Nevertheless, after damage, the Notch pathway is once again deployed in chicken supporting cells and the differentiating hair cells that they generate (Stone and Rubel, 1999; Daudet et al., 2009). It will therefore be of great interest to identify the epigenetic state of Notch pathway genes and their targets in mature mammalian supporting cells. It should also be noted that the co-expression of Jag1 and Sox2, which is seen in sensory patches from their first appearance (Kiernan et al., 2005b; Pan et al., 2010, 2013; Neves et al., 2011, 2012), is maintained in adult mouse supporting cells (Oesterle et al., 2008). It is thus formally possible that low levels of Notch signaling may persist in the adult cochlea and may maintain expression of these two genes by lateral induction. If this is the case, such signaling does not appear to confer competence for regeneration on supporting cells.

A recent study demonstrated that a small but significant number of new hair cells could be generated from supporting cells by treating noise-damaged animals with gamma secretase inhibitors (Mizutari et al., 2013), leading to a partial restoration of function. How can we reconcile these results with our data in the present study? First, it is possible that the Notch pathway can continue to regulate hair cell and supporting cell fate in the adult animal when expressed at significantly lower levels. We feel this is unlikely since binding of the Notch-ICD-MAML-RBP complex to its target sites in the genome is likely to be severely compromised at low concentrations of Notch-ICD (Ong et al., 2006). Second, it is possible that a sub-population of supporting cells continue to express Notch pathway components at significant levels, but that these would not be detected when analyzing gene expression in the entire cochlea. In our single cell analysis of 123 P21 pillar cells and Deiters’ cells, we were able to detect a very small number of cells in which Notch pathway components were expressed at comparable levels to their neonatal counterparts (Figure 6), and levels of Hes5 and the hair cell marker Atoh1 are more than 10-fold lower in the adult, even after noise damage (Figure 7). This suggests that if such cells persist in the adult cochlea, they are present in extremely small numbers. Finally, it is also possible that a second, Notch-independent pathway that can be targeted by gamma secretase inhibitors is operating in a small number of mature supporting cells. The effect of inhibiting this second pathway would be overshadowed by Notch inhibition in the neonatal cochlea, but might be uncovered in the adult cochlea when the Notch pathway is no longer active. It should also be noted that the noise damage protocol (Liu et al., 2011) used in our study—98 dB for 4 h—is significantly less severe than the protocol used by Mizutari et al. (116 dB for 2 h). However, the large and significant drop in Atoh1 and Hes5 levels we observe in undamaged adult tissue compared to neonatal animals still supports our observed down-regulation of Notch pathway genes in the first postnatal week.

In conclusion, our results suggest that the canonical Notch pathway is not active to any significant degree in the adult organ of Corti, and that the down-regulation of signaling occurs prior to the onset of hearing. The Notch pathway can therefore be viewed as a developmental scaffold for the organ of Corti—it is partly necessary for establishing the pattern and proportion of hair cells and supporting cells, but not necessary to maintain this pattern once it has been established. This suggests that inhibition of Notch signaling in the adult organ of Corti in the absence of other manipulations is unlikely to promote significant numbers of new hair cells, and that alternative or supplementary therapeutic interventions should be considered.

Acknowledgments

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Notch down-regulation in the cochlea

Maass et al.


Notch signaling: a simple pathway becomes complex.


Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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