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## ***Atoh1* directs hair cell differentiation and survival in the late embryonic mouse inner ear**

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### **Abstract**

*Atoh1* function is required for the earliest stages of inner ear hair cell development, which begins during the second week of gestation. *Atoh1* expression in developing hair cells continues until early postnatal ages, but the function of this late expression is unknown. To test the role of continued *Atoh1* expression in hair cell maturation we conditionally deleted the gene in the inner ear at various embryonic and postnatal ages. In the organ of Corti, deletion of *Atoh1* at E15.5 led to the death of all hair cells. In contrast, deletion at E16.5 caused death only in apical regions, but abnormalities of stereocilia formation were present throughout the cochlea. In the utricle, deletion at E14.5 or E16.5 did not cause cell death but led to decreased expression of myosin VIIa and failure of stereocilia formation. Furthermore, we show that maintained expression of *Barhl1* and *Gfi1*, two transcription factors implicated in cochlear hair cell survival, depends upon continued *Atoh1* expression. However, maintained expression of *Pou4f3* and several hair cell-specific markers is independent of *Atoh1* expression. These data reveal novel late roles for *Atoh1* that are separable from its initial role in hair cell development.

### **Keywords**

Hearing; Cochlea; Development; Vestibular system; Differentiation

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## Introduction

Hair cell production begins in the ventromedial otocyst, where prosensory cells are specified and subsequently give rise to hair and supporting cells (Barald and Kelley, 2004; Fritzscht et al., 2011; Groves and Fekete, 2012). In the mouse organ of Corti these cells become post-mitotic from E12.5 to E15.5 in an apical-to-basal gradient (Ruben, 1967). Paradoxically, cochlear hair cell differentiation begins in the mid-basal turn and progresses in the opposite direction (Jahan et al., 2010, 2013; Rubel, 1978). Hair cells of the vestibular system are born over a much longer time period (E12.5–P2) and differentiate in a roughly central-to-peripheral direction from E13.5 through the first postnatal week (Burns et al., 2012; Ruben, 1967; Sans and Chat, 1982; Takumida and Harada, 1984). *Sox2*, *Eya1*, *Six1* and *Atoh1* (*Math1*) have been implicated in initial hair cell specification, while *Barhl1*, *Gfi1*, *Hes6*, *NeuroD* and *Pou4f3* (*Brn3c*, *Brn3.1*) are expressed by and/or required for hair cell maturation and survival (Ahmed et al., 2012; Kim et al., 2001; Qian et al., 2006; Wallis et al., 2003; Xiang et al., 1998).

*Atoh1* encodes a basic helix-loop-helix (bHLH) transcription factor necessary for hair cell production but not required for establishment of the sensory epithelium (Bermingham et al., 1999; Chen et al., 2002; Kawamoto et al., 2003; Woods et al., 2004; Zheng and Gao, 2000). *Atoh1* expression in the cochlea begins at E13.5, progressing in basal-to-apical and medial-to-lateral directions (Dabdoub et al., 2008; Jahan et al., 2010; Matei et al., 2005; Pan et al., 2012; Woods et al., 2004). Interestingly, mouse cochlear hair cells express *Atoh1* until P3-P7 and possibly into adulthood (Lanford et al., 2000; Lumpkin et al., 2003; Matei et al., 2005; Scheffer et al., 2007). *Atoh1* expression in the vestibular system starts at E12.5, spreads throughout the sensory epithelium by E14.5, and is down-regulated during the first postnatal week (Bermingham et al., 1999; Shailam et al., 1999). In *Atoh1*-null mice, sensory precursor cells of the cochlea remain undifferentiated and undergo apoptosis from E15.5 to E17.5 in a basal-to-apical gradient that follows that of normal maturation (Basch et al., 2011; Chen et al., 2002; Fritzscht et al., 2005; Pan et al., 2011). Recent use of conditional deletion techniques in “self-terminating” *Atoh1*<sup>Cre/flox</sup> mice showed that limited cochlear hair cell differentiation occurs even with transient *Atoh1* expression (Pan et al., 2012). However, this approach was limited by inability to control the duration of *Atoh1* expression and by stochastic effects secondary to the *Cre* driver that was used.

To explore additional functions of *Atoh1* during inner ear development we used a tamoxifen-inducible *Cre* allele to conditionally delete *Atoh1* in developing hair cells at different embryonic and postnatal ages, allowing us to determine how varying durations of *Atoh1* expression affected hair cell survival/differentiation. We demonstrate that *Atoh1* deletion following specification of hair cells leads to cell death or disrupts differentiation depending upon the timing of *Atoh1* gene deletion. These data demonstrate that *Atoh1* functions in multiple phases of hair cell development and suggest that late expression is crucial for normal hair cell maturation and viability.

## Material and methods

### Mice

All mice were housed at the Case Western Reserve University or Children’s Hospital of Pittsburgh of UPMC Animal Care Facilities in accordance with IACUC guidelines. Generation of *Atoh1*<sup>CreERT2</sup>, *Atoh1*<sup>flox</sup> and *ROSA*<sup>LacZ</sup> (JAX #007914) mice was described previously (Fujiyama et al., 2009; Shroyer et al., 2007; Soriano, 1999). *ROSA*<sup>LacZ</sup> mice were maintained on a congenic C57BL/6J strain background, while *Atoh1*<sup>CreERT2</sup> and *Atoh1*<sup>flox</sup> mice were maintained on mixed genetic backgrounds. Timed matings were used to obtain embryonic ages, with noon of the plug date defined as E0.5. Conditional deletion of

*Atoh1* occurred in *Atoh1<sup>CreERT2/flox</sup>* mice following tamoxifen administration; *Atoh1<sup>+flox</sup>* littermates served as controls.

### Tamoxifen administration

Pregnant dams received a single 250 mg/kg oral gavage dose of tamoxifen (Sigma) at E14.5, E15.5, E16.5, E17.5, P1 or P3. Embryos were subsequently harvested 1–4 days later at E16.5, E18.5, P3, or P5.

### Tissue preparation

Pregnant dams were sacrificed and embryos harvested into cold 1X phosphate-buffered saline (PBS). Temporal bones were either dissected directly into cold 4% paraformaldehyde (PFA) (embryonic, P0–P5) or dissected after transcardial perfusion with 4% PFA (embryonic, P0–P5 and adult), fixed overnight in 4% PFA, washed in 1X PBS and stored at 4 °C in PBS (for immunohistochemistry) or 0.4% PFA (for in situ hybridization). Cochleae and utricles were dissected and processed for frozen sectioning or were analyzed in wholemount. Three–seven cochleae and 3–5 utricles from at least 3 animals of each genotype were examined for each experiment.

### Immunohistochemistry

Whole cochleae or whole utricles were blocked for 30 min at room temperature (RT) in 1X PBS/ 0.3% Triton X-100/3% non-fat dry milk (PBST-NFDM) (cochleae) or 2% bovine serum albumin/0.8% normal goat serum/0.5% Triton X-100 (utricles) and incubated at 4 °C overnight in the same solution containing primary antibodies at the following dilutions: rabbit anti-myosin VI (25–6790, Proteus Bioscience) 1:500; rabbit anti-myosin VIIa (Proteus Biosciences, Ramona, CA) 1:500; chicken anti- $\alpha$ -galactosidase (ab9361, Abcam) 1:500; Alexa Fluor 488-conjugated phalloidin (A12379, Invitrogen) 1:100; Alexa Fluor 647-conjugated phalloidin (A22287, Invitrogen) 10  $\mu$ g/ml; rabbit anti-cleaved caspase 3 (9661S, Cell Signaling) 1:100. Donkey Cy3-, Alexa 488- and Alexa 594-conjugated secondary antibodies (Jackson ImmunoResearch, 1:500 dilution) in blocking solution were applied for 30 min at RT. Some tissue was counterstained with 1  $\mu$ g/ml DAPI (4',6'-diamidino-2-phenylindole; Sigma-Aldrich) for 10 min. Slides were mounted in Prolong Gold (Invitrogen) and imaged using Zeiss LSM 510 confocal, Olympus FV-1000 confocal, Nikon Eclipse 800 epifluorescence or Leica DM5500B epifluorescence microscopes. For confocal imaging, Z-series were constructed from images obtained with a 20  $\times$ , 40  $\times$  or 63  $\times$  lens. Images were converted to RGB stacks using ImageJ (NIH).

### In situ hybridization

Plasmids containing cDNAs for *Atoh1* and *Pou4f3* were used to generate antisense RNA probes by *in vitro* transcription. Following fixation cochleae were dehydrated in graded methanol and stored at –20 °C. Rehydrated cochleae were digested with 20  $\mu$ g/ml proteinase K (Ambion, Austin, TX, USA) for 20 min and stored overnight at 60 °C with riboprobe in hybridization solution containing 50% (v/v) formamide, 50% (v/v) 2X saline sodium citrate (Roche) and 6% (w/v) dextran sulfate. Ears of mutant mice and control littermates were processed in the same reaction tubes to maintain consistency. Cochleae were washed and incubated overnight with anti-digoxigenin antibody (Roche Diagnostics GmbH, Mannheim, Germany) conjugated with alkaline phosphatase. Signal was developed in Nitroblue phosphate/5-bromo, 4-chloro, 3-indolyl phosphate (BM purple substrate, Roche Diagnostics, Germany) and cochleae were mounted flat in glycerol and viewed on a Nikon Eclipse 800 microscope using differential interference contrast microscopy; images were captured with Metamorph software.

## Cell counts, length and density measurements

Total numbers of cells were counted on photographs of immunostained, wholemount tissues; no correction factors were applied. Cochlear length was determined by using ImageJ to draw a line along the row of inner hair cells from the base of the cochlea past the apical-most hair cells to the cochlear apex (“cochlear length line”). Organ of Corti length was calculated using the same method by tracing from the basal-most to the apical-most myosin VI+ hair cell. For counts by cochlear region the “cochlear length line” was divided into equal basal, middle and apical portions and the total number of hair cells was counted in each region.

Whole utricles were triple-labeled for myosin VIIa, phalloidin and DAPI or  $\beta$ -galactosidase, phalloidin and DAPI. Cell density was determined by counting the number of DAPI-stained nuclei in 3 centrally- and 3 peripherally-located 1120  $\mu\text{m}^2$  boxes drawn on z-stack photographs within the myosin VIIa+ territory (examples of box locations are shown in Fig. 5). The number of myosin VIIa+ cells was counted in these boxes and divided by the total DAPI+ nucleus number to calculate the percentage of cells that expressed myosin VIIa. Phalloidin-labeled hair bundles on myosin VIIa+ cells were qualitatively categorized as long, short or absent and the percentage of myosin VIIa+ cells in each category was calculated.

Statistical comparisons were made using unpaired, two-tailed Student's *t*-tests and single factor ANOVA (Microsoft Excel).

## RNA extraction, cDNA synthesis, and RT-PCR

Fresh organs of Corti were dissected away from the spiral ganglion and greater epithelial ridge, pooled by genotype (14–22/age) and homogenized using a mortar and pestle. RNA was extracted (74104, Qiagen) and reverse-transcribed (205311, Qiagen). PCR primers (Table 1) were designed to target exon sequences and cDNA was amplified using the following protocols:

*Atoh1*: 94 °C (30 s), 60 °C (30 s), 72 °C (60 s) for 10 cycles  
 94 °C (30 s), 50 °C (30 s), 72 °C (60 s) for 30 cycles  
 All other genes: 95 °C (45 s), 58 °C (45 s), 72 °C (90 s) for 40 cycles.

## Results

To study *Atoh1* function in the late embryonic and early postnatal inner ear we conditionally deleted the gene using an *Atoh1*<sup>CreERT2</sup> knock-in mouse line that faithfully recapitulates endogenous *Atoh1* expression (Fujiyama et al., 2009). A single tamoxifen dose administered to pregnant dams at E15.5 resulted in  $\beta$ -galactosidase expression in  $97 \pm 3\%$  of cochlear hair cells ( $n=3$  cochleae) in E18.5 *Atoh1*<sup>CreERT2</sup>; *ROSA*<sup>LacZ</sup> embryos (Fig. 1A). Furthermore, tamoxifen administration at E16.5 efficiently excised an *Atoh1*<sup>fllox</sup> allele (Shroyer et al., 2007): *Atoh1* mRNA transcripts were undetectable by *in situ* hybridization on whole cochleae (Fig. 1C and D) and vestibular organs (Fig. 1E and F) and RT-PCR performed on organ of Corti total RNA (Fig. 1B) obtained from E18.5 *Atoh1*<sup>CreERT2/fllox</sup> embryos. These data indicate that the *Atoh1*<sup>CreERT2</sup> allele we used for these experiments produces complete or near complete excision of floxed sequences at the *ROSA* and *Atoh1* loci at the ages we tested.

## Continued *Atoh1* expression is necessary for survival and differentiation of cochlear hair cells

Cochlear hair cells become post-mitotic between E12.5 and E15.5, and the earliest-born hair cells (those in the mid-basal turn) begin to differentiate at E13.5 (Ruben, 1967; Woods et al., 2004). To determine the role of *Atoh1* just after the last cochlear hair cells became post-mitotic we administered tamoxifen to pregnant dams at E15.5 and harvested embryos at E16.5 (Fig. 2). E16.5 *Atoh1<sup>CreERT2/flox</sup>* embryos had 25% fewer myosin VI+ hair cells compared to control littermates ( $1163 \pm 714$ ,  $n=3$  cochleae vs.  $1544 \pm 80$ ,  $n=4$  cochleae,  $p=0.003$ ), and the length of the organ of Corti was decreased by 9% ( $2630 \pm 50 \mu\text{m}$  vs.  $2876 \pm 60 \mu\text{m}$ ;  $n=3$  cochleae/genotype,  $p=0.035$ ). This cell loss was not secondary to changes in overall cochlear length (*Atoh1<sup>CreERT2/flox</sup>*  $3436 \pm 62 \mu\text{m}$  vs. control  $3497 \pm 59 \mu\text{m}$ ;  $n=3$  cochleae/genotype,  $p=0.51$ ). To determine whether hair cell loss was regionally variable we divided cochleae longitudinally into three equal parts and counted the number of myosin VI + hair cells in each region. We found 19% ( $491 \pm 31$  vs.  $616 \pm 13$ ;  $p=0.009$ ), 23% ( $391 \pm 18$  vs.  $507 \pm 21$ ;  $p=0.007$ ) and 33% ( $281 \pm 24$  vs.  $422 \pm 39$ ;  $p=0.025$ ) decrease in hair cell number in the base, middle and apex of *Atoh1<sup>CreERT2/flox</sup>* ( $n=3$  cochleae) vs. control littermate ( $n=4$  cochleae) embryos (Fig. 2D). Third row outer hair cells and scattered inner hair cells were most obviously affected (Fig. 2B–B'). In addition, the regular spacing between outer hair cells was disrupted in *Atoh1<sup>CreERT2/flox</sup>* embryos (Fig. 2B–B'). Strikingly, cochleae ( $n=7$ ) from E18.5 *Atoh1<sup>CreERT2/flox</sup>* embryos that received tamoxifen at E15.5 had no myosin VI+ cells and lacked DAPI +nuclei in the typical hair cell position (Fig. 2C–C' and data not shown). These data suggested that *Atoh1*-null hair cells died between E16.5 and E18.5. To verify this, cochleae from E16.5 *Atoh1<sup>CreERT2/flox</sup>* embryos and littermate controls dosed with tamoxifen at E15.5 were immunostained for activated caspase 3. Multiple activated caspase 3+ cells were seen throughout the organ of Corti of *Atoh1<sup>CreERT2/flox</sup>* embryos, but no hair cells were labeled in control littermates (Fig. 2E–F'). Thus, deletion of *Atoh1* at E15.5 led to rapid hair cell demise throughout the cochlea, suggesting that *Atoh1* is necessary for hair cell survival shortly (1–3 days) after hair cells become post-mitotic.

To determine whether *Atoh1* was necessary for hair cell survival at later embryonic ages we administered tamoxifen to pregnant dams at E16.5 and harvested embryos at E18.5 (Fig. 3). The number of myosin VI+ hair cells was decreased by 50% ( $1099 \pm 81$  vs.  $2160 \pm 56$ ;  $n=3$  cochleae/genotype,  $p=0.0004$ ) in *Atoh1<sup>CreERT2/flox</sup>* vs. control littermates; we verified that these hair cells underwent Cre-mediated recombination in *Atoh1<sup>CreERT2/flox</sup>; ROSA<sup>LacZ</sup>* embryos (Fig. 3E–E'). The length of the organ of Corti was decreased by 15% ( $3557 \pm 55 \mu\text{m}$  vs.  $4212 \pm 51 \mu\text{m}$ ,  $n=3$  cochleae/genotype,  $p=9.7 \times 10^{-4}$ ), but overall cochlear length was unchanged ( $4504 \pm 31 \mu\text{m}$  vs.  $4458 \pm 24 \mu\text{m}$ ,  $n=3$  cochleae/genotype,  $p=0.30$ ). Looking by region, we found that hair cell numbers were reduced by 56% ( $323 \pm 73$  vs.  $728 \pm 19$ ;  $p=0.0059$ ) in the middle turn and 88% ( $83 \pm 4$  vs.  $716 \pm 10$ ;  $p=5.4 \times 10^{-7}$ ) in the apex of cochleae from *Atoh1<sup>CreERT2/flox</sup>* embryos compared to control littermates ( $n=3$  cochleae/genotype) (Fig. 3F). Activated caspase 3 immunostaining was also seen in these regions in *Atoh1<sup>CreERT2/flox</sup>* embryos but not in control littermates (Fig. 3G, G', G'', H, H', and H''), suggesting that these cells were undergoing apoptosis. In contrast, myosin VI immunostaining was present in four rows of hair cells in the lower middle and basal turns (Fig. 3A, A', C, C'), and we found no decrease in the number of myosin VI+ hair cells in the basal turn of *Atoh1<sup>CreERT2/flox</sup>* embryos ( $693 \pm 13$  vs.  $716 \pm 37$ ,  $n=3$  cochleae/genotype;  $p=0.60$ ; Fig. 2F). However, phalloidin labeling revealed that essentially all of the remaining hair cells in the middle and basal turns had grossly disorganized and fragmented hair bundles while those in the apical turn lacked hair bundles completely (compare Fig. 3B–B' to D–D'). This phenotype could not be explained by activation of cell death programs in the lower middle and basal turns since there were no activated caspase 3+ cells in these regions

(Fig. 3G, G', H, and H'). Cell loss and stereocilia phenotypes were also seen in E18.5 embryos that received tamoxifen at E17.5 except that four continuous rows of hair cells extended farther apically than in those that received tamoxifen at E16.5 (data not shown). Taken together, these data suggest that apical hair cells still require *Atoh1* for survival at E16.5 and E17.5, while basal hair cells require *Atoh1* for stereocilia formation at those ages.

Cochlear hair cells stop expressing detectable amounts of *Atoh1* mRNA between P3 and P7 (Lanford et al., 2000; Lumpkin et al., 2003; Scheffer et al., 2007). To determine whether *Atoh1* was required for auditory hair cell survival or maturation following birth, *Atoh1*<sup>CreERT2/flox</sup> pups and control littermates were given tamoxifen at P1 or P3 and cochleae were harvested two days later (P3 or P5, respectively). No hair cell loss or hair bundle abnormalities were seen in *Atoh1*<sup>CreERT2/flox</sup> pups at either age (Fig. 4 and data not shown). These data indicate that the function of postnatal *Atoh1* expression is different from that of late embryonic expression in that postnatal expression is not necessary for short-term hair cell survival or stereocilia formation/maintenance.

### Continued *Atoh1* expression is necessary for differentiation of vestibular hair cells

We next examined the utricle to determine whether *Atoh1* deletion produced similar effects in vestibular hair cells. In contrast to the cochlea, utricular hair cells become post-mitotic over a long period of time (E12.5–P2) in a predominantly central-to-peripheral gradient (Burns et al., 2012; Ruben, 1967; Sans and Chat, 1982). We first verified that tamoxifen administration to *Atoh1*<sup>CreERT2</sup>; *ROSA*<sup>LacZ</sup> embryos at E14.5 labeled essentially all hair cells by E18.5 (1538 ± 60 -gal+, *n*=2 utricles vs. 1559 ± 17 myosin VIIa+ cells, *n*=4 utricles; *p*=0.67). To determine potential effects of *Atoh1* deletion at times when some hair cells were being born while others were differentiating, we administered tamoxifen at E14.5 or E16.5 and harvested utricles at E18.5. We found that *Atoh1*<sup>CreERT2/flox</sup> embryos exposed to tamoxifen at either age had statistically significant decreases in the number of myosin VIIa+ cells and the number of cells with a phalloidin+stereociliary bundle compared to littermate controls (Table 2; Fig. 5). These effects were more prominent with tamoxifen administration at E14.5 (Table 2; Fig. 5D–F, J–L). In addition, the percentage of myosin VIIa+ cells with a stereociliary bundle was decreased to about the same extent compared to controls following tamoxifen administration at either age (Table 2). Furthermore, hair bundles of surviving hair cells visualized by phalloidin labeling appeared thinner in *Atoh1*<sup>CreERT2/flox</sup> embryos than in littermate controls (Fig. 5B, B', E, E', H, H', K, and K'). These data suggest that conditional deletion of *Atoh1* disrupts utricular hair cell differentiation.

We next sought to determine whether hair cell survival was affected and/or whether there was regional variability akin to that seen in the cochlea. To this end, we chose to expand our analysis of E18.5 *Atoh1*<sup>CreERT2/flox</sup> embryos exposed to tamoxifen at E14.5 because more pronounced effects were seen at this age (Table 2). We analyzed 6 regions of equal size (3 central, 3 peripheral; see Fig. 5 for examples of locations) in utricles from *Atoh1*<sup>CreERT2/flox</sup> embryos and control littermates to determine whether regional variability was present. We found no differences in the numbers of DAPI+cell nuclei/100 μm<sup>2</sup> located in the central or peripheral regions of *Atoh1*<sup>CreERT2/flox</sup> embryos and control littermates (Table 3). In contrast, the number of myosin VIIa+ cells was decreased both centrally and peripherally (13% vs. 27%) in *Atoh1*<sup>CreERT2/flox</sup> embryos. We also found that the number of -galactosidase+cells (Fig. 5M; 1495 ± 39, *n*=2 utricles) was much higher than the number of myosin VIIa+ cells (1076 ± 36, *n*=4 utricles; *p*=0.0021) in E18.5 *Atoh1*<sup>CreERT2/flox</sup>; *ROSA*<sup>LacZ</sup> embryos that received tamoxifen at E16.5 but similar to the number of myosin VIIa+ cells in control littermates (1525 ± 20, *n*=4 utricles; *p*=0.48). These data suggest that cell death was not responsible for the decreased numbers of myosin VIIa+ cells seen in *Atoh1*<sup>CreERT2/flox</sup> embryos. We next qualitatively classified myosin VII+ hair cells into

three groups based on the length (long, short or absent; examples are shown in Fig. 5K -L ) of their stereociliary bundles. We found no difference in the percentage of myosin VIIa+ cells with long stereocilia in central or peripheral regions (Table 3). However, decreases of 13% (central) and 28% (peripheral) in the number of hair cells with short stereocilia were found in *Atoh1<sup>CreERT2/flox</sup>* embryos. In addition, the percentage of myosin VIIa+ cells without stereocilia increased by 15% and 24%, respectively. Thus, the effects of *Atoh1* deletion on myosin VIIa expression and hair bundle production/development were more severe in peripheral than in central regions.

### Conditional *Atoh1* deletion affects cochlear hair cell gene expression

Given the stereocilia abnormalities seen in surviving hair cells following *Atoh1* deletion at E14.5 (utricle), E16.5 and E17.5 (cochlea) we next sought to determine whether there were qualitative changes in the expression of candidate genes involved in hair and supporting cell specification, differentiation and maturation. We administered tamoxifen to pregnant dams at E16.5, isolated RNA from pooled E18.5 *Atoh1<sup>CreERT2/flox</sup>* or control embryo organs of Corti, and performed RT-PCR (Fig. 6A). Genes upstream of *Atoh1* (*Eya1*, *Six1*, *Sox2*), those expressed by supporting cells (*Jag1*, *Hes1*, *Prox1*), hair cell markers (*Myo6*, *Myo7A*, *CDH23*) and the hair cell differentiation factor *Pou4f3* continued to be expressed in cochleae from treated *Atoh1<sup>CreERT2/flox</sup>* embryos. In contrast, expression of *Barhl1* and *Gfi1* was absent, suggesting that *Atoh1* is required for continued expression of these genes. We verified these results for *Barhl1* and *Pou4f3* using *in situ* hybridization ( $n=4$  cochleae/genotype). No expression of *Barhl1* was detected in cochleae from E18.5 *Atoh1<sup>CreERT2/flox</sup>* embryos that received tamoxifen at E16.5 (Fig. 6B and C), while *Pou4f3* was expressed by remaining hair cells in the cochlea and vestibular system (Fig. 6D-E ).

### Discussion

*Atoh1* has been viewed to function as a proneural gene important for specification and/or as a differentiation initiation factor in various cell types (Bermingham et al., 1999; Matei et al., 2005; Pan et al., 2012). Our data suggest that *Atoh1* serves at least two additional functions during cochlear hair cell development: an intermediate stage function in hair cell survival and a late stage function in hair cell differentiation/maturation. These latter two functions need not be mutually exclusive and are likely to overlap temporally because of the maturation gradients (basal-to-apical, medial-to-lateral) inherent to cochlear development. These gradients also likely explain the increasing basal-to-apical phenotypic severity seen in the cochleae of *Atoh1<sup>CreERT2/flox</sup>* mice. Alternatively, *Atoh1* might function primarily as a survival factor after specification is complete and differentiation has started, and the disorganized stereocilia we observe in the cochlea of late knockout embryos may represent a non-specific marker of hair cell degeneration. This interpretation is consistent with data from other models of hair cell loss (Jahan et al., 2012; Soukup et al., 2009) but seems unlikely here because basal hair cells of *Atoh1<sup>CreERT2/flox</sup>* mice are negative for activated caspase 3, unlike their apical counterparts. However, given the short survival times in our studies we cannot rule out the possibility that these defective hair cells degenerate postnatally as they do in another model of delayed *Atoh1* deletion (Pan et al., 2012). Unfortunately, treated *Atoh1<sup>CreERT2/flox</sup>* mice survived only 2–3 days postnatally secondary to near complete *Atoh1* deletion in the gut, which ultimately resulted in their death (data not shown).

Our data are consistent with and extend those obtained in an *Atoh1* “self-terminating” mutant, where hair cell death and stereo-cilia disorganization were observed when a constitutively-expressed transgenic *Atoh1<sup>Cre</sup>* allele was used to delete *Atoh1* expression (Pan et al., 2012). An advantage of our system was that it allowed us to exert precise temporal control of *Atoh1* deletion by using a strong inducible knock-in *Atoh1<sup>CreERT2</sup>*

allele, and to therefore separate early from late effects of *Atoh1* deletion in a way that was impossible to achieve in that and other previously-described models (Pan et al., 2011).

Interestingly, our data suggest that continued expression of *Atoh1* plays slightly different roles in the utricle and cochlea. In both areas *Atoh1*<sup>CreERT2/flox</sup> mice have stereocilia abnormalities ranging from abnormal appearance to failure of formation. On the other hand utricular hair cells, unlike their cochlear counterparts, do not require continued *Atoh1* expression for survival. This disparity could arise from the different rate at which hair cells differentiate in the two structures. Some apical cochlear hair cells begin to differentiate 4–5 days after their terminal mitoses (Jahan et al., 2010, 2013; Rubel, 1978), and our data suggest that continued *Atoh1* expression during this “limbo” period is crucial for their survival. In contrast, stereocilia development in the utricle begins at E13.5, just one day after the first utricular hair cells become post-mitotic (Takumida and Harada, 1984). Thus, it could be that *Atoh1* is a survival factor for only as long as it takes to initiate differentiation, an interpretation consistent with our observation that basal cochlear hair cells die after E15.5 but not E16.5 or E17.5 tamoxifen administration. Another possibility is that deletion of *Atoh1* changes cellular interactions in the utricle, leading to changes in precursor allocation. For example, lateral inhibition plays an important role in supporting cell specification (Kelley, 2006), so disrupted differentiation of hair cells could inhibit supporting cell differentiation, leading to persistence of precursors that subsequently become “reassigned” to a hair cell identity. This explanation presupposes utricular hair cell death followed by transdifferentiation of supporting cells and/or increased mitotic activity of precursors. The former explanation is plausible given that utricular hair cells continue to be generated for two weeks after mitotic activity ceases, likely through trans-differentiation of supporting cells (Burns et al., 2012). However, in our system this seems unlikely given that we did not see decreased overall cell numbers (Table 3) and/or evidence of more cell divisions (data not shown).

Our data also provide further evidence for central-to-peripheral progression of utricular hair cell development. We saw larger numbers of hair cells with mature stereocilia in the central regions of E18.5 control and *Atoh1*<sup>CreERT2/flox</sup> embryos, consistent with this interpretation. Furthermore, peripheral areas of *Atoh1*<sup>CreERT2/flox</sup> embryonic utricles had fewer myosin VIIa<sup>+</sup> hair cells than central areas, and fewer of the peripherally located hair cells had stereociliary bundles (Table 3). Central regions of *Atoh1*<sup>CreERT2/flox</sup> embryonic utricles also had decreased numbers of myosin VIIa<sup>+</sup> hair cells, and fewer of these cells had stereociliary bundles, than in central regions of control littermates. So, while hair cell differentiation in general progresses from center to periphery, new cells must continue to be generated and differentiate centrally even after hair cell generation has begun in the periphery. This interpretation agrees well with previous birth-dating studies (Sans and Chat, 1982).

Another important aspect of our study is the demonstration that *Barhl1* and *Gfi1*, two transcription factors important for cochlear hair cell survival and differentiation, rely on *Atoh1* for sustained expression. Previous evidence suggested that both genes lie downstream of *Atoh1* (Chellappa et al., 2008; Shroyer et al., 2005). *Barhl1* is first expressed in hair cells at E14.5 (Li et al., 2002; Pauley et al., 2008) and *Gfi1* at E15.5 (Hertzano et al., 2004; Wallis et al., 2003); thus, expression of both genes initiates prior to *Atoh1* deletion in *Atoh1*<sup>CreERT2/flox</sup> mice that received tamoxifen at E16.5. Because most middle turn and all basal turn hair cells survive until E18.5 in this paradigm yet no *Barhl1* or *Gfi1* expression is seen at E18.5, we conclude that *Atoh1* is necessary for maintained expression of these genes. This conclusion is in line with a recent knock-in mouse model where *Atoh1* was replaced by *Neurog1* and no expression of *Barhl1* was observed in surviving hair cells (Jahan et al., 2012). *Gfi1* has been described as a downstream target of *Pou4f3* because it is not expressed in hair cells of *Pou4f3*<sup>ddl/dl</sup> mice (Hertzano et al., 2007; Hertzano et al.,



2004). Our data show that *Gfi1* expression is lost in *Atoh1<sup>CreERT2</sup>* mice treated with tamoxifen at E16.5 despite continued expression of *Pou4f3*. This shows that *Pou4f3* expression alone is not sufficient to maintain *Gfi1* expression, suggesting either that *Pou4f3* is necessary only for initiation of *Gfi1* expression or that both *Atoh1* and *Pou4f3* are required for maintenance of expression. This second possibility could be tested by conditionally deleting *Pou4f3* during late embryonic development. Interestingly, our data also suggest that *Gfi1*'s role in hair cell development might, like that of *Atoh1*, change over time. Previous evidence suggested that *Gfi1* acts as a hair cell survival factor because *Gfi1*-null mice lose virtually all hair cells in a basal-to-apical gradient of increasing severity between E17.5 and P3 (Pauley et al., 2008; Wallis et al., 2003). Our analysis was limited to embryonic ages, but we saw no evidence of hair cell death in the middle and basal turns of *Atoh1<sup>CreERT2/flox</sup>* mice that received tamoxifen at E16.5 or later. These observations suggest that a short period of *Gfi1* expression might be sufficient for hair cell survival, an interpretation supported by experiments conducted in cultured organ of Corti where downregulation of *Gfi1* by *Prox1* overexpression at E15.5, but not P1, caused hair cell death (Kirjavainen et al., 2008). As with *Atoh1* itself, we interpret this to mean that initial expression of *Gfi1* is required for survival, but the requirement for *Gfi1* changes over developmental time. Hair cell death in *Barhl1*-null mice begins after P6 and is slowly progressive over several months (Li et al., 2002); further experiments will be necessary to determine if this occurs in *Atoh1<sup>CreERT2/flox</sup>* mice.

Our data also provide *in vivo* evidence that several cochlear hair cell-specific genes including *Pou4f3* do not rely on *Atoh1* for maintained expression. Previous work demonstrated that sequences upstream of the *Pou4f3* locus can drive luciferase expression in the presence of *Atoh1*, suggesting that *Atoh1* might directly regulate *Pou4f3* (Masuda et al., 2011; Masuda et al., 2012). However, another *in vitro* study demonstrated that ectopic expression of *Eya1* and *Six1* in cochlear explants directed expression of *Pou4f3* either alone or together with *Atoh1*, suggesting that regulation of the two genes might be independent of one another (Ahmed et al., 2012). Our results agree with and extend this second study by showing that *Atoh1* is not necessary for maintained *Pou4f3* expression *in vivo*, demonstrating that *Pou4f3* operates in a parallel pathway. Furthermore, we demonstrate that *Atoh1* expression is not necessary for maintained expression of *CDH23*, *Myo6*, or *Myo7a*, all of which are first expressed in hair cells between E14.5 and E16.5 (Lagziel et al., 2005; Xiang et al., 1998). This leaves three possibilities. First, *Atoh1* might be directly required for initial expression of these markers, but maintenance is controlled by other transcription factors. Certainly this is possible in our system, as *Atoh1* is expressed throughout the organ of Corti by E15.5 (Woods et al., 2004) and we looked at marker gene expression following *Atoh1* deletion at E16.5. Second, *Atoh1* might upregulate a set of transcription factors that then, in turn, directly control expression of *CDH23*, *Myo6*, and *Myo7a*. Finally, it is possible that *Atoh1* does not regulate these genes at all. This possibility is supported by the finding that myosin VI expression was induced in *Atoh1*-positive and -negative “hair cell-like” cells induced by ectopic *Eya1* and *Six1* expression (Ahmed et al., 2012). Furthermore, in that study essentially all of the myosin VI-positive cells also expressed *Pou4f3*, suggesting that this gene is capable of regulating at least some aspects of hair cell differentiation in the absence of *Atoh1*. This interpretation is also consistent with the finding that small numbers of “hair cell-like” cells can form even in the complete absence of *Atoh1* expression (Du et al., 2007; Pan et al., 2011). Future work will need to address the genetic pathways that directly control marker expression in hair cells.

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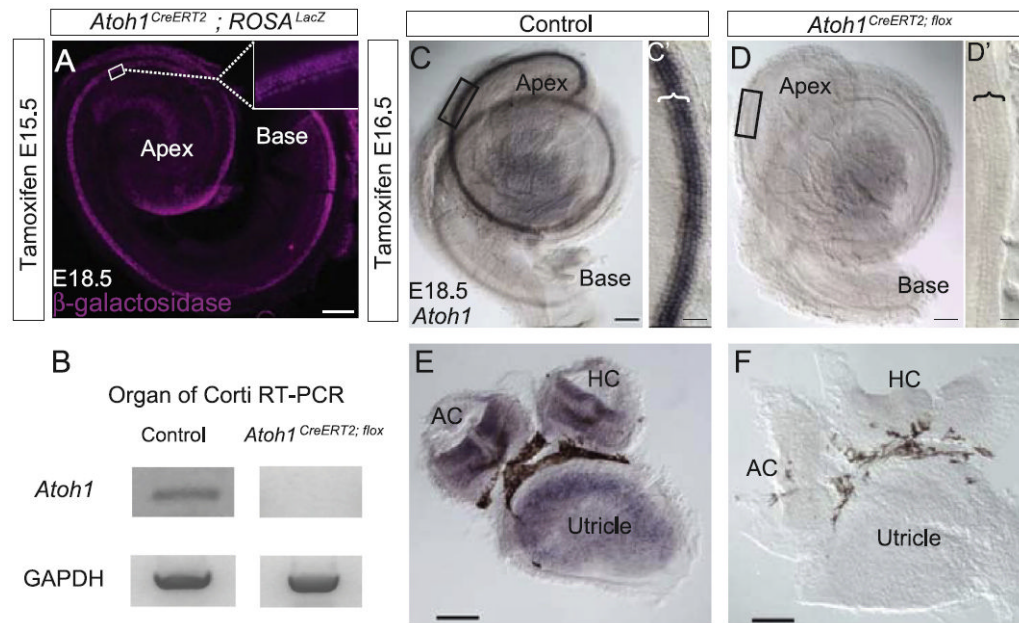
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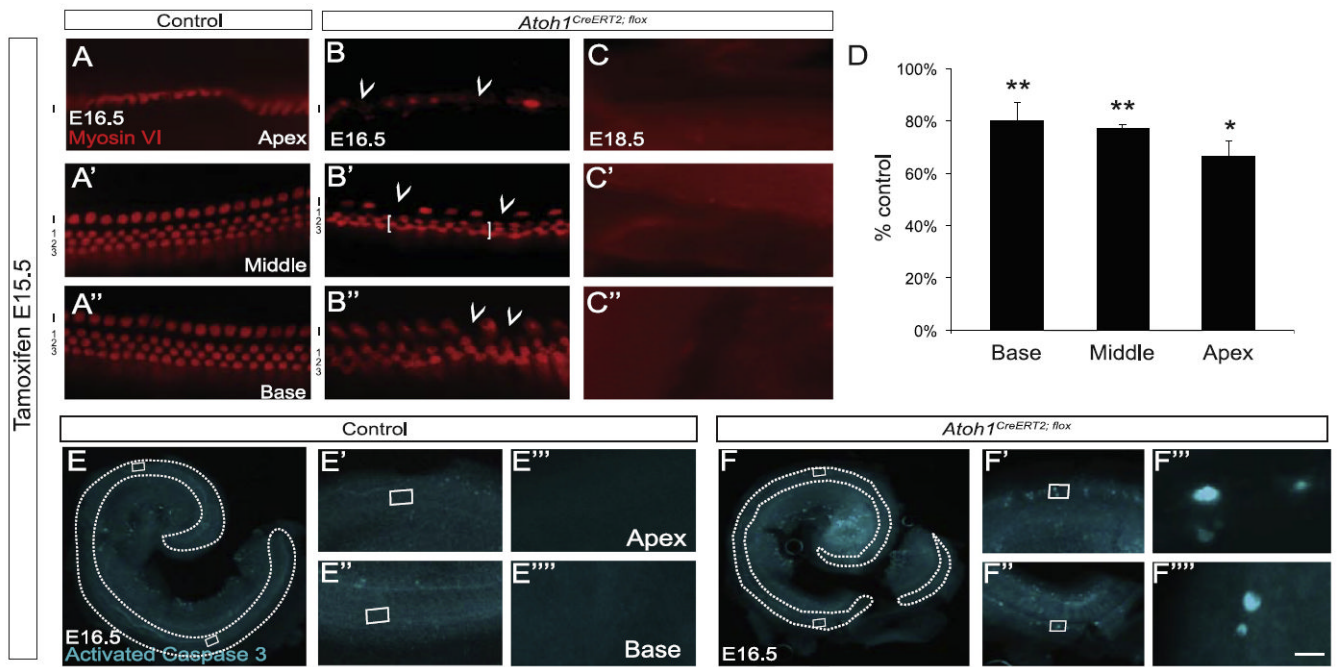
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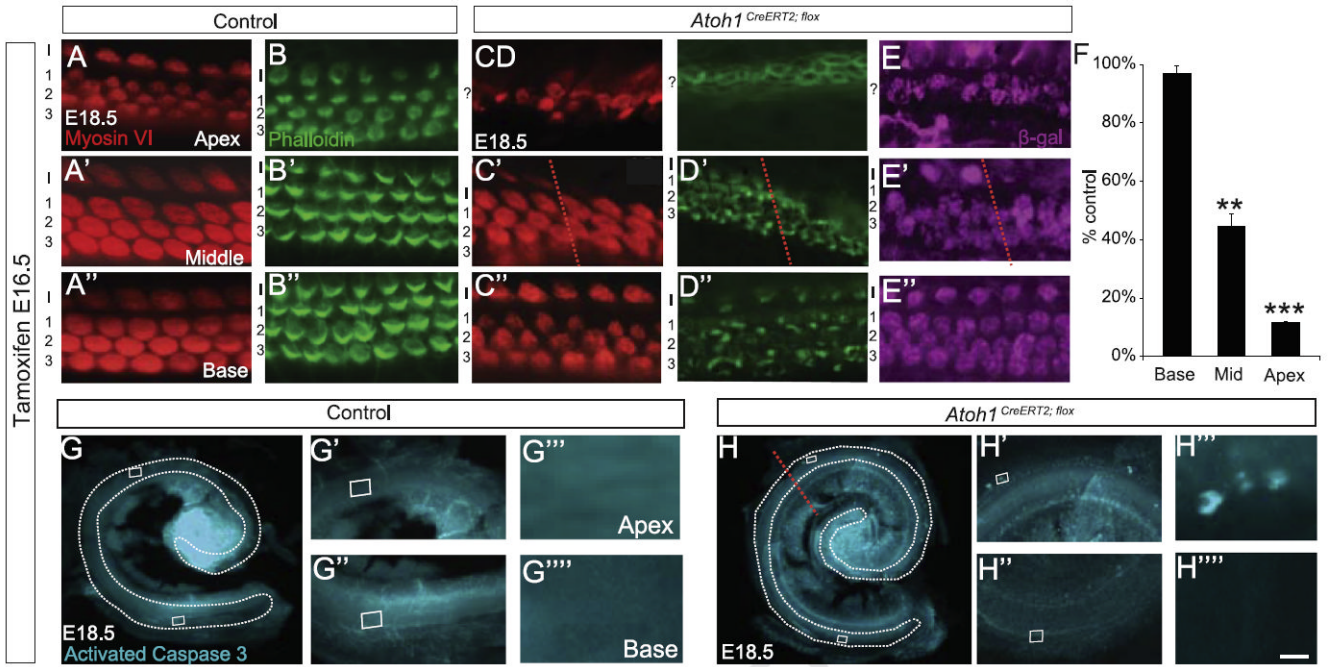
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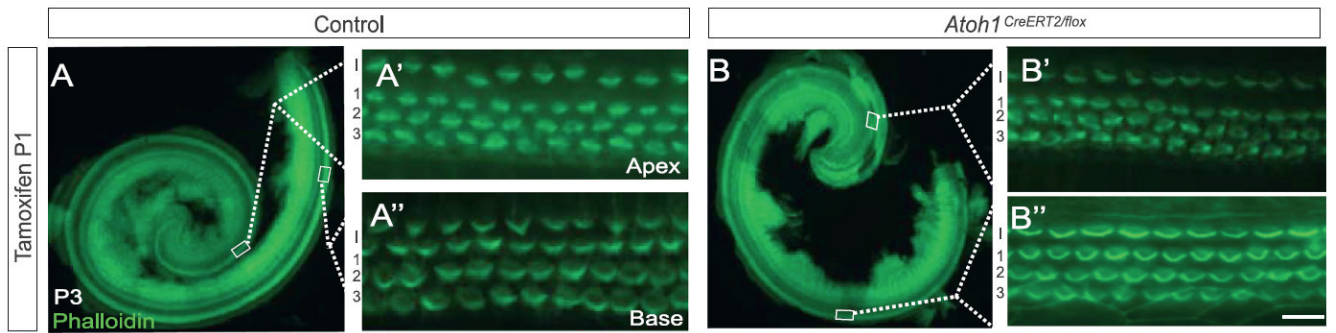
**Fig. 1.** Tamoxifen administration activates *Atoh1<sup>CreERT2</sup>* in nearly all cochlear hair cells and efficiently deletes an *Atoh1<sup>flox</sup>* allele. (A) Cochlea from E18.5 *Atoh1<sup>CreERT2/+</sup>; ROSA<sup>LacZ</sup>* embryo treated with tamoxifen at E15.5 and immunostained for  $\beta$ -galactosidase. Box shows location of inset. Nearly every hair cell is labeled. (B) RT-PCR for *Atoh1* on organ of Corti total RNA isolated from *Atoh1<sup>CreERT2/flox</sup>* verifies *Atoh1* deletion. (C–F) *In situ* hybridization for *Atoh1* on cochleae (C, D) and vestibular organs (E, F) from E18.5 control (C, C', and E) and *Atoh1<sup>CreERT2/flox</sup>* (D, D', and F) embryos following tamoxifen administration at E16.5. No labeling is present in the *Atoh1<sup>CreERT2/flox</sup>* embryonic cochlea or vestibular system. Boxes show locations of C' and D'; brackets identify organ of Corti. AC – anterior crista, HC – horizontal crista. Scale bar: 500  $\mu$ m (A); 100  $\mu$ m (C–F); 25  $\mu$ m (C', D').



**Fig. 2.** Cochlear hair cells die following *Atoh1* deletion at E15.5. Pregnant dams received tamoxifen at E15.5. (A–C) Cochleae from control littermate (A) and *Atoh1<sup>CreERT2/flox</sup>* (B–C) embryos immunostained for myosin VI. (B–B') E16.5 *Atoh1<sup>CreERT2/flox</sup>* mice lacked outer hair cells (1–3, brackets in B) predominantly in the third row and scattered inner hair cells (I, arrowheads) throughout the cochlea. No hair cells were present at E18.5 (C–C'). (D) Myosin VI+ hair cell counts by region in *Atoh1<sup>CreERT2/flox</sup>* embryos as percent of control littermate numbers ( $n=3-4$  cochleae/genotype). \* $p<0.05$ , \*\* $p<0.01$  compared to control. (E–F) Activated caspase 3 immunostaining of cochleae from E16.5 embryos demonstrated apoptotic cell death in the organ of Corti (outlined) of *Atoh1<sup>CreERT2/flox</sup>* (F–F') but not littermate controls (E–E'). Scale bar: 15  $\mu\text{m}$  (A–C), 500  $\mu\text{m}$  (E, F); 50  $\mu\text{m}$  (E', E'', F', and F''); 5  $\mu\text{m}$  (E''', E''', F''', and F''').

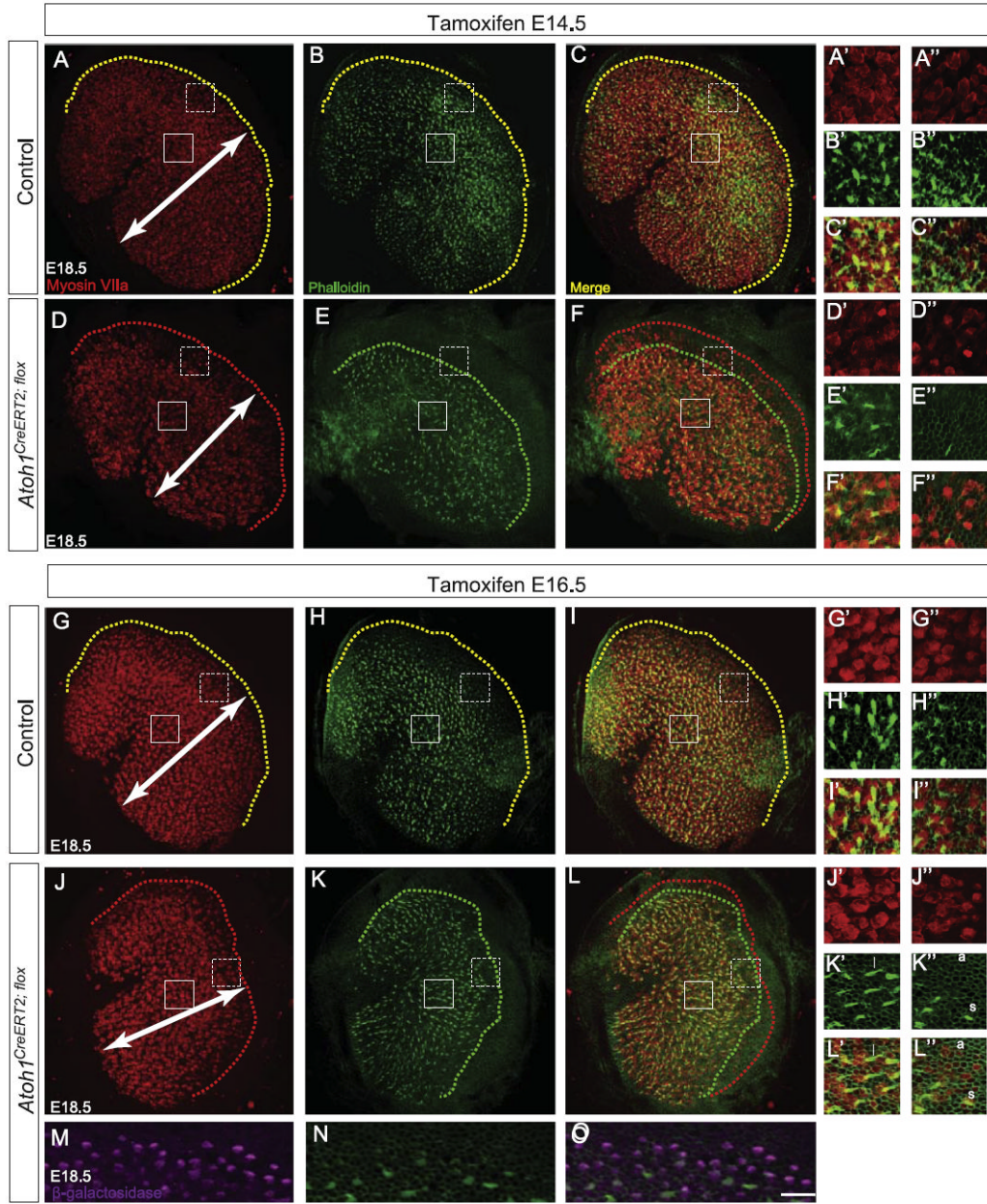


**Fig. 3.** *Atoh1* deletion at E16.5 causes hair cell loss and disrupts cochlear hair cell stereocilia formation. (A–D ) Cochleae from E18.5 littermate control (A–B ) and *Atoh1*<sup>CreERT2/flox</sup> (C–D ) embryos treated with tamoxifen at E16.5 and immunostained with myosin VI, -galactosidase or labeled with phalloidin (A–A , B–B , C–C , D–D and E–E are from different animals). *Atoh1*<sup>CreERT2/flox</sup> embryos have fewer hair cells from the mid-middle turn (red lines in C , D , E and H) to the apex while these cells are present in normal numbers from the mid-middle turn to the base. However, remaining hair cells have disorganized or no stereocilia (D–D ). Remaining hair cells also show evidence of recombination (E–E ). (F) Hair cell counts by cochlear region graphed as percent of control littermate numbers show significant loss of myosin VI+ hair cells in the apex and middle turn but not the base of *Atoh1*<sup>CreERT2/flox</sup> embryos ( $n=3$  cochleae/genotype). \*\* $p<0.001$ , \*\*\* $p<1 \times 10^{-6}$ . G–H ) Immunostaining for activated caspase 3 shows cell death in the apex, but not the base, of *Atoh1*<sup>CreERT2/flox</sup> embryos. Scale bar: 15  $\mu\text{m}$  (A–E ); 500  $\mu\text{m}$  (G, H); 50  $\mu\text{m}$  (G , G , H , and H ); 5  $\mu\text{m}$  (G , G , H , and H ).



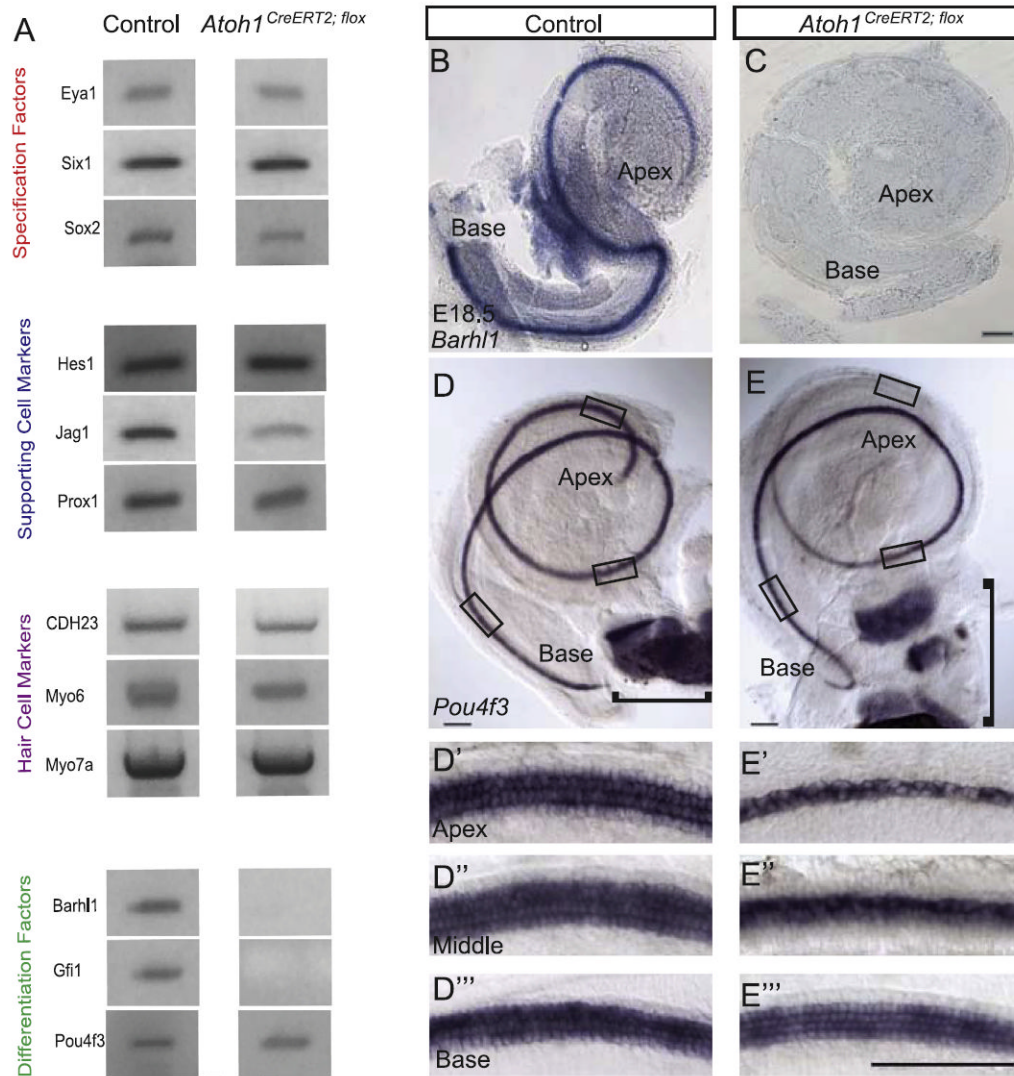
**Fig. 4.** *Atoh1* deletion at P1 does not affect hair cell number or stereocilia formation. Pups were injected with tamoxifen at P1 and cochleae harvested at P3. Phalloidin labeling revealed no loss of hair cells or stereocilia abnormalities. Scale bar: 500  $\mu\text{m}$  (A, B); 15  $\mu\text{m}$  (A', A'', B', and B'').





**Fig. 5.** *Atoh1<sup>CreERT2/flox</sup>* embryos have fewer myosin VIIa+ cells and fewer stereociliary bundles in the utricle. Pregnant dams were gavaged with tamoxifen at either E14.5 (A–F ) or E16.5 (G–L ) and utricles harvested at E18.5. Immunostaining for myosin VIIa (A, D, G, and J) and labeling with phalloidin (B, E, H, and K) revealed decreased mediolateral extent (arrows) of the hair cell field in *Atoh1<sup>CreERT2/flox</sup>* embryos (D–F, J–L) compared to control littermates (A–C, G–I). Yellow dotted lines in utricles from control littermates (A–C, G–I) show that hair cells with stereocilia are found right up to the border of the hair cell field. In contrast, red (D, F, J, and L) and green (E, F, K, and L) dotted lines in utricles from *Atoh1<sup>CreERT2/flox</sup>* embryos show that many peripherally-located hair cells lack stereocilia. Solid and dotted boxes indicate regions shown in A–L and A–L, respectively, and are representative of the locations of “central” and “peripheral” regions of interest for cell

counts (see text). Fewer myosin VIIa+ hair cells are seen in these regions (A, A', D, D', G, G', J, and J'), and more of these hair cells lack stereocilia in *Atoh1<sup>CreERT2/flox</sup>* embryos than in controls (B–C, E–F, H–I, and K–L). Examples of long (l), short (s) and absent (a) stereociliary bundles are shown in K–L. Immunostaining for  $\beta$ -galactosidase (M) shows that *Atoh1<sup>CreERT2/flox</sup>; ROSA<sup>LacZ</sup>* embryos have many cells that have undergone recombination, but only a small number of these have phalloidin+ hair bundles (N, O). Scale bar: 30  $\mu$ m (A–L); 12  $\mu$ m (M–O); 10  $\mu$ m (A'–L').



**Fig. 6.** *Atoh1* deletion at E16.5 abolishes cochlear expression of *Barhl1* and *Gfi1* but not several other genes. Pregnant dams received tamoxifen at E16.5 and embryos were harvested at E18.5. (A) Qualitative RT-PCR of organ of Corti mRNA from control and *Atoh1*<sup>CreERT2/flox</sup> embryos demonstrates that expression of *Barhl1* and *Gfi1* is lost in *Atoh1*<sup>CreERT2/flox</sup> mice. Upstream specification factors, hair cell markers, supporting cell markers and *Pou4f3* continue to be expressed. (B, C) *In situ* hybridization for *Barhl1* confirms absence of expression in *Atoh1*<sup>CreERT2/flox</sup> embryonic cochleae. (D–E) *Pou4f3* continues to be expressed in all regions of *Atoh1*<sup>CreERT2/flox</sup> embryonic cochleae where hair cells are present. *Pou4f3* expression is also maintained in the vestibular system (brackets). Scale bar: 100  $\mu$ m.

**Table 1**

RT-PCR primers.

Gene	Forward primer	Reverse primer
<i>Atoh1</i>	TCTGCTGCATTCTCCCGAGC	CTCTGGGGGTTACTCGGTGC
<i>Barhl1</i>	GAACCGCAGGACTAAATGGAA	GAGCGCCGAGTAATTGCCT
<i>Cadherin 23</i>	ACTCCCTGGTCACTTGCTATG	TGGTGAAGAAAGGTAGTCGGT
<i>Eya1</i>	TAACAGCTCGCCGTATCCAG	GTCCAGATGAACACTCTCTCA
GAPDH	GTCATCATCTCCGCCCTTCTGC	GATGCCTGCTTCACCACCTTCTTG
<i>Gfi1</i>	AGAAGGCGCACAGCTATCAC	GGCTCCATTTTCGACTCGC
<i>Hes1</i>	TCAACACGACACCGGACAAAC	ATGCCGGGAGCTATCTTTCTT
<i>Jag1</i>	ATGCAGAACGTGAATGGAGAG	GCGGGACTGATACTCCTTGAG
<i>Myo6</i>	CCACAATGTCAAAGTTCGGTACA	GGCATCGTCCCAAGAGATTTTC
<i>Myo7a</i>	GGGGACTATGTATGGATGGACC	TGATGTGCGTGGCATTCTGAG
<i>Pou4f3</i>	ATGCGCCGAGTTTGTCTCC	GGGCTTGAACGGATGATTCTTG
<i>Prox1</i>	TCGAACGTACTCCGCAAGC	TGCCACCGTTTTTGTTCATGT
<i>Six1</i>	GAAAGGGAGAACACCGAAAACA	GTGGCCCATATTGCTCTGGA
<i>Sox2</i>	CCTACTCACTGCTCGACCTG	TTGCACGGGATCATGTAGCC

**Table 2**

Utricular hair cells require *Atoh1* for survival and stereocilia formation. Total numbers of immunostained cells were counted in the indicated number of utricles harvested from E18.5 embryos and reported as average number  $\pm$  SEM. Reported decreases are relative to littermate controls. Values for E14.5 and E16.5 tamoxifen administration are compared within genotype at the bottom of the Table.

	Control littermate	<i>Atoh1</i> <sup>CreERT2/flox</sup>	Decrease	<i>p</i> -value ( <i>t</i> -test)
<b>Tamoxifen at E14.5</b>	<b><i>n</i>=4 utricles</b>	<b><i>n</i>=4 utricles</b>		
Myosin VIIa+	1559 $\pm$ 17	881 $\pm$ 56	43%	2.5 $\times$ 10 <sup>-5</sup>
Phalloidin+hair bundle	965 $\pm$ 25	394 $\pm$ 17	59%	1.4 $\times$ 10 <sup>-6</sup>
% Phalloidin+/Myosin VIIa+	62 $\pm$ 1%	45 $\pm$ 1%	17%	8.4 $\times$ 10 <sup>-5</sup>
<b>Tamoxifen at E16.5</b>	<b><i>n</i>=4 utricles</b>	<b><i>n</i>=4 utricles</b>		
Myosin VIIa+	1525 $\pm$ 20	1076 $\pm$ 36	29%	3.6 $\times$ 10 <sup>-5</sup>
Phalloidin+hair bundle	946 $\pm$ 24	544 $\pm$ 23	42%	2.1 $\times$ 10 <sup>-5</sup>
% Phalloidin+/Myosin VIIa+	62 $\pm$ 1%	51 $\pm$ 3%	11%	0.014
<b>Tamoxifen E14.5 vs. E16.5</b>	<b><i>p</i>-value (<i>t</i>-test)</b>	<b><i>p</i>-value (<i>t</i>-test)</b>		
Myosin VIIa+	0.25	0.03		
Phalloidin+hair bundle	0.60	0.0020		
% Phalloidin+/Myosin VIIa+	0.97	0.13		

**Table 3**

*Atoh1<sup>CreERT2/fox</sup>* embryonic utricles have regionally-variable differentiation defects but no change in cell density. Pregnant dams received tamoxifen at E14.5 and embryos were harvested at E18.5. Total numbers of DAPI+ nuclei in the regions of interest (450–500 cells; see Methods) were counted for cell density calculations (DAPI+nuclei/100  $\mu\text{m}^2$ ). Hair bundles were qualitatively scored as long, short or absent. All numbers are reported as average  $\pm$  SEM; percent change is relative to littermate controls.

	Control littermate	<i>Atoh1<sup>CreERT2/fox</sup></i>	% Change	<i>p</i> -value ( <i>t</i> -test)
<b>DAPI+ nuclei/100 <math>\mu\text{m}^2</math></b>	<b><i>n</i>=2 utricles</b>	<b><i>n</i>=2 utricles</b>		
Central	7.1 $\pm$ 0.2	7.1 $\pm$ 0.3	0%	0.97
Peripheral	7.1 $\pm$ 0.3	6.8 $\pm$ 0.2	-4%	0.43
<b>Myosin VIIa+/DAPI+</b>	<b><i>n</i>=4 utricles</b>	<b><i>n</i>=4 utricles</b>		
Central	74 $\pm$ 4%	61 $\pm$ 3.8%	-13%	0.04
Peripheral	70 $\pm$ 3.8%	43 $\pm$ 3.2%	-27%	$3.4 \times 10^{-4}$
<b>Hair bundles (phalloidin+/myosin VIIa+)</b>	<b><i>n</i>=4 utricles</b>	<b><i>n</i>=4 utricles</b>		
<b>Long</b>				
Central	29 $\pm$ 1.6%	27 $\pm$ 5.9%	-2%	0.70
Peripheral	4.9 $\pm$ 2.1%	8.2 $\pm$ 2.1%	+3.3%	0.27
<b>Short</b>				
Central	36 $\pm$ 1.6%	23 $\pm$ 3.3%	-13%	0.003
Peripheral	50 $\pm$ 3.9%	22 $\pm$ 2.5%	-28%	$6.6 \times 10^{-6}$
<b>Absent</b>				
Central	35 $\pm$ 1.6%	50 $\pm$ 3.4%	+15%	$6.7 \times 10^{-4}$
Peripheral	45 $\pm$ 4.0%	69 $\pm$ 2.6%	+24%	$4.5 \times 10^{-5}$