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Generation of *Atoh1-rtTA* transgenic mice: a tool for inducible gene expression in hair cells of the inner ear

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Atoh1 is a basic helix-loop-helix transcription factor that controls differentiation of hair cells (HCs) in the inner ear and its enhancer region has been used to create several HC-specific mouse lines. We generated a transgenic tetracycline-inducible mouse line (called *Atoh1-rtTA*) using the *Atoh1* enhancer to drive expression of the reverse tetracycline transactivator (rtTA) protein and human placental alkaline phosphatase. Presence of the transgene was confirmed by alkaline phosphatase staining and rtTA activity was measured using two tetracycline operator (TetO) reporter alleles with doxycycline administered between postnatal days 0–3. This characterization of five founder lines demonstrated that *Atoh1-rtTA* is expressed in the majority of cochlear and utricular HCs. Although the tetracycline-inducible system is thought to produce transient changes in gene expression, reporter positive HCs were still observed at 6 weeks of age. To confirm that *Atoh1-rtTA* activity was specific to *Atoh1*-expressing cells, we also analyzed the cerebellum and found rtTA-driven reporter expression in cerebellar granule neuron precursor cells. The *Atoh1-rtTA* mouse line provides a powerful tool for the field and can be used in combination with other existing Cre recombinase mouse lines to manipulate expression of multiple genes at different times in the same animal.

Conditional gene expression enables the temporal study of genes during development and in adulthood. This method also allows for cell-type specific gene manipulation and alleviates embryonic lethality which can occur when certain genes are overexpressed or deleted in the germline. The Cre-mediated recombination system (Cre/loxP) is the most common method to delete or ectopically express genes in a cell-type specific manner, with temporal control of gene expression achieved by fusion of Cre recombinase with a modified tamoxifen-inducible estrogen receptor (CreERTM or CreER^{T2}) (reviewed in¹). Tetracycline-inducible mouse models (Tet-On or Tet-Off) can also be used for conditional gene expression and in contrast to Cre/loxP, gene expression changes are transient^{2,3}. In some cases one needs to manipulate expression of multiple genes in two different cell types or in the same cell type but at different ages. This can be achieved by combining Cre/loxP and tetracycline-inducible mouse models. Unfortunately there are few tetracycline-inducible mouse lines with cell-type specific expression available, thus warranting the development of new models.

The Tet-On system uses a reverse tetracycline transactivator (rtTA) protein that binds to the promoter and activates transcription of a second transgene which contains a tetracycline operator (TetO). The rtTA protein can only bind to TetO and activate transcription in the presence of doxycycline (a more potent analogue of tetracycline). Once doxycycline is removed, transcription will cease since rtTA can no longer bind to TetO, allowing for transient control of gene expression (Figure 1A)^{2,3}. Cell-type specific expression can be achieved using specific promoters or enhancers to express the rtTA protein. This mechanism is distinct from the Cre/loxP system, and thus the two methods of conditional gene expression can be combined.

Atoh1, also called *Math1* in mice, is the mammalian homolog of the *Drosophila* gene, *atonal*, and is a basic helix-loop-helix transcription factor that controls differentiation of hair cells (HCs) in the inner ear⁴ and proliferation of granule neuron precursor cells in the cerebellum⁵. The 1.7 kb enhancer element located in the 3' untranslated region of *Atoh1*⁶ has been used to generate several mouse lines: *Atoh1-Cre*⁷, *Atoh1-CreER*^{TM,8}, *Atoh1-CreER*^{T2,9}, and *Atoh1-eGFP*¹⁰. Because each of these mouse models has Cre or eGFP expression in HCs and

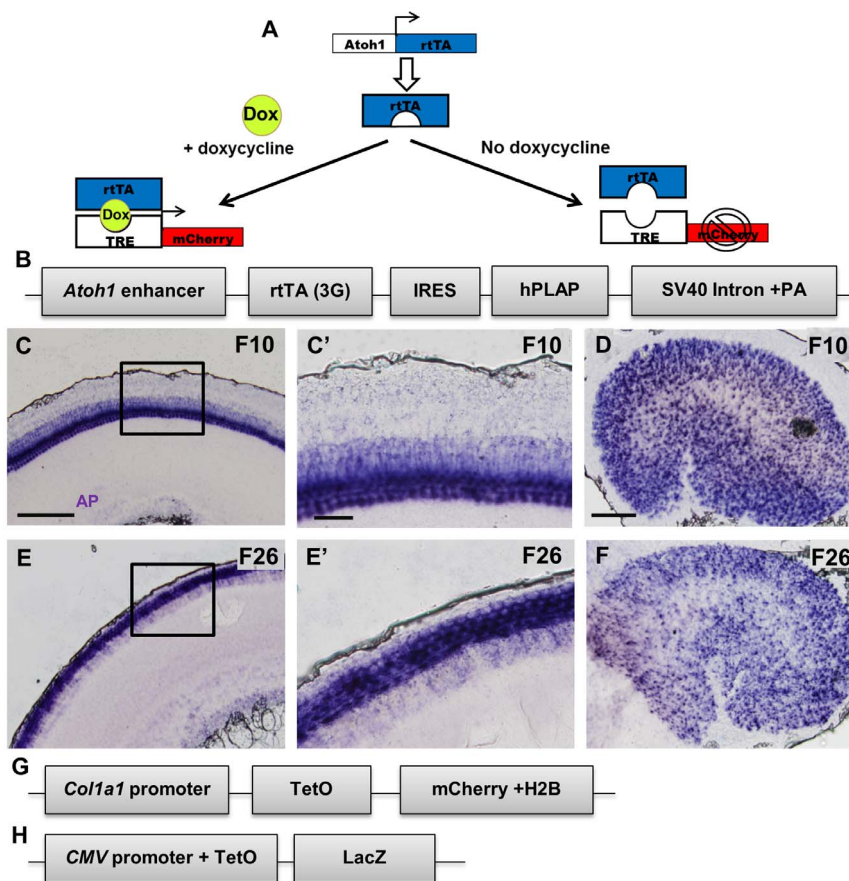


Figure 1 | Components of the Tet-On system and alkaline phosphatase staining. (A) Diagram explaining the Tet-On system. (B) Modified *Atoh1-CreER-IRES-hPLAP-SV40 Intron*-polyadenylation construct⁸ showing replacement of the *CreER* sequence with the 3G *rtTA* sequence. Representative alkaline phosphatase (AP) staining images of F10 and F26 showing the presence of the *Atoh1-rtTA* transgene in the apical turn of the P0 cochlea (C, E) and utricle (D, F). (C' and E') High magnification images of region labeled by the black squares in (C) and (E). Scale bars: 100 μm in (C–F) and 20 μm in (C') and (E'). Schematic of the *TetO-mCherry* (G) and *TetO-LacZ* (H) reporter alleles.

cerebellar granule cells, we used the same enhancer element to generate a HC-specific Tet-On mouse model. Here, we describe the generation and characterization of an *Atoh1-rtTA* mouse line in the inner ear.

Results

The *CreER* sequence in the *Atoh1-CreER-internal ribosome entry site (IRES)-human placental alkaline phosphatase (hPLAP)-SV40 intron*-polyadenylation construct (*Atoh1-CreER-IRES-hPLAP-SV40-polyA*) was replaced with a 3G *rtTA* (Figure 1B). This 3rd generation *rtTA* has reduced levels of basal expression and increased sensitivity to doxycycline compared to Tet-On and Tet-On Advanced systems¹¹. After pronuclear injection, seven founders were produced and the presence of the transgene was confirmed by Southern blot and PCR analysis. All founders went through germline transmission. However, two founder lines did not breed well; thus we assessed *rtTA* activity in five founder lines (F7, F10, F12, F23, and F26), each of them showing expression of hPLAP in HCs of the cochlea and utricle at postnatal day (P) 0 (Figure 1C–F).

We used two *rtTA* reporter alleles since their reporter activity varied. The *TetO-mCherry* reporter is a knock-in allele where the TetO element and *mCherry* coding sequence fused to histone H2B were inserted downstream of the endogenous *collagen type 1, alpha 1 (Col1a1)* promoter (Figure 1G)¹². This reporter is robust since the *Col1a1* promoter is strongly expressed in epithelial cells¹³, and the *mCherry* molecule provides bright endogenous fluorescence that is photostable¹⁴. In contrast, the *TetO-LacZ* reporter is a transgenic

allele where *LacZ* is under the control of the human cytomegalovirus early promoter (CMV) fused to the TetO element (Figure 1H)¹⁵. *LacZ* expression is variable, requiring X-gal staining or an anti- β -galactosidase (β gal) antibody for detection.

Reporter alleles were first tested for basal activity at P3 with *TetO-reporter*^{+ve} mice administered doxycycline between P0–P3, but in the absence of the *rtTA* allele. In *TetO-mCherry*^{+ve} mice, no *mCherry*^{+ve} cells were seen in the sensory region of the cochlea or utricle (Figure 2A, B). However there was an apical-to-basal gradient of increasing *mCherry*^{+ve} cells in the spiral ganglion (SGN) region of the cochlea (Figure 2A–A') as well as several *mCherry*^{+ve} cells in the utricular stroma (Figure 2B'). In *TetO-LacZ*^{+ve} mice, no *LacZ*^{+ve} cells were observed in the cochlea or SGN; however, in the utricle, several *LacZ*^{+ve} supporting cells (SCs) were observed, but no HCs or stromal cells were labeled (Figure 2C–C'). The presence of reporter^{+ve} cells in the absence of the *rtTA* protein has been reported previously in other organ systems¹⁵.

We also tested for leakiness of *rtTA* activity in the absence of doxycycline using *Atoh1-rtTA*^{+ve}; *TetO-mCherry*^{+ve} or *Atoh1-rtTA*^{+ve}; *TetO-LacZ*^{+ve} mice from the F7 lineage analyzed at P3. No *mCherry*^{+ve} cells were observed in the sensory regions of the cochlea or utricle; however, there was an apical-to-basal gradient of increasing *mCherry*^{+ve} cells in the SGN region (Figure 2D–D') and *mCherry*^{+ve} cells in the utricular stroma (Figure 2E–E'), similar to the basal activity seen in *TetO-mCherry*^{+ve} controls that received doxycycline but lacked the *rtTA* allele (Figure 2A–B'). No *LacZ*^{+ve} cells were detected in *Atoh1-rtTA*^{+ve}; *TetO-LacZ*^{+ve} cochlea

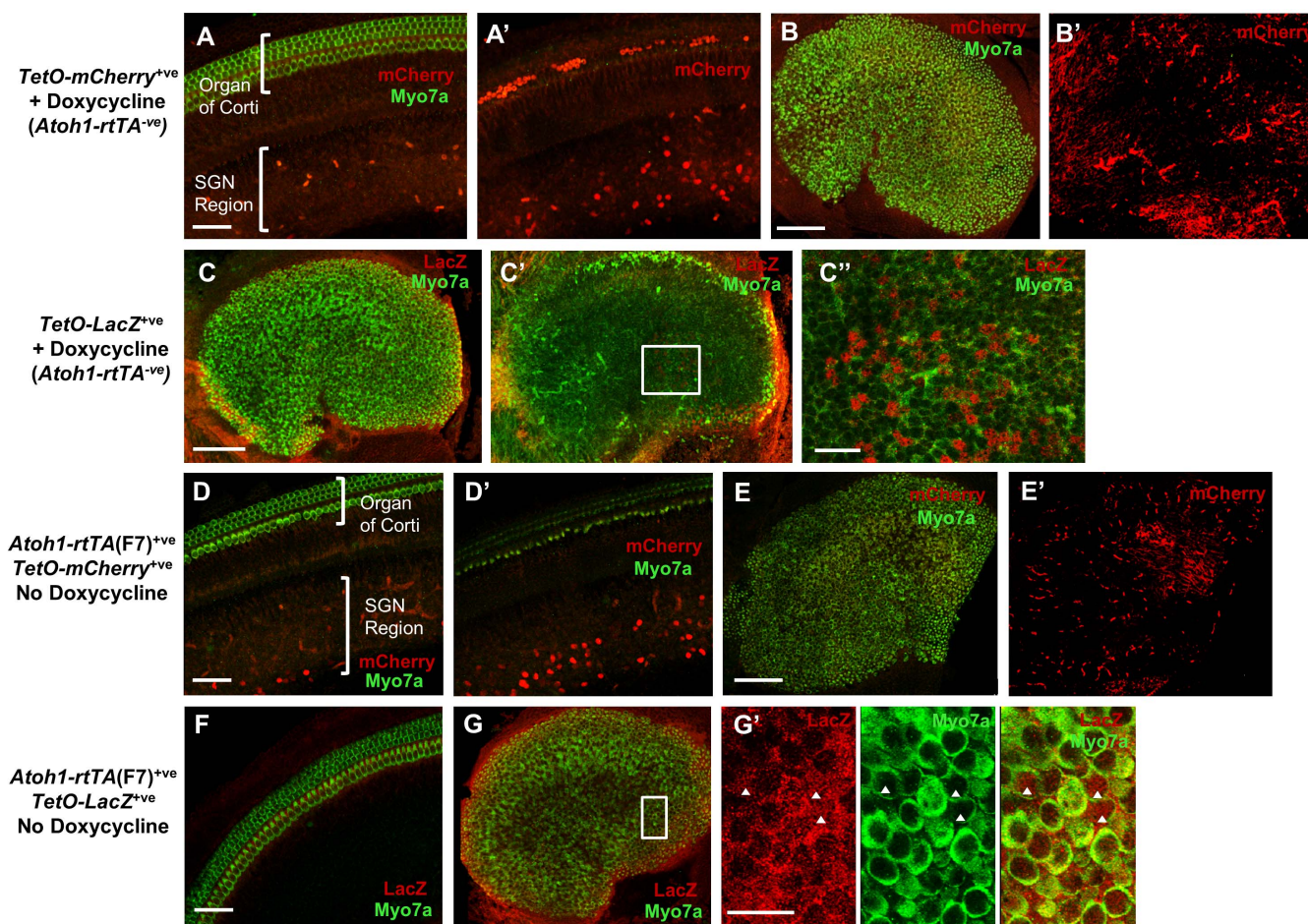


Figure 2 | Basal activity of *TetO-reporters* and *Atoh1-rtTA(F7)* activity in the absence of doxycycline. Representative confocal images of a *TetO-mCherry*^{+/ve} (*Atoh1-rtTA*^{-ve}) control cochlea (A–A′) and utricle (B–B′) at P3 from mice that were administered doxycycline to test for basal expression of the *TetO-mCherry* reporter. mCherry^{+/ve} cells were detected in the SGN region of the cochlea (A–A′) and in the utricular stroma (B–B′). Note in A′ the labeled cells in the organ of Corti are red blood cells. No mCherry^{+/ve} cells were detected in the sensory region of either the cochlea or utricle. (C–C′′) Representative confocal images of a *TetO-LacZ*^{+/ve} (*Atoh1-rtTA*^{-ve}) control utricle at P3 from mice that were administered doxycycline to test for basal expression of the *TetO-LacZ* reporter. Some LacZ^{+/ve} SCs but no LacZ^{+/ve} HCs were detected. (C′′) High magnification images of the region outlined by the white square in C′. Representative confocal images of an un-induced *Atoh1-rtTA(F7)*^{+/ve}; *TetO-mCherry*^{+/ve} control cochlea (D–D′) and utricle (E–E′) at P3 that did not receive doxycycline. mCherry^{+/ve} (red) cells were detected in the SGN region of the basal turn of the cochlea (D–D′) and in the utricular stroma (E–E′). No mCherry^{+/ve} cells were detected in the sensory region of either the cochlea or utricle. Representative confocal image of an un-induced *Atoh1-rtTA(F7)*^{+/ve}; *TetO-LacZ*^{+/ve} control cochlea (F) and utricle (G–G′) at P3 that did not receive doxycycline. Several HCs (myo7a^{+/ve}, green) expressed LacZ (red) in the medial region of the utricle (G–G′) and no LacZ^{+/ve} cells were detected in the cochlea (F). (G′) High magnification image of the region outlined by the white square in (G). Arrowheads denote LacZ^{+/ve} HCs. Myo7a (green) was used to label HCs in most panels. Scale bar: 50 μm in (A–A′), (D–D′), and (F), 100 μm in (B–C′), (E), and (G), and 20 μm in (C′′) and (G′).

(Figure 2F), while in the utricle some LacZ^{+/ve} SCs were observed and a small percentage of HCs in the medial region were LacZ^{+/ve} (Figure 2G–G′).

Each of the *Atoh1-rtTA* founder lines was bred with a *TetO-mCherry* or a *TetO-LacZ* reporter mouse and double-transgenic offspring (*Atoh1-rtTA*^{+/ve}; *TetO-reporter*^{+/ve}) administered doxycycline between P0–P3 were evaluated at P3 for rtTA activity. The five *Atoh1-rtTA* founders bred with the *TetO-mCherry* reporter showed robust expression of mCherry^{+/ve} cochlear HCs: F7 = 99.6 ± 0.1%, F10 = 99.7 ± 0.2%, F12 = 98.2 ± 0.4%, F23 = 69.6 ± 11.5%, and F26 = 94.4 ± 1.6% (mCherry^{+/ve} HCs expressed as a percentage of total HCs, *n* = 3, Figure 3A–E′, K). There was no significant difference in the number of mCherry^{+/ve} cells between inner and outer HCs or across cochlea turns for any founder line. In addition, the intensity of the mCherry reporter was very consistent across all turns of the cochlea and in all founders (Figure 3A–E′). However, there were variations in the cell type-specificity of mCherry expression (Table 1). F26 was the only

founder in which mCherry expression was specific to HCs. F7, F10, and F23 each had mCherry^{+/ve} HCs in all turns of the cochlea. In apical and middle turns, mCherry expression was only observed in HCs. However in the basal turn of these founders, there were some mCherry^{+/ve} inner phalangeal cells (IPHCs) (a SC subtype that surrounds inner HCs) and a few mCherry^{+/ve} cells in the greater epithelial ridge (GER). Lastly, F12 had HC-specific mCherry labeling only in the apex. In addition to mCherry^{+/ve} HCs, the middle turn had few mCherry^{+/ve} IPHCs, which increased in number in the basal turn.

The cochlea of *Atoh1-rtTA*; *TetO-LacZ* mice also showed variability among the five founders: F7 = 77.0 ± 11.0%, F10 = 85.8 ± 3.3%, F12 = 84.1 ± 4.6%, F23 = 63.5 ± 6.8%, and F26 = 78.7 ± 2.8% (LacZ^{+/ve} HCs expressed as a percentage of total HCs, *n* = 3–4, Figure 3F–J′, L). There was no significant difference in the number of LacZ^{+/ve} cells between inner and outer HCs or across cochlear turns in any founder. All founders had LacZ expression in other cells besides HCs (Table 1). In F7 and F26, LacZ expression was the most

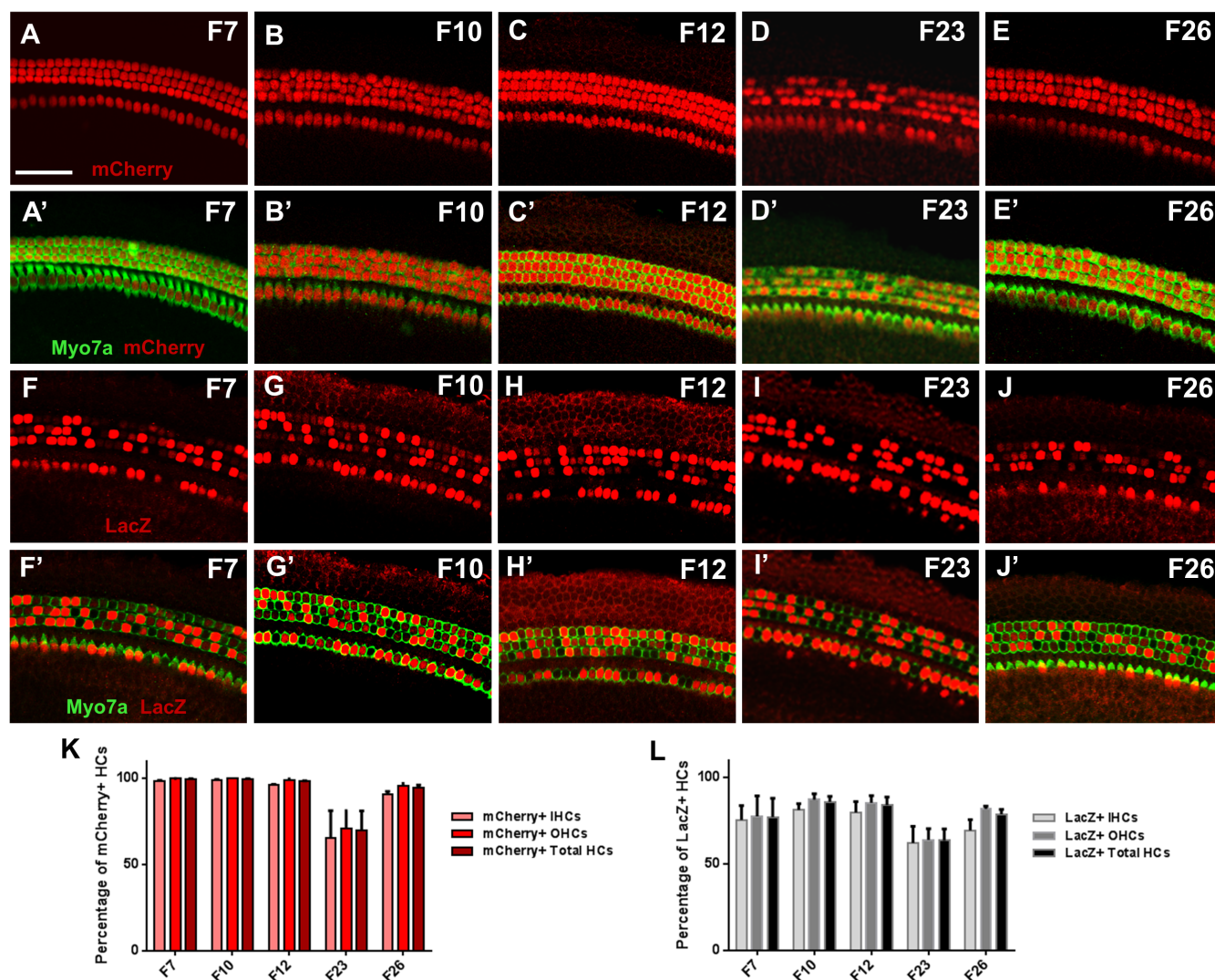


Figure 3 | *Atoh1-rtTA* activity in the cochlea. Representative confocal images of $mCherry^{+ve}$ (red) cells in the middle turn of each founder line breed with the *TetO-mCherry* reporter at P3 (A–E) and corresponding merged images where HCs were labeled with myosin VIIa (Myo7a, green) (A'–E'). Representative confocal images of $LacZ^{+ve}$ (red) cells in the middle turn of each founder line breed with the *TetO-LacZ* reporter at P3 (F–J) and corresponding merged images where HCs were labeled with Myo7a (green) (F'–J'). (K) Percentage of $Myo7a^{+ve}$ cells that express mCherry in each founder line, normalized to the number of total $Myo7a^{+ve}$ cells in the same region. (L) Percentage of $Myo7a^{+ve}$ cells that express LacZ in each founder line, normalized to the number of total $Myo7a^{+ve}$ cells in the same region. Data are expressed as mean \pm S.E.M for an n of 3–4. IHC, inner hair cells; OHC, outer hair cells. Scale bar: 50 μ m.

specific to HCs with both lines having few $LacZ^{+ve}$ IPhCs in the basal turn. There were also some $LacZ^{+ve}$ cells in the SGN region of the basal turn for F26. In F23, LacZ expression was specific to HCs only in the apical turn with a moderate amount of $LacZ^{+ve}$ IPhCs and cells in the GER in the middle turn that increased in the basal turn. Lastly, F10 had some $LacZ^{+ve}$ IPhCs and GER cells in all three cochlear turns, while F12 had an apical-to-basal gradient of increasing quantities of $LacZ^{+ve}$ IPhCs. In contrast to the consistent mCherry

intensity, LacZ intensity varied from cell to cell across all founders (Figure 3F–J').

In *Atoh1-rtTA*; *TetO-mCherry* utricles, the percentage of total $mCherry^{+ve}$ HCs were: F7 = $78.2 \pm 0.6\%$, F10 = $85.6 \pm 0.5\%$, F12 = $73.7 \pm 4.1\%$, F23 = $48.6 \pm 6.4\%$, and F26 = $79.4 \pm 3.6\%$ ($mCherry^{+ve}$ HCs expressed as a percentage of total HCs, $n = 3$, Figure 4A–E, K). Several of the *Atoh1-rtTA*; *TetO-mCherry* founders showed a significant difference in the percentage of $mCherry^{+ve}$ HCs

Table 1 | Summary of *Atoh1-rtTA* activity in non-hair cells of the cochlea

	F7	F10	F12	F23	F26
TetO-mCherry	Base = Few GER, Several IPhC	Base = Few GER, Several IPhC	Middle = Few IPhC Base = Moderate IPhC	Base = Few GER, Several IPhC	N/A
TetO-LacZ	Base = Few IPhC	Apex, Middle, and Base = Few IPhC and GER	Apical to basal gradient of increasing IPhCs	Middle = Few IPhC and GER Base = Moderate IPhC and GER	Base = Few IPhC, Few SGC

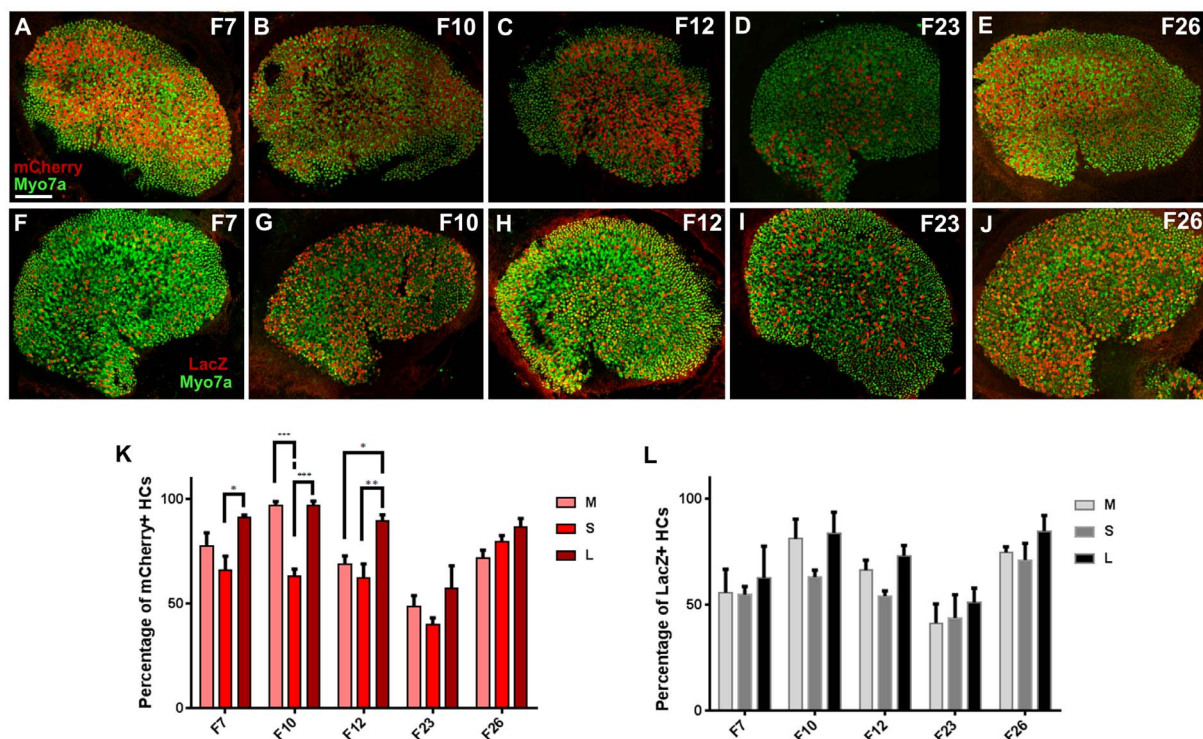


Figure 4 | *Atoh1-rtTA* activity in the utricle. (A–E) Representative confocal images of mCherry⁺ve (red) cells and Myo7a-labeled HCs (green) in the utricle for each founder line bred with the *TetO-mCherry* reporter at P3. (F–J) Representative confocal images of LacZ⁺ve (red) cells and Myo7a-labeled HCs (green) in the utricle for each founder line bred with the *TetO-LacZ* reporter at P3. (K) Percentage of Myo7a⁺ve cells that express mCherry in utricular regions of each founder line, normalized to the number of total Myo7a⁺ve cells in the same region. (L) Percentage of Myo7a⁺ve cells that express LacZ in utricular regions of each founder line, normalized to the number of total Myo7a⁺ve cells in the same region. Data are expressed as mean \pm S.E.M for an n of 3. Statistical differences between regions were obtained using a two-way ANOVA followed by a Tukey's posthoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. M, medial; S, striolar; L, lateral Scale bar: 100 μ m.

in different regions of the utricle (Figure 4K). This included a difference between striolar and lateral regions in F7, F10, and F12 and a difference between medial and striolar regions in F10. All founders had varying numbers of mCherry⁺ve SCs that were found across all regions of the utricle with F7 and F10 having the fewest and F12 having the most. Lastly, the mCherry intensity varied from cell to cell across all founders (Figure 4A–E). We also observed mCherry⁺ve HCs in the lateral and superior cristae.

In contrast, fewer numbers of labeled cells were detected in *Atoh1-rtTA*; *TetO-LacZ* utricles: F7 = 57.7 \pm 9.9%, F10 = 76.0 \pm 7.3%, F12 = 64.5 \pm 0.6%, F23 = 45.4 \pm 8.8%, and F26 = 76.8 \pm 5.8% (LacZ⁺ve HCs expressed as a percentage of total HCs, $n = 3$, Figure 4F–J, L). There was no significant difference in the number of LacZ⁺ve HCs among utricular regions, however all founder lines had LacZ⁺ve SCs with F7 and F26 having the fewest and F23 and F12 having the most. LacZ intensities also varied with all founders having a mosaic expression pattern throughout the utricle (Figure 4F–J). We also observed LacZ⁺ve HCs in the lateral and superior cristae.

During postnatal development of the cerebellum, *Atoh1* is expressed in proliferating granule neuron precursors (GNPs) of the external granule layer⁵. We therefore assessed mCherry expression in cerebella of the five *Atoh1-rtTA*; *TetO-mCherry* transgenic lines at P3, after doxycycline treatment was administered from P0–P3. We observed mCherry⁺ve GNPs in all transgenic lines with F7, F12, and F26 having the most labeled cells (Figure 5A–J'). In un-induced negative controls, which did not receive doxycycline, no mCherry⁺ve cells were observed in the cerebellum (Figure 5P–P'). This finding confirms that *Atoh1-rtTA* activity occurs in *Atoh1*-expressing cells in the cerebellum. We also examined the rest of the brain and only observed mCherry⁺ve cells in the hippocampus

of F12, F23, and F26 (Figure 5K–O'). No other brain region had mCherry expression. This misexpression of mCherry in the hippocampus of three founder lines matches the eGFP expression seen in *Atoh1-eGFP* mice which also use the *Atoh1* enhancer element to drive expression of eGFP¹⁶. However *Atoh1-eGFP* mice have eGFP expression in the cortex which was not observed in our model¹⁶.

Additional control experiments were performed with *Atoh1-rtTA* mice of the F7 lineage which showed strong and consistent rtTA activity in cochlear and utricular HCs and was HC-specific in apical and middle turns of the cochlea. These included measuring transient expression of TetO reporters in inner ear tissues and the hearing ability of mice containing the newly generated *Atoh1-rtTA* allele.

The Tet-On system is designed to produce transient changes in gene expression since the rtTA protein can no longer bind to TetO and activate transcription once doxycycline is removed^{2,3}. To measure transient effects in our system, we used *Atoh1-rtTA* mice of the F7 lineage bred with *TetO-mCherry* or *TetO-LacZ* reporter mice and administered doxycycline from P0–P3. The cochlea and utricle were analyzed at P7, P21, and 6 weeks of age. In *Atoh1-rtTA*⁺ve; *TetO-LacZ*⁺ve mice, the majority of cochlear and utricular HCs retained expression of LacZ at all ages tested and no HC loss was detected (Figure 6A–B). A similar expression pattern of mCherry⁺ve HCs was seen in *Atoh1-rtTA*⁺ve; *TetO-mCherry*⁺ve mice. However outer HC loss was detected at P21 which worsened at 6 weeks of age, but inner HCs and utricular HCs remained intact (Figure 6C–E). Since the Tet-On system requires the presence of both the rtTA protein and doxycycline for gene expression to occur (Figure 1A) and the *Atoh1-rtTA* transgene we generated uses the *Atoh1* enhancer element to drive expression of both rtTA and hPLAP (Figure 1B), we performed alkaline phosphatase staining in the cochlea and utricle at 6 weeks

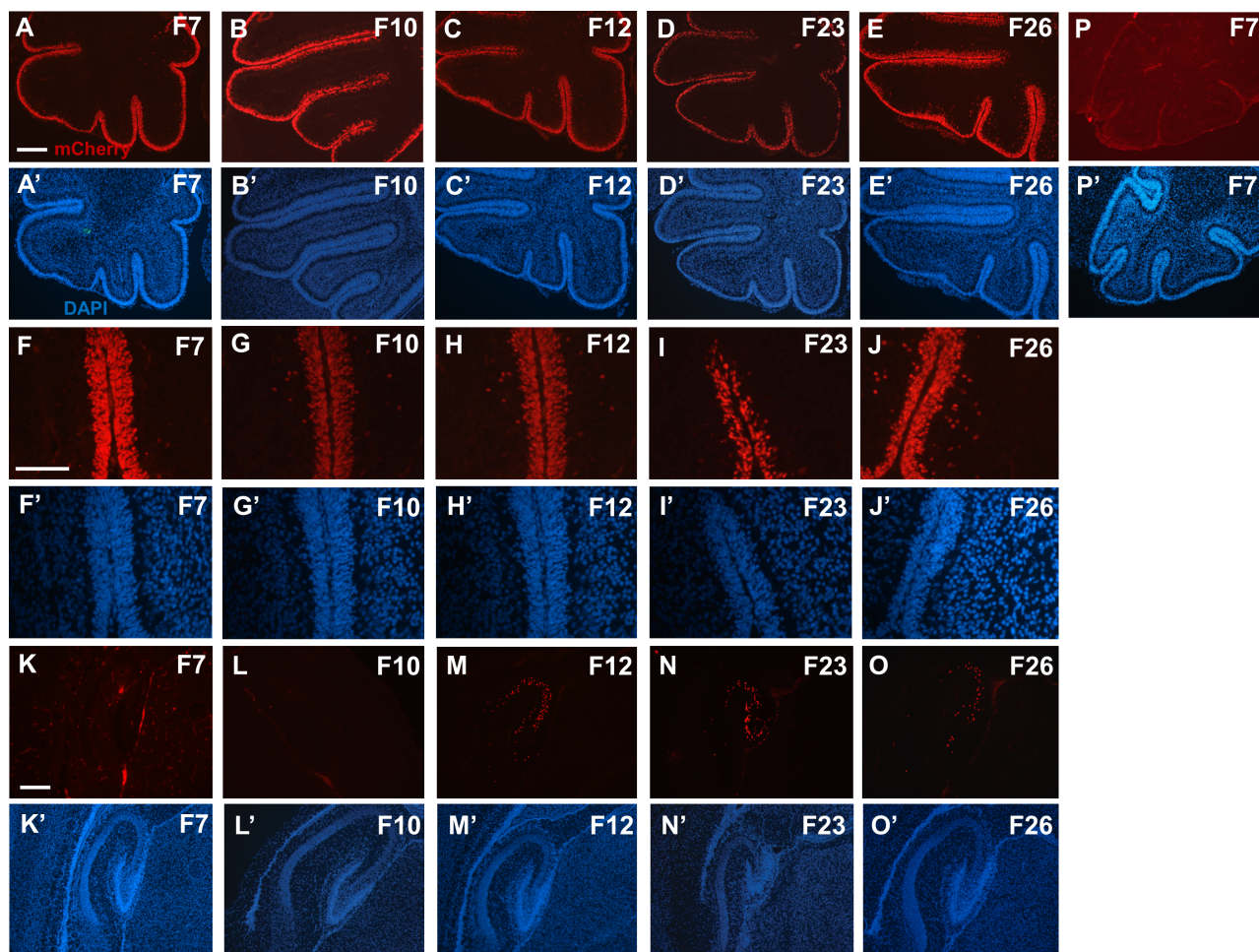


Figure 5 | *Atoh1-rtTA* activity in the cerebellum and hippocampus. Representative images of mCherry⁺ (red) cells in the cerebella (A–J') and hippocampus (K–O') of each founder line at P3 after doxycycline administration from P0–P3. (P–P') un-induced control images of *Atoh1-rtTA*(F7)⁺; *TetO-LacZ*⁺ cerebella without doxycycline induction. Nuclei are labeled by DAPI (blue). Scale bars: 200 μ m.

of age to determine whether expression of the transgene persists. Faint alkaline phosphatase staining was present in the utricle of *Atoh1-rtTA*⁺ mice from the F7 lineage (Figure 6G) suggesting that the transgene is still expressed at 6 weeks of age. However no staining was observed in the cochlea (Figure 6F).

Finally we measured the hearing ability of *Atoh1-rtTA* mice using evoked auditory brainstem response (ABR) to investigate whether the *Atoh1-rtTA* transgene had any negative effects on cochlear function. ABR was performed on 3 month old *Atoh1-rtTA*⁺; *TetO-mCherry*⁺ mice of the F7 lineage without administration of doxycycline and littermate controls that lacked both the *rtTA* and *mCherry* alleles. There was no significant difference in ABR responses between groups at any of the frequencies tested (Figure 7). Thus we conclude that *Atoh1-rtTA* mice have normal hearing and the *Atoh1-rtTA* transgene had no effect on genes required for hearing function.

Discussion

We have created a new transgenic mouse model using the tetracycline-inducible system to target HCs in the inner ear and provided a thorough characterization of rtTA activity in five founder lines using two *TetO-reporter* alleles. Differences in rtTA activity among founders were expected since the *Atoh1-rtTA* mouse lines are transgenics. Thus each founder likely has a different insertion site and a different copy number of the transgene. In summary, F7, F10, F12, and F26 showed rtTA activity in the majority of cochlear HCs (94–99% with *TetO-mCherry* and 77–85% with *TetO-LacZ*), while

F23 was less robust, labeling 70% of HCs with *TetO-mCherry* and 64% with *TetO-LacZ*. F26 was the most specific to HCs, but had highly variable LacZ intensity levels which may suggest a lower level of rtTA expression. LacZ intensity levels were more robust in F7, F10, and F12, but *Atoh1-rtTA* activity was also detected in other cell types. F7 showed non-HC reporter expression only in the basal turn, while F10 and F12 labeled other cells in all turns of the cochlea. Therefore F7 provides strong and consistent rtTA activity in cochlear HCs and was HC-specific in apical and middle turns of the cochlea.

In control experiments for basal activity of the reporter lines and leakiness of the un-induced *Atoh1-rtTA* allele, there was a similar expression pattern of mCherry⁺ cells in the SGN of the cochlea, mCherry⁺ cells in the utricular stroma, as well as some LacZ⁺ SCs in the utricle. The presence of reporter⁺ cells in the absence of the rtTA protein has been reported previously in other organ systems¹⁵ and thus these reporter⁺ cells can be ignored in the characterization of the founder lines since they are not related to *Atoh1-rtTA*. The *TetO-mCherry* and *TetO-LacZ* reporter lines are still useful to measure *Atoh1-rtTA*-driven activity since no HCs were labeled in the cochlea or utricle and only a few SCs were labeled by LacZ in the utricle. However we observed a small percentage of LacZ⁺ HCs in the medial region of the utricle in un-induced *Atoh1-rtTA*⁺; *TetO-LacZ*⁺ mice from the founder 7 lineage suggesting that a few utricular HCs can express LacZ without doxycycline. Yet no labeled HCs were observed when the *TetO-mCherry* reporter was used in un-induced negative control experiments. Taken together this data

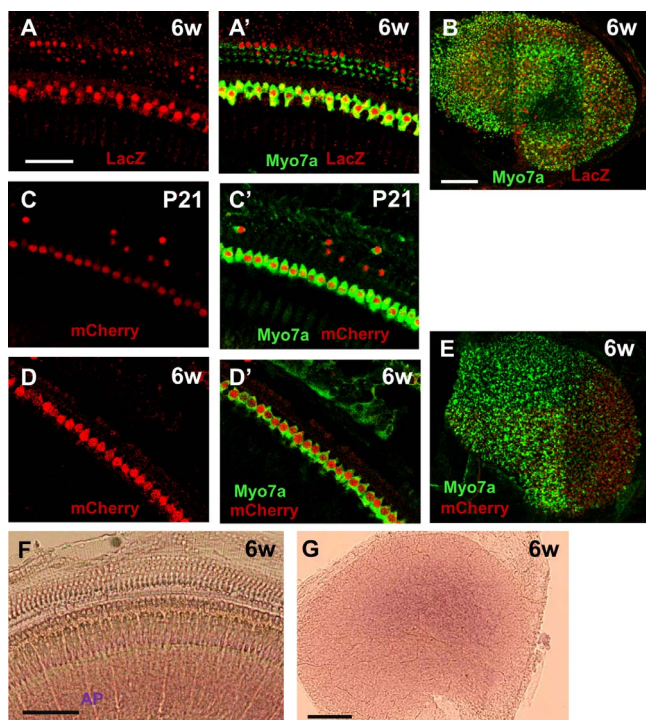


Figure 6 | TetO-reporter expression was still present at 6 weeks of age. Representative confocal images of LacZ⁺ve (red) cells in the middle cochlear turn (A) and utricle (B) of *Atoh1-rtTA(F7)*^{+/ve}; *TetO-LacZ*^{+/ve} mice at 6 weeks of age after doxycycline treatment ended at P3. (A') is a merged image with HCs labeled by myosin VIIa (*Myo7a*, green). Representative confocal images of mCherry⁺ve (red) cells in the middle cochlear turn of *Atoh1-rtTA(F7)*^{+/ve}; *TetO-mCherry*^{+/ve} mice at P21 (C) and 6 weeks of age (D) and in the utricle at 6 weeks of age (E) after doxycycline treatment ended at P3. (C', D') are merged images with HCs labeled by myosin VIIa (*Myo7a*, green). Representative alkaline phosphatase (AP) staining images at 6 weeks of age in the cochlea (F) and utricle (G) of *Atoh1-rtTA* mice from the F7 lineage. Scale bars: 50 μ m in (A–A'), (C–D) and 100 μ m in (B, E–G).

illustrates the importance of using more than one reporter line to characterize a new Tet-On mouse line.

In the cochlea, several founder lines showed mCherry or LacZ expression in cells other than HCs including IPhCs and cells in the GER. This may have been caused by a low level of *Atoh1* expression when doxycycline was administered or by transgene insertion effects. Others have shown that *Atoh1* is expressed in SCs, although this expression becomes undetectable by embryonic day 17^{17,18}. Therefore, some IPhCs at P0–P3 may still have *Atoh1* expression that is below the level of detection. However the cochlea matures in a gradient from base to apex^{19–22} with *Atoh1* expression persisting for the longest period of time in the apex. In contrast we observed more mCherry⁺ve and LacZ⁺ve IPhCs in middle and basal turns which does not match this pattern. In addition not all founder lines showed mCherry⁺ve IPhCs with the same doxycycline induction paradigm. Thus a more likely explanation is that local regulatory elements located near the *Atoh1-rtTA* transgene insertion site could have caused expression of rtTA in IPhCs.

In addition several of the founders showed a significant difference in the percentage of mCherry⁺ve HCs in different regions of the utricle. These regional differences may have been caused by differences in the timing of cell cycle exit and subsequent HC differentiation²³, by differences in postnatal addition of HCs among regions²⁴, or by transgene insertion effects. However no regional differences were observed when using the *TetO-LacZ* reporter making transgene insertion effects the most likely explanation. Similarly three of the five founder lines had mCherry⁺ve cells in the hippocampus where *Atoh1* is not normally

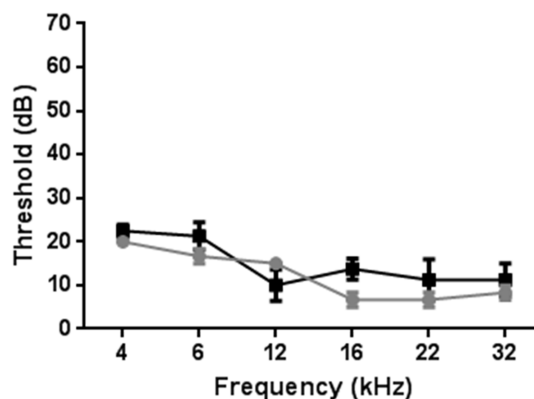


Figure 7 | Hearing ability of *Atoh1-rtTA(F7)* mice. ABR was performed on 3 month old *Atoh1-rtTA(F7)*^{+/ve}; *TetO-mCherry*^{+/ve} mice without administration of doxycycline (grey circles) and littermate controls that lacked both the *rtTA* and *mCherry* alleles (black squares). Data are expressed as mean \pm S.E.M for an *n* of 3–4. There were no statistical differences between groups.

expressed. This was also observed in the *Atoh1-eGFP* transgenic line¹⁶ and was likely caused by transgene insertion effects.

Since the tetracycline-inducible system is designed to produce transient changes in gene expression and the half-life of LacZ is 24–48 hours in mammalian cells²⁵, it is surprising that both reporters remained robustly expressed at 6 weeks of age when doxycycline treatment ended at P3. (The half-life of mCherry is unknown.) The Tet-On system requires the presence of both the rtTA protein and doxycycline for expression of TetO-driven genes. Others have demonstrated that endogenous *Atoh1* is rapidly downregulated in the inner ear during the first postnatal week⁴. However expression of eGFP was observed in cochlear HCs at 6 weeks of age in the *Atoh1-eGFP* mouse²⁶ which uses the same *Atoh1* enhancer element to drive expression of eGFP as our *Atoh1-rtTA* transgene. Using alkaline phosphatase staining, we detected weak expression of the *Atoh1-rtTA* transgene in utricular HCs at 6 weeks of age, but not in cochlear HCs. In the *Atoh1-rtTA* transgene, hALAP expression is controlled by IRES-mediated translation while rtTA expression is controlled by 5' cap-dependent translation. Others have shown that IRES-mediated translation is less efficient²⁷ thus it is possible that rtTA is expressed in cochlear HCs when hALAP is below the level of detection. Another possible explanation for the persistent expression of the TetO-reporters is that the rtTA/doxycycline/TetO complex may be very stable and resistant to degradation.

In support of our findings for persistent TetO-reporter expression, a *Sox10-rtTA* allele was recently characterized in the inner ear using the *TetO-LacZ* reporter and LacZ expression was also observed at 8 weeks of age after cessation of doxycycline treatment at P2²⁸. Analogous to the blood-brain barrier, the blood-labyrinth barrier functions to protect the inner ear from substances circulating in the blood. Since the blood-labyrinth barrier develops in rodents during the first two postnatal weeks²⁹, it is possible that a large amount of doxycycline entered the cochlear and vestibular systems before it was formed and then became trapped. In addition doxycycline is heavily protein bound (>90%)³⁰ which could contribute to a long half-life in inner ear tissues and other drugs have been shown to remain in the inner ear for a much longer time than in serum, brain, or other organs³¹. In support of our hypothesis, doxycycline treatment administered at P14 failed to induce rtTA-driven gene expression in the inner ear using the *Rosa26-loxP-stop-loxP-rtTA-IRES-eGFP* mouse line³². While the *Sox10-rtTA* model showed decreased rtTA-driven activity when doxycycline was administered at P14, no effect was observed at P28 unless doxycycline was combined with the loop diuretic furosemide, which alters the properties of the blood-laby-



rinth barrier²⁸. In addition others have shown that tetracycline, which is structurally similar to doxycycline, cannot cross the blood-brain barrier³¹. Taken together, these data suggest that doxycycline has limited ability to cross the blood-labyrinth barrier and supports our hypothesis that the persistent expression of rtTA-driven reporter expression in *Atoh1-rtTA* mice is caused by sequestration of doxycycline in inner ear tissues. In light of our observations, it is uncertain whether transient gene expression occurred when other *rtTA* alleles were used in inner ear studies such as the *Rosa26-loxP-stop-loxP-rtTA-IRES-eGFP* allele^{32–34} and the *Pax2-rtTA* allele³⁵. To determine if similar long lasting effects occur, each *rtTA* allele should be tested with a *TetO-reporter* line specifically in inner ear tissues using the same doxycycline induction paradigm used in experimental studies.

It was also surprising that outer HC loss occurred in *Atoh1-rtTA^{+/ve}; TetO-mCherry^{+/ve}* mice at P21 and 6 weeks of age. Outer HCs are known to be more susceptible to death than inner HCs when exposed to multiple types of insults including noise or ototoxic drugs^{36–40}. However to our knowledge the death of outer HCs by expression of a reporter gene has not been previously reported. This cell death may have occurred due to a large amount of mCherry mRNA or protein that accumulated in the cell between P3 and P21 which overwhelmed the transcriptional/translational machinery at the detriment of genes required for outer HC survival. Alternatively the mCherry protein could directly induce cell death pathways in outer HCs. In addition, in the *TetO-mCherry* allele, *mCherry* was knocked into the endogenous *Colla1* locus¹² and thus only one copy of *Colla1* is expressed which may contribute to the outer HC death we observed. However no outer HC loss was observed in *TetO-mCherry* mice without doxycycline induction at 3 months of age as demonstrated by the normal ABR measurements we obtained. Thus one copy of *Colla1* is not toxic but the combination of one copy of *Colla1* with mCherry expression could be.

In conclusion, the *Atoh1-rtTA* mouse produces rtTA activity in the majority of HCs in the cochlea and utricle and in GNP cells of the cerebellum during neonatal ages. Therefore it is a valuable tool for future experiments especially when used in combination with existing Cre and CreER mouse models to modulate expression of multiple genes in two distinct cell types or in the same cell type at different times. While transient control of gene expression was not achieved with the *Atoh1-rtTA* mouse model, this is likely a property of the inner ear's inability to remove doxycycline efficiently and has implications for other tetracycline-inducible mouse models used in inner ear research. Our studies also highlight potential problems with long term expression of the mCherry reporter in outer HCs.

Methods

Generation of *Atoh1-rtTA* transgenic mice. The *Atoh1-CreER-IRES-hPLAP-SV40-polyA* construct⁸ was modified to replace the *CreER* sequence with a *Tet-On 3G rtTA* sequence (Clontech, cat# 631159). Founder lines were obtained and Southern blot was performed as previously described⁸. Briefly, Southern blot analysis used the XbaI restriction enzyme and a probe that recognizes the *Atoh1* enhancer sequence. Genotyping primers were designed with the forward primer in the *Atoh1* enhancer element and the reverse primer in the *rtTA* sequence (5' TCGTCAAGGGCGTCGGTCCG 3' and 5' CTGGCAGAGGCTCTGGCC 3').

Characterization of *Atoh1-rtTA* transgenic mice. Expression of the transgene was detected at P0 using alkaline phosphatase staining (NBT/BCIP ready to use tablets (Roche cat#11697471001)). Temporal bones were post-fixed for 1 hour in 4% paraformaldehyde, then transferred to 10 mM PBS. After dissection, samples were heated at 65°C for 1 hour in 10 mM PBS then cooled to room temperature before the addition of the alkaline phosphatase staining reagent (1 NBT/BCIP tablet dissolved in 10 ml double-distilled water). The enzymatic reaction occurred at room temperature in the dark for 30 to 90 minutes followed by washes in PBS and mounting to slides.

To characterize rtTA activity, we bred five founder lines with two different TetO-reporter lines: *TetO-mCherry* (The Jackson Laboratory, stock #14592) and *TetO-lacZ* (The Jackson Laboratory, stock #2621). TetO reporter expression was induced with doxycycline administered in the food (2000 mg/kg, Harlan Laboratories) to the nursing mother from P0–P3 as well as an injection of doxycycline (100 mg/kg, intraperitoneal (IP), Fisher) administered to the pups at P1. Samples were collected at P3 and post-fixed overnight in 4% paraformaldehyde. Routine immunostaining

procedures⁴¹ were used with the following primary antibodies: anti-βgal (1 : 500, cat #AB9361 Abcam) and anti-myosin VIIa (1 : 200, cat #25-6790 Proteus BioSciences). All secondary antibodies were Alexa-conjugated from Invitrogen and were used at a 1 : 1000 dilution. mCherry was directly visualized on 12 μm brain cryosections stained with DAPI (1 : 1000, Invitrogen). Images of the cochlea and utricle were taken using a Zeiss LSM 700 or a Leica SP5 confocal microscope. Images of the brain were taken using a Zeiss Axioskop 2 plus with an AxioCanHRC camera. All animal work was performed in accordance with the guidance and approval from the Institutional Animal Care and Use Committee at St. Jude Children's Research Hospital and Southern Illinois University, School of Medicine.

Quantification. Inner ear HCs were identified by myosin VIIa labeling. In the cochlea, the number of LacZ^{+/ve} HCs was counted in two, 200 μm regions, randomly chosen from each cochlear turn. Since the mCherry reporter had more robust expression, the entire cochlea was scanned and mCherry negative HCs were counted. For the utricle, the number of labeled HCs in 50 μm × 50 μm squares was counted at nine locations spaced along the anterior/posterior axis of the medial, striolar, and lateral regions as previously described⁴². Reporter^{+/ve} cells were averaged across turns or utricle regions and expressed as a percentage of total HCs per organ. The *n* value throughout the paper refers to one ear per animal.

Auditory Brainstem Response. Mice were anesthetized using Avertin (600 mg/kg, IP) and ABR was performed as previously described⁴¹.

Statistical Analysis. All data are presented as mean ± S.E.M. Statistical analyses were performed using Graphpad Prism 6.02 (Graphpad Software Inc.). Comparison of labeled HCs from different groups (i.e. inner vs. outer HCs, across cochlear turns, and among utricle regions) and ABR threshold values across frequencies were determined using a two-way ANOVA followed by either Sidak's or Tukey's multiple comparisons test.

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Author contributions

B.C.C., J.A.D., F.Z., M.F.R. and J.Z. developed the approach; B.C.C., J.A.D., J.B. and F.Z. performed experiments and data analysis; B.C.C., J.B., F.Z., M.F.R. and J.Z. wrote or edited the manuscript text. All authors reviewed the manuscript prior to submission.

Additional information

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