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Quantitative Analysis of Supporting Cell Subtype Labeling among CreER Lines in the Neonatal Mouse Cochlea

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Abstract

Four CreER lines that are commonly used in the auditory field to label cochlear supporting cells (SCs) are expressed in multiple SC subtypes, with some lines also showing reporter expression in hair cells (HCs). We hypothesized that altering the tamoxifen dose would modify CreER expression and target subsets of SCs. We also used two different reporter lines, *ROSA26^{tdTomato} and CAG-eGFP,* to achieve the same goal. Our results confirm previous reports that *Sox2^{CreERT2}* and *Fgfr3-iCreER^{T2}* are not only expressed in neonatal SCs, but also in HCs. Decreasing the tamoxifen dose did not reduce HC expression for *Sox2^{CreERT2}*, but changing to the *CAG-eGFP* reporter decreased reporter-positive HCs 7-fold. However, there was also a significant decrease in the number of reporter-positive SCs. In contrast, there was a large reduction in reporter-positive HCs in *Fgfr3-iCreER^{T2}* mice with the lowest tamoxifen dose tested, yet only limited reduction in SC labeling. The targeting of reporter expression to inner phalangeal and border cells was increased when *Plp-CreER^{T2}* was paired with the *CAG-eGFP* reporter, however the total number of labeled cells decreased. Changes to the tamoxifen dose or reporter line with *Prox1^{CreERT2}* caused minimal changes. Our data demonstrate that modifications to the tamoxifen dose or the use of different reporter lines may be successful in narrowing the numbers and/or types of cells labeled, but each CreER line responded differently. When the *ROSA26^{tdTomato}* reporter was combined with any of the four CreER lines, there was no difference in the number of tdTomato-positive cells after one or two injections of tamoxifen given at birth. Thus tamoxifen-mediated toxicity could be reduced by only giving one injection. While the

CAG-eGFP reporter consistently labeled fewer cells, both reporter lines are valuable depending on the goal of the study.

Keywords: Plp-CreER, Sox2-CreER, Fgfr3-iCreER, Prox1-CreER, mouse genetics, Cre/loxP

Introduction

The mammalian cochlea is a highly organized structure containing sensory hair cells (HCs) surrounded by supporting cells (SCs), which are divided into subtypes based on location (Figure 1A). Cells of the greater epithelial ridge (GER), inner phalangeal cells (IPhCs), and border cells (BCs) are located medial to inner HCs (IHCs). Inner pillar cells (IPCs) and outer pillar cells (OPCs) separate IHCs and outer HCs (OHCs). Deiters' cells (DCs) surround OHCs with Hensen cells (HeCs) and Claudius cells (CCs) located lateral to the last row of OHCs (Raphael and Altschuler, 2003). While HCs and SCs are distinct cell types, they are derived from the same pool of progenitor cells (Yang et al., 2010; Cai et al., 2013; Driver et al., 2013).

Much research has focused on HCs including studies of planar cell polarity (Denman-Johnson and Forge, 1999; Lewis and Davies, 2002), mechanotransduction (Hudspeth and Corey, 1977; LeMasurier and Gillespie, 2005), otoprotection (Huang et al., 2000; Lefebvre et al., 2002), and regeneration (Corwin and Warchol, 1991; Rubel et al., 1995). While less is known about SCs, several functions have been described including protection of HCs from excitotoxicity (Spicer and Schulte, 1996; Kikuchi et al., 2000;

Boettger et al., 2002; Furness et al., 2002), phagocytosis of dying HCs (Abrashkin et al., 2006; Taylor et al., 2008; Anttonen et al., 2014), and sealing the epithelial surface after HC death (McDowell et al., 1989; Raphael and Altschuler, 1991a, b). Studies have also shown that SCs release factors which promote the formation of synapses and survival of HCs and auditory nerves (Pirvola et al., 1992; Flores-Otero et al., 2007; Sugawara et al., 2007; Tritsch et al., 2007; Gomez-Casati et al., 2010; Tritsch and Bergles, 2010; Zuccotti et al., 2012). In the neonatal mouse cochlea, SCs are the source of regenerated HCs (Bramhall et al., 2014; Cox et al., 2014). These functions have been attributed to SCs as a group, with little known about the function of individual SC subtypes, primarily because tools are limited.

Genetically-modified mouse models that target cochlear cells have provided critical tools to increase our understanding of inner ear physiology and response to damage. The CreER/loxP system, which allows cell type-specific and temporal control of gene expression, has been used to delete or over-express genes, to label cell populations for fate-mapping, and to ablate specific cell types. Here, we used four CreER alleles known to target broad populations of SCs. The CreER enzyme driven by both the *Fgfr3-iCreER^{T2}* and *Sox2^{CreERT2}* lines is also expressed in neonatal HCs (Cox et al., 2012; Bramhall et al., 2014; Walters et al., 2015). We hypothesized that modifying the tamoxifen induction paradigm and/or changing the paired reporter line would label subpopulations of SCs as well as decrease HC labeling in *Fgfr3-iCreER^{T2}* and *Sox2^{CreERT2}* lines. This hypothesis is based, in part, on previous studies showing that the *ROSA26^{tdTomato}* reporter produces a brighter fluorescent protein that is more stable than the fluorophores produced by other reporter lines such as *CAG-eGFP* or *Rosa26^{LacZ}*

(Madisen et al., 2010). With the *Fgfr3-iCreER^{T2}* line, a 10-fold reduction in tamoxifen or changing to the *CAG-eGFP* reporter produced a large reduction of labeled OHCs. However, the number of reporter-positive SCs also decreased. Reducing the tamoxifen dose in *Sox2$^{CreERT2+/-}$::ROSA26$^{tdTomato/+}$* mice produced a minimal effect. However, changing to the *CAG-eGFP* reporter reduced reporter-positive HCs and SCs. *Plp-CreER^{T2}* is largely expressed in IPhCs/BCs of the neonatal cochlea with some PCs/DCs also labeled. Reduction in tamoxifen dose had a minimal effect when the *ROSA26^{tdTomato}* reporter was used. However, using the *CAG-eGFP* reporter with a reduced tamoxifen dose, fewer PCs/DCs were labeled, but this also decreased the number of reporter-positive IPhCs/BCs. As previously described, *Prox1^{CreERT2}* is limited to PCs/DCs in the neonatal cochlea, with relatively fewer IPCs labeled. Alterations in tamoxifen dose caused few changes in labeled cells.

Materials and Methods

Mice

Sox2^{CreERT2} (stock #17593; Arnold et al., 2011), *Plp-CreER^{T2}* (stock # 5975; Doerflinger et al., 2003), and *ROSA26$^{CAG\text{-}loxP\text{-}stop\text{-}loxP\text{-}tdTomato}$ (ROSA26^{tdTomato})* mice, also referred to as Ai14, (stock #7914; Madisen et al., 2010) were obtained from The Jackson Laboratory (Bar Harbor, ME). *Fgfr3-iCreER^{T2}* mice (Rivers et al., 2008; Young et al., 2010) were provided by Dr. William Richardson (University College London, UK), *Prox1^{CreERT2}* mice (Srinivasan et al., 2007) were provided by Dr. Guillermo Oliver (St. Jude Children's Research Hospital, Memphis, TN) and *CAG-loxP-stop-loxP-eGFP (CAG-eGFP)* mice (Nakamura et al., 2006) were provided by Dr. Jeffery Robbins

(Cincinnati Children's Hospital, Cincinnati, OH). Genotyping for all mouse lines was performed by Transnetyx, Inc. (Cordova, TN). Mice of both genders were used and all animal work was performed in accordance with approved animal protocols from the Institutional Animal Care and Use Committee at Southern Illinois University School of Medicine.

Tamoxifen Injections

CreER recombination was induced by intraperitoneal (IP) injections of tamoxifen (Sigma-Aldrich – St. Louis, MO) dissolved in 100% corn oil. Mice received one injection per day on either postnatal day (P) 0, P1, or on both P0 and P1 with the dose per injection ranging from 0.3 mg/40 g to 5 mg/40 g. Stock solutions were diluted so that the total volume of each injection remained the same. To measure CreER leakiness, samples that were CreER-positive and reporter-positive but did not receive tamoxifen were analyzed as controls. Samples were evaluated between P5-P7.

Immunostaining

Samples were post-fixed in 4% paraformaldehyde overnight (Polysciences, Inc – Warrington, PA) and subsequently stored in 10 mM Phosphate Buffered Saline (Sigma-Aldrich – St. Louis, MO) at 4° C. Cochleae were dissected into apical, middle, and basal sections using a whole-mount or surface preparation method. Routine immunostaining was performed on free-floating cochlear turns as previously described (Montgomery and Cox, 2016) with the following primary antibodies: anti-myosin VIIa (1:200, cat #25-6790, Proteus BioSciences – Ramona, CA), anti-Sox2 (1:500, cat #sc-17320, Santa Cruz – Dallas, TX), anti-GFP (1:1000, cat #ab13970, Abcam –

Cambridge, MA), and anti-GFP conjugated to Alexa 488 (1:50 cat#A21311, Invitrogen – Grand Island, NY). All secondary antibodies were Alexa-conjugated from Invitrogen (Waltham, MA) and were used at a 1:1000 dilution. Images were taken using a Leica SP5 confocal microscope and image analysis was performed using Leica LAS AF LITE software.

Cell counts

HCs and SCs were identified by immunostaining for myosin VIIa and Sox2, respectively. Myosin VIIa is expressed in the cytoplasm of HCs and Sox2 is expressed in SC nuclei, while both tdTomato and eGFP are expressed throughout the cell in both cytoplasm and nuclei. SC subtypes that expressed either the tdTomato or eGFP reporter were quantified in two randomly chosen 200 µm regions per cochlear turn, averaged, and expressed as a percentage of labeled cells compared to total cells. For the *Fgfr3-iCreER^{T2}* and *Sox2^{CreERT2}* lines, all labeled SC subtypes were pooled into one value. For the *Plp-CreER^{T2}* and *Prox1^{CreERT2}* lines, SC subtypes were counted individually for IPCs, OPCs, DCs, and IPhC/BCs.

Reporter labeling in HCs was detected in *Fgfr3-iCreER^{T2}* and *Sox2^{CreERT2}* lines. For the *ROSA26^{tdTomato}* reporter, the entire organ of Corti was imaged, measured, and divided into six equal sections for quantification of tdTomato-positive HCs to determine if a gradient of tdTomato expression was present. For the *CAG-eGFP* reporter, eGFP-positive HCs were quantified in 2 sections: a 250 µm region at the most apical tip (where the majority of eGFP-positive cells were found) and the rest of the organ of

Corti. Data for both reporter lines are expressed as a percentage of the reporter-positive HCs compared to the total HCs within each region.

Statistical analysis

All data are presented as mean ± SEM. One-way or two-way ANOVA followed by Tukey's post-hoc tests, Student's *t*-test, and Pearson's correlations were performed using Graphpad Prism 6.0 2 (Graphpad Software Inc – La Jolla, CA).

Results

The CreER/loxP system allows cell-type specific gene expression through the use of cell-type specific promoters that drive expression of the Cre enzyme. Temporal control is achieved by fusing a modified estrogen receptor (ER) to Cre, which restricts the CreER protein to the cytoplasm. Only in the presence of tamoxifen will CreER translocate to the nucleus for excision of loxP sites (Feil et al., 1996; Hayashi and McMahon, 2002). Using four independent CreER mouse lines (*Fgfr3-iCreER^{T2}, Sox2^{CreERT2}, Plp-CreER^{T2}*, and *Prox1^{CreERT2}*) that have previously been shown to label broad populations of SCs in the neonatal cochlea, we sought to label subpopulations of SCs by altering the tamoxifen induction paradigm and/or reporter line.

The *ROSA26^{tdTomato}* reporter line was developed to improve the fluorescent labeling of cells in a Cre-dependent manner over standard reporter lines such as *CAG-eGFP*. It

uses two ubiquitously expressed promoters (*ROSA26* and *CAG*) to drive expression of tdTomato, as well as the woodchuck hepatitis virus post-transcriptional regulatory element (*WPRE*) to enhance mRNA stability. Endogenous fluorescence of tdTomato is ~2.8 times brighter than eGFP and is readily detected, whereas as detection of eGFP often requires amplification with immunostaining prior to imaging (Madisen et al., 2010; Madisen et al., 2015). Therefore different patterns of reporter labeling are seen when *ROSA26*^{tdTomato} and *CAG-eGFP* reporter lines are used with the same CreER allele and tamoxifen induction paradigm. We also tested for Cre leakage, which can occur when some of the CreER enzyme enters the nucleus in the absence of tamoxifen. To determine the leakiness of the~~se~~ four CreER lines, we performed control experiments using CreER-positive::reporter-positive mice that did not receive tamoxifen.

Fgfr3-iCreER^{T2}

Fibroblast growth factor receptor 3 (*Fgfr3*) is necessary for development of PCs in the organ of Corti (Colvin et al., 1996). At P0, *Fgfr3* expression is thought to be confined to PCs and DCs in the lateral compartment of the cochlea (Peters et al., 1993; Mueller et al., 2002; Pirvola et al., 2002; Hayashi et al., 2007). However recent single-cell RNA-seq analyses have detected a low level of *Fgfr3* expression in some HCs at P1-2 (Burns et al., 2015; Waldhaus et al., 2015). The *Fgfr3-iCreER^{T2}* mouse line is a transgenic allele, where CreER is driven by the *Fgfr3* promoter (Young et al., 2010). Previous characterization of the *Fgfr3-iCreER^{T2}* allele showed that after tamoxifen injection at neonatal ages, reporter expression was detected in the vast majority of PCs and DCs

within the organ of Corti (Cox et al., 2012). Specifically when *Fgfr3-iCreER$^{T2/+}$::ROSA26$^{tdTomato/+}$* mice were injected with tamoxifen (3 mg/40 g, IP) at both P0 and P1, 100% of PCs and DCs expressed tdTomato. However, there were also 25-75% of OHCs labeled (Cox et al., 2012). To verify this expression pattern and to establish a basis for comparison, we repeated this induction protocol in *Fgfr3-iCreER$^{T2/+}$::ROSA26$^{tdTomato/+}$* mice and found that 94.4 % ± 5.5% of PCs and DCs were labeled throughout the organ of Corti (Figure 1B, Table 1). Similar to previously published results, this dosing paradigm also showed robust labeling of OHCs (43.8% ± 2.9%; Figure 2B, Table 1). To investigate whether there was a gradient of HC labeling across cochlear turns, the organ of Corti was divided into six sections of equal length (Figure 2A) and the percentage of labeled OHCs was counted within each section. The percentage of tdTomato+ OHCs declined in an apical to basal gradient (98.5% ± 0.7% in the first segment of apex and 13.2% ± 3.1% in the last segment of the base; Pearson correlation coefficient r^2 = 0.81, P = 0.049; Figure 2B, Table 1). Three tdTomato-positive IHCs were detected in the apical tip of one sample (data not shown). In addition, no tdTomato-positive SCs or HCs were detected in controls that were not treated with tamoxifen (Figure 1B-E, 2B-E).

To attenuate HC labeling and target only PCs and DCs, Cre-mediated recombination was induced with a 10-fold lower dose of tamoxifen (0.3 mg/40 g, IP) at P1 only. The percentage of tdTomato-positive HCs was reduced to 3.9% ± 2.2% of total OHCs across the entire cochlea (Figure 2B, F-M, Table 1). Yet the apical to basal gradient remained with 14.6% ± 10.6% OHCs labeled in the first apical section and 0.2% ± 0.2%

in the most basal section (Pearson correlation coefficient r^2 = 0.81, P = 0.049; Figure 2B, F-M, Table 1). However, this dosing paradigm also reduced total PC/DC labeling to 77.7% ± 4.9% (Figure 1B, F-H"', Table 1).

Because HC labeling was still present with the reduced tamoxifen dose, *Fgfr3-iCreER^{T2}* mice were next paired with the *CAG-eGFP* reporter. Using the previously published tamoxifen induction paradigm (3 mg/40 g, IP at P0/P1) in *Fgfr3-iCreER$^{T2/+}$::CAG-eGFP$^{+/loxP}$* mice, we observed 37.8% ± 3.4% eGFP-positive PCs and DCs throughout the cochlea (Figure 1B, L-N"', Table 1). However, similar to the 0.3 mg/40 g tamoxifen paradigm in *Fgfr3-iCreER$^{T2/+}$::ROSA26$^{tdTomato/+}$* mice, 5.8% ± 2.6% eGFP-positive OHCs were still detected (Figure 3B, F-M, Table 1). Because the apical tip of the cochlea contained a large number of eGFP-positive HCs which quickly declined, the cochlea was divided into two sections for quantification: the first 250 µm of the apex and the remainder of the cochlea (Figure 3A). Upon quantification, there was a significant difference between the two regions with the largest amount of OHC labeling in the most apical section (40.5% ± 6.1% compared to 4.4% ± 2.5%; paired Student's *t* test *t*(2)= 9.822, *P* =0.0102); Figure 3B, Table 1). No eGFP-positive SCs or HCs were observed in control animals that were not treated with tamoxifen (Figure 1B, I-K, 3B-E).

Sox2^{CreERT2}

Sox2 is a transcription factor expressed in all SCs throughout the organ of Corti as well as in immature HCs (Hume et al., 2007; Dabdoub et al., 2008). Low levels of *Sox2*

expression have been detected in some HCs at P1-2 using single-cell RNA-seq analyses (Burns et al., 2015; Waldhaus et al., 2015). The *Sox2*^{CreERT2} mouse line is a knock-in allele, where CreER was inserted into the endogenous *Sox2* locus (Arnold et al., 2011). *Sox2*^{CreERT2} expression has previously been reported in both HCs and SCs in the neonatal organ of Corti (Bramhall et al., 2014; Walters et al., 2015). When paired with the *ROSA26*^{tdTomato} reporter line and tamoxifen (3 mg/40 g, IP) given at P0/P1, more than 85% of SCs and more than 50% of HCs throughout the cochlea were labeled (Walters et al., 2015). To determine whether HC labeling could be reduced, we performed a tamoxifen dose response analysis. For comparison, we repeated the dosing paradigm from Walters et al., (2015). In *Sox2*$^{CreERT2+/-}$*::ROSA26*$^{tdTomato/+}$ mice with tamoxifen (3 mg/40 g, IP) injected at P0/P1, 100% ± 0.0% of SCs from the GER to HeCs throughout the cochlea expressed tdTomato (Figure 4A, Table 1). 35.3% ± 4.8% tdTomato-positive HCs were also observed which appeared to decrease in a gradient from apical to basal turn of the cochlea (Figure 5A, Table 1). To quantify this gradient, we divided the organ of Corti into six segments of equal length as before (Figure 2A). No difference was observed between the percentage of IHCs and OHCs labeled in any of the six segments (Figure 5A). However, the percentage of tdTomato-positive HCs was 97.2% ± 1.7% in the most apical segment which declined to 2.7%± 1.2% in the most basal segment (Figure 5A, Table 1; Pearson correlation coefficient r^2 = 0.996, P = 0.00002). In addition, *Sox2*$^{CreERT2+/-}$*::ROSA26*$^{tdTomato/+}$ mice showed some Cre leakiness with approximately 20 tdTomato-positive SCs detected in the entire cochlea in controls that did not receive tamoxifen (Figure 4A-D, 5A-D).

To determine if a reduced tamoxifen dose leads to a reduced number of labeled HCs, *Sox2*$^{CreERT2+/-}$*::ROSA26*$^{tdTomato/+}$ mice were injected with tamoxifen (3 mg/40 g, IP) at P0 only. Again, 99.9% ± 0% of SCs throughout the cochlea were labeled (Figure 4A, E-G"', Table 1). There was minimal change in the percentage of tdTomato-labeled HCs (34.5% ± 4.7%) with no significant reduction seen compared to the dual injection of tamoxifen (Figure 5A, E-L, Table 1).

We next used *CAG-eGFP*$^{+/loxP}$ mice to investigate whether a different reporter would minimize HC labeling. *Sox2*$^{CreERT2+/-}$*::CAG-eGFP*$^{+/loxP}$ mice were injected with tamoxifen (3 mg/40 g, IP) at P1 only. Unlike the *ROSA26*^{tdTomato} reporter, no eGFP-positive cells were observed in controls that did not receive tamoxifen (Figure 4A, H-J, 6A-D). As predicted, there was a large reduction in the total number of eGFP-positive HCs with the majority of the labeled cells located in the apex (Figure 6A, E-L). Therefore, we quantified eGFP-positive HCs using the same parameters used for *FGFR3-iCreER*$^{T2/+}$::*CAG-eGFP*$^{+/loxP}$ samples (Figure 3A). Throughout the entire organ of Corti, only 3.2%± 0.4% HCs were labeled with eGFP (Figure 6A, Table 1), which is far less than the number of tdTomato-positive HCs observed with tamoxifen induction at either P0 only (34.5%± 4.7%) or P0/P1 (35.3%± 4.8% Figure 5A, Table 1). Within the first 250 µm of the apical tip in *Sox2*$^{CreERT2+/-}$*::CAG-eGFP*$^{+/loxP}$ mice, 44.8% ± 10.8% of HCs were eGFP-positive, however, only 1.6% ± 0.5% eGFP-positive HCs were observed throughout the remainder of the cochlea (paired Student's *t* test $t(3.967)= 2$, $P =0.0581$, Figure 6A, Table 1). Parallel to the reduction in eGFP-positive HCs, fewer SCs were labeled with eGFP throughout the cochlea (66.0% ± 5.9% compared to 100%± 0.0%

tdTomato-positive SCs with tamoxifen given at P0/P1 and 99.9% ± 0.0% tdTomato-positive SCs with tamoxifen given at P0 only; Figure 4A, K-M"', Table 1).

Plp-CreER^{T2}

Proteolipid protein (*Plp)* is expressed in oligodendrocytes and Schwann cells of the central and peripheral nervous systems (Fuss et al., 2000; Mallon et al., 2002). In the mammalian cochlea, *Plp* is expressed in PCs and DCs in the late embryonic stage (Morris et al., 2006). *Plp-CreER^{T2}* is a transgene that has previously been reported to label IPhCs and BCs, as well as some PCs and DCs in the neonatal organ of Corti (Doerflinger et al., 2003; Gomez-Casati et al., 2010; Cox et al., 2012; Mellado Lagarde et al., 2014). Specifically, *Plp-CreER$^{T2/+}$::ROSA26$^{eYFP/+}$* mice injected with tamoxifen (3 mg/40 g, IP) at P0/P1 had 47% eYFP-positive IPhs/BCs, 3.4% eYFP-positive PCs, and 5.2% eYFP-positive DCs with no statistical differences detected among cochlear turns (Liu et al., 2014). When paired with the *ROSA26^{LacZ}* reporter and tamoxifen (33 mg/kg, IP) given from P0-P7, *Plp-CreER^{T2}* was expressed only in SCs with the majority of labeled cells being IPhCs/BCs, but no quantification was given (Gomez-Casati et al., 2010). In *Plp-CreER$^{T2/+}$::Rosa26$^{tdTomato/+}$* mice given tamoxifen (3 mg/40 g, IP) at P0/P1, tdTomato expression was observed in 50% of IPhCs/BCs in the apex, 80% of IPhCs/BCs in the middle and base, and 5-10% of PCs and DCs throughout the whole cochlea (Cox et al., 2012). Similar to Cox et al. (2012), Mellado Lagarde et al., (2014) found fewer tdTomato-positive IPhCs/BCs in the apex (25.9%) compared to middle and basal turns (71.9% and 86.1%, respectively) with the same tamoxifen induction

paradigm. In order to set a standard with which to compare alternate tamoxifen dosing paradigms, we repeated the tamoxifen dose published in Cox et al., (2012) and Mellado Lagarde et al., (2014). Similar to the previous reports, there was an apical to basal gradient of increasing numbers of tdTomato-positive SCs (Figure 7A, Table 1). Additionally, fewer IPhCs/BCs expressed tdTomato in the apex (48.2% ± 4.7%) compared to the middle (79.9% ± 2.5%), and base (89.2% ± 3.7%; one-way ANOVA $F(1.313, 3.940)=71.03$, $P=0.0010$ with Tukey's multiple comparisons test: Figure 8A, Table 1). Interestingly, this paradigm also showed SC labeling lateral to IHCs in PCs and DCs. Specifically, 13.5% ± 4.6% of IPCs and 10.6% ± 2.0% OPCs were labeled throughout the organ of Corti (Figure 8A-D, Table 1). There also appeared to be a gradient for DC labeling with fewer tdTomato-positive DCs in the apex (5.8% ± 1.9%) and middle (9.8% ± 2.8%) than in the base (29.3% ± 4.7%; one-way ANOVA $F(1.193, 3.579)=39.29$, $P=0.0044$ with Tukey's multiple comparisons test; Figure 8D, Table 1).

We attempted to label only IPhCs/BCs by reducing the tamoxifen dose to one injection given at either P0 only or P1 only, while maintaining the same concentration (3 mg/40 g). Expression of tdTomato in IPhCs/BCs of *Plp-CreER*$^{T2/+}$*::Rosa26*$^{tdTomato/+}$ mice that received tamoxifen at P0 only did not differ from tamoxifen injections given at P1 only or at both P0/P1 (Figure 7E-J, 8A, Table 1). There was also no difference in the number of tdTomato-positive IPCs, OPCs, and DCs with a P0 only or P1 only injection compared to the P0/P1 injection, (Figure 7E-J, 8A-D, Table 1).

Because reduction in tamoxifen dose did not reduce labeling of PCs/DCs, we next generated *Plp-CreER$^{T2/+}$::CAG-eGFP$^{+/loxP}$* mice. When Cre recombination was induced with tamoxifen (3 mg/40 g, IP) at P0/P1, fewer IPhC/BC were labeled by eGFP than tdTomato in all turns of the cochlea (apex = 21.8% ± 10.9% eGFP-positive IPhCs/BCs compared to 48.2% ± 4.7% tdTomato-positive IPhCs/BCs; middle = 28.2% ± 2.1% eGFP-positive IPhCs/BCs compared to 79.9% ± 2.5% tdTomato-positive IPhCs/BCs; base = 38.1% ± 3.9% eGFP-positive IPhCs/BCs compared to 89.2% ± 3.7% tdTomato-positive IPhCs/BCs; two-way ANOVA $F(2, 45)=30.39$, $P<0.0001$ with Tukey's multiple comparisons test; Figure 7Q-S, 8A, Table 1). There was no difference in IPC, OPC, or DCs labeling between eGFP and tdTomato reporter lines with tamoxifen induction (3 mg/40 g, IP) at P0/P1 (Figure 7Q-S, 8B-D, Table 1).

Since minimal changes in the *Plp-CreER^{T2}* expression pattern occurred with the *CAG-eGFP$^{+/loxP}$* reporter, we also investigated a lower dose of tamoxifen to reduce PC/DC labeling. Reducing the amount of tamoxifen given to *Plp-CreER$^{T2/+}$::CAG-eGFP$^{+/loxP}$* mice to a single injection (3 mg/40 g, IP) at P0 only reduced the number of eGFP-positive cells only in specific turns of the cochlea. The eGFP-positive IPhCs/BCs in the middle turn were reduced (24.3% ± 5.9%) compared to *Plp-CreER$^{T2/+}$::Rosa26$^{tdTomato/+}$* mice (79.9% ± 2.5%; two-way ANOVA $F(6, 45)=97.06$, $P<0.0001$ with Tukey's multiple comparisons test; Figure 7N-P, 8A, Table 1). OPCs were also reduced in the middle turn (0.0% ± 0.0% eGFP-positive OPCs compared to 15.7% ± 3.4% tdTomato-positive OPCs; two-way ANOVA $F(6, 60)=8.524$, $P<0.0001$ with Tukey's multiple comparisons test) and DCs were reduced in the base (5.9% ± 4.8% eGFP-positive DCs compared to

29.3% ± 4.7% tdTomato-positive DCs; two-way ANOVA $F(6, 45)=6.313$, $P<0.0001$ with Tukey's multiple comparisons test; Figure 7N-P, 8D, Table 1). Unlike *Fgfr3-iCreER^{T2}* and *Sox2^{CreERT2}*, neither *Plp-CreER$^{T2/+}$::Rosa26$^{tdTomato/+}$* nor *Plp-CreER$^{T2+/-}$::CAG-eGFP$^{+/loxP}$* mice showed reporter expression in HCs throughout the cochlea. Finally, no tdTomato-positive or eGFP-positive cells were detected in *Plp-CreER^{T2}* control mice which did not receive tamoxifen (Figure 7A-D, K-M, 8A-D).

Prox1^{CreERT2}

Prox1 is a transcription factor that is expressed in IPCs, OPCs, and DCs throughout the neonatal organ of Corti, but is downregulated by ~P14 (Bermingham-McDonogh et al., 2006). The *Prox1^{CreERT2}* mouse line is a knock-in allele, where CreER was inserted into the endogenous *Prox1* locus (Srinivasan et al., 2007). Previously, Yu et al. (2010) characterized *Prox1^{CreERT2}* mice using *ROSA26^{LacZ}* and *ROSA26^{eYFP}* reporters and induced Cre recombination with tamoxifen injections (3 mg/40 g, IP) at P0 and P1. LacZ and eYFP expression were found to only label PCs and DCs, however a small number of cells were labeled (~13% in the apex, ~10% in the middle, and ~7% in the base, Yu et al., 2010). When the same tamoxifen induction paradigm was used with the *Rosa26^{tdTomato}* reporter, tdTomato also labeled only PCs and DCs, but many more cells were labeled (Mellado Lagarde et al., 2013). As a basis for comparison, we replicated this paradigm and quantified tdTomato expression at P6. As previously reported, *Prox1^{CreERT2}* expression was limited to PCs and DCs and no HC labeling was observed. The tdTomato labeling of PCs and DCs combined was higher in the apex (71.1% ±

1.2%) and middle (57.2% ± 1.5%) compared to basal turns (44.3% ± 3.3%; one-way ANOVA $F(1.018, 2.036)=37.08$, $P=0.0248$ with Tukey's multiple comparisons test; Figure 9A). Similar to the previous report, there was differential labeling among SC subtypes. Specifically, tdTomato was expressed in fewer IPCs (25.1%± 2.9%) compared to OPCs (70.1%± 2.6%) and DCs (76.1% ± 0.1%; one-way ANOVA $F(1.122, 2.245)=141.1$, $P=0.0045$ with Tukey's multiple comparisons test; Figure 9N, Table 1). In addition, the number of tdTomato-positive IPCs was higher in the apex (45.9% ± 1.6%) compared to the base (7.4% ± 1.2%; one-way ANOVA $F(1.021, 2.042)=35.11$, $P=0.0260$ with Tukey's multiple comparisons test; Figure 9N, Table 1). There was no difference in tdTomato-positive OPCs across turns; however, significantly fewer DCs expressed tdTomato in the base (61.7% ± 4.0%) compared to the apex (89.6% ± 3.0%;) and middle (80.7% ± 0.6%; one-way ANOVA $F(1.302, 2.603)=18.53$, $P=0.0299$ with Tukey's multiple comparisons test; Figure 9P, Table 1). Our results are quite similar to Mellado-Lagarde et al. (2013). No tdTomato-positive cells were detected in *Prox1*$^{CreERT2+/-}$*::Rosa26*$^{tdTomato/+}$ control samples that did not receive tamoxifen (Figure 9A-D, N-P).

Because so few IPCs were labeled with this induction paradigm, we attempted to increase the number of tdTomato-positive IPCs by using a higher dose of tamoxifen. In *Prox1*$^{CreERT2+/-}$*::Rosa26*$^{tdTomato/+}$ mice injected (IP) with 5 mg/40 g tamoxifen at P0/P1, we again observed fewer tdTomato-positive IPCs (24.5% ± 0.9%) than OPCs (71.4% ± 1.3%) or DCs (79.5%± 0.4%; one-way ANOVA $F(1.038, 2.077)=927.6$, $P=0.0009$ with Tukey's multiple comparisons test; Figure 9K-P, Table 1) across the whole cochlea. We

did not observe an increase in tdTomato labeling in IPCs at this dose (24.5% ± 0.9%) compared to the 3 mg/40 g dose (25.1%± 2.9%), nor did increasing the dose increase tdTomato expression in OPCs or DCs (Figure 9K-M, O-P, Table 1). Interestingly, however, 17 OHCs and 1 IHC in the apical tip of one sample expressed tdTomato with the 5 mg/40 g dose (Figure 9Q-S).

We next attempted to eliminate IPC labeling to make *Prox1*^{CreERT2} label just OPCs and DCs using two different adjustments to the tamoxifen regimen. In *Prox1*$^{CreERT2+/}$ *::Rosa26*$^{tdTomato/+}$ mice injected with tamoxifen (3 mg/40 g, IP) at P0 only, there were no differences in the number of tdTomato-positive IPCs, OPCs, or DCs compared to the P0/P1 injection with the same dose (Figure 9H-J, N-P, Table 1). Similarly, when the tamoxifen dose was reduced to 0.75 mg/40 g (IP) at P0 only, there was no significant difference in the number of tdTomato-positive IPCs (16.8% ± 4.0%) compared to the 3 mg/40 g dose given at P0/P1 (25.1% ± 2.9%), however this number was significantly lower compared to the 3 mg/40 g dose given at P0 only (33.4% ± 2.4%; one-way ANOVA $F(4, 10)=25.88$, $P<0.0001$ with Tukey's multiple comparisons test, Figure 9E-G, N, Table 1). Additionally, tdTomato expression was reduced in OPCs (37.9% ± 2.5%; one-way ANOVA $F(4, 10)= 201.3$, $P<0.0001$ with Tukey's multiple comparisons test) and DCs (41.5% ± 2.4%; one-way ANOVA $F(4, 10)= 519.5$, $P<0.0001$ with Tukey's multiple comparisons test; Figure 9E-G, O-P, Table 1) compared to 3 mg/40 g given at either P0 (77.7% ± 3.4% OPCs and 77.9 ± 2.4% DCs) or P0/P1 (70.1% ± 2.6% OPCs and 76.1% ± 0.1% DCs).

Discussion

Advances in the science of genetic modification have produced an arsenal of tools that can be used to study the expression or deletion of specific genes in specific cell types. This has allowed the development of more targeted therapeutics, as well as broadened our understanding of living organisms on a cellular and genetic level. To better employ these tools, we should understand their capabilities and limitations. To that end, we have endeavored to provide a guide for the use of four CreER mouse lines that are commonly used in the investigation of the neonatal organ of Corti. We were able to reduce HC labeling and increase labeling of SC subpopulations by reducing the tamoxifen dose when *Fgfr3-iCreER^{T2}* mice were paired with the tdTomato reporter. In addition, changing to the *CAG-eGFP* reporter achieved this goal for both *Fgfr3-iCreER^{T2}* and *Sox2^{CreERT2}* mice; however this also reduced the total amount of SC labeling. Both the remaining two CreER lines only labeled SCs with the previously used tamoxifen doses and no changes in tamoxifen dose altered the expression pattern in *Prox1^{CreERT2}* mice. However, a handful of HCs in the apical tip of the cochlea were labeled when we increased the tamoxifen dose. Altering tamoxifen dose had little impact on *Plp-CreER^{T2}* activity. Although when paired with the *CAG-eGFP* reporter, the number of labeled PCs/DCs decreased, fewer IPhCs/BCs labeled as well. Importantly, we learned that for all CreER lines a single dose of tamoxifen at P0 is equivalent to doses given at both P0 and P1 using the tdTomato reporter. This is an important finding because reduced tamoxifen exposure may reduce toxicity and mortality.

The four CreER lines we chose to study use promoters expressed at high levels in postnatal SCs to drive expression of CreER and therefore their expression patterns were previously expected to label only those cell types. However, low levels of *Fgfr3* and *Sox2* expression have been detected in some HCs at P1-2 using single-cell RNA-seq analyses (Burns et al., 2015; Waldhaus et al., 2015), which correspond with our results using *Sox2*^{CreERT2} and *Fgfr3-iCreER*^{T2}. Interestingly, *Fgfr3-iCreER*^{T2} HC expression occurred primarily in OHCs, with only a handful of labeled IHC in the apical tip. *Fgfr3* expression begins at approximately embryonic day (E) 15.5 in the region where PCs, DCs, and OHCs will form (Peters et al., 1993; Pirvola et al., 1995; Mueller et al., 2002; Hayashi et al., 2007). Therefore it is possible that *Fgfr3* is not expressed in the progenitor cells that give rise to IHCs. Alternatively, since *Fgfr3-iCreER*^{T2} is a transgenic allele, expression could be affected by the location of the transgene insertion site in the genome. Importantly, reducing the tamoxifen dose 10-fold (from 3 mg/40 g given at both P0/P1 to 0.3 mg/40 g given at P1 only) caused a significant reduction in the amount of labeled HCs (from ~44% to ~ 4%), while there was a minimal reduction in PC and DC labeling. In contrast, *Sox2*^{CreERT2} activity was detected in both IHCs and OHCs. This was expected since *Sox2* is expressed throughout the developing cochlea (Kiernan et al., 2005; Hume et al., 2007; Dabdoub et al., 2008). In addition, immature HCs are known to express *Sox2* in the first postnatal week (Kiernan et al., 2005; Hume et al., 2007; Walters et al., 2015) which overlaps with the ages when tamoxifen was injected. When the *Rosa26*^{tdTomato} reporter was paired with *Sox2*^{CreERT2}, the number of tdTomato-positive HCs did not change with varying doses of tamoxifen. Switching to the

CAG-eGFP reporter was able to reduce HC expression (from ~ 35% to ~3%), however it also reduced SC labeling (~100% to ~66%).

Two of the four CreER lines investigated (*Sox2*^{CreERT2} and *Fgfr3-iCreER*^{T2}) expressed an apical to basal decreasing gradient of reporter expression in HCs with both *ROSA26*^{tdTomato} and *CAG-eGFP* reporter lines. *Prox1*^{CreERT2} also showed an apical to basal decreasing gradient of reporter expression in SC subtypes. Cochlear HCs and SCs are derived from the same pool of progenitor cells (Yang et al., 2010; Cai et al., 2013; Driver et al., 2013) and the organ of Corti is still maturing during the first postnatal week. Therefore genes necessary for progenitor cells are being downregulated, while those required for the transition from a progenitor cell fate to a HC or SC fate are being upregulated during the first few days after birth. *Atoh1* is one of the first genes to be expressed prior to HC commitment and is upregulated at ~E13.5 in the basal turn of the cochlea (Woods et al., 2004). Between ~E13.5 and E15.5, *Atoh1* expression extends throughout the rest of the cochlea allowing IHCs to differentiate first, followed by OHCs and then SCs (Kelley, 2007). Therefore, cells in the basal turn of the cochlea are approximately two days more mature than cells in the apical turn. Genes that are expressed in early cochlear differentiation like *Sox2*, *Prox1*, and *Fgfr3* would have higher expression levels in the less mature cells of the apical turn at P0, matching the gradient of CreER activity we observed. However, the lack of gradient seen for SCs in *Sox2*^{CreERT2} mice likely occurred because expression of *Sox2* persists in SCs throughout the life of the animal (Oesterle et al., 2008). Interestingly, *Plp-CreER*^{T2} showed the opposite gradient with increased numbers of labeled IPhCs/BCs and DCs in middle and

basal turns compared to the apex. Maturation of the organ of Corti also occurs in a wave from the base to the apex of the cochlea and therefore genes required for differentiation are first expressed in the base. Little is known about how *Plp* expression patterns change in the developing cochlea. However our data may suggest that *Plp* is expressed later than *Sox2*, *Prox1*, and *Fgfr3* and only in cells that have committed to a SC fate as part of the maturation process.

Of note, *Prox1*^{CreERT2} labeled more OPCs and DCs compared to IPCs. This was somewhat surprising since PROX1 is detected in all IPCs using immunostaining (Bermingham-McDonogh et al., 2006; Cox et al., 2014), however there are known differences between IPCs located in the medial compartment of the cochlea and OPCs/DCs located in the lateral compartment. For example, *Hes5* is detected in OPCs, DCs, and IPhCs/BCs, but not IPCs (Cox et al., 2014) and *CD44* is expressed only in OPCs (Hertzano et al., 2010). Similarly, *Lgr5* is expressed in IPCs, IPhCs/BCs, and the third row of DCs, but not in the rest of the DC pool or in OPCs (Chai et al., 2011; Shi et al., 2012). Taken together, even though IPCs and OPCs are both classified as PCs and have similar morphologies, they are very distinct cell types. Another interesting result obtained with *Prox1*^{CreERT2} was the expression of the tdTomato reporter in a handful of HCs in the apical tip when the highest tamoxifen dose (5 mg/40 g, P0/P1) was given. In the embryonic cochlea, *Prox1* is expressed in progenitor cells beginning at ~E14.5 and is downregulated in cells as they take on a HC fate. By ~E18.5 *Prox1* is not detectable in HCs using immunostaining (Bermingham-McDonogh et al., 2006); however, the sensitivity of antibodies is known to be limited and low levels of *Prox1* may persist in

neonatal HCs of the apical turn that is revealed when a high dose of tamoxifen is combined with the *ROSA26*^{tdTomato} reporter.

Interestingly, *Sox2*^{CreERT2} showed differential Cre leakiness when paired with the two different reporter lines. Cre leakiness refers to the phenomenon where some CreER molecules are able to enter the nucleus and excise loxP sites in the absence of tamoxifen. With the *Rosa26*^{tdTomato} reporter, there were several tdTomato-positive SCs in the organ of Corti, but no eGFP-positive SCs were detected when the *CAG-eGFP*$^{+/loxP}$ reporter was used. Because *Rosa26*^{tdTomato} produces a more stable and brighter fluorescent protein than *CAG-eGFP*$^{+/loxP}$ (Madisen et al., 2010), it is possible that smaller quantities of CreER enzyme that translocated into the nucleus in the absence of tamoxifen were sufficient to induce tdTomato expression. However, no tdTomato-positive cells were detected in the controls for *Fgfr3-iCreER*^{T2}, *Plp-CreER*^{T2}, or *Prox1*^{CreERT2}.

For this study, we used two reporter lines to assess the activity of four CreER lines in the neonatal organ of Corti. However, each floxed allele is different because the location of the loxP sites within the gene and the distance between loxP sites affects the efficiency of the Cre enzyme to bind and excise DNA located between loxP sites (Sauer and Henderson, 1989; Kuhn et al., 1995; Feltri et al., 1999; Kellendonk et al., 1999). While our study is primarily useful for fate-mapping purposes, it is difficult to translate the reporter expression patterns we measured to other floxed alleles. Some alleles may be similar to the *ROSA26*^{tdTomato} reporter, while others may closely resemble the *CAG-*

eGFP reporter line. This illustrates the importance of using more than one reporter line to understand the expression pattern of any CreER line used to delete or overexpress a gene. In addition, it would be wise to use immunostaining to confirm that the gene deletion/overexpression pattern matches the reporter data. Overall, the best method for CreER induction when using the *ROSA26*^{tdTomato} reporter appears to be one injection of tamoxifen at 3 mg/40 g given at P0 because it induces similar CreER activity as two injections without the increased risk of toxicity. Depending on the goal of the study, either the *ROSA26*^{tdTomato} reporter or *CAG-eGFP* reporter is valuable, however the *CAG-eGFP* labeled fewer cells with all CreER lines investigated.

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Conflict of Interest

All authors declare no conflict of interest on the present manuscript.

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Figure 1. *Fgfr3-iCreER^{T2}* is expressed in the majority of PCs/DCs. A, Cross-sectional view of the mouse organ of Corti with SC subtypes highlighted in different colors. The tunnel of Corti is partially open to represent the immature state of the neonatal cochlea. GER, greater epithelial ridge; SGN, spiral ganglion neurons. **B**, Quantification of total reporter-positive SCs in *Fgfr3-iCreER$^{T2/+}$::ROSA26$^{tdTomato/+}$* mice or *Fgfr3-iCreER$^{T2/+}$::CAG-eGFP$^{+/loxP}$* mice given different doses of tamoxifen (TAM). Cells were quantified in two randomly chosen 200 µm regions per cochlear turn, averaged, and expressed as a percentage of labeled cells compared to total cells. (***P*<0.01, ****P*<0.001 as determined by a two-way ANOVA $F(3,24)=274.5$ with a

Tukey's post-hoc test) (N =3-4)**.** Representative confocal images of tdTomato (red) expression in *Fgfr3-iCreER$^{T2/+}$::ROSA26$^{tdTomato/+}$* controls without tamoxifen (**C-E**) and those given various doses of tamoxifen (**F-H'''**). Representative confocal images of eGFP (green) expression in *Fgfr3-iCreER$^{T2/+}$::CAG-eGFP$^{+/loxP}$* controls without tamoxifen (**I-K**) and those given various doses of tamoxifen (**L-N'''**). Optical cross sections showing that the location of tdTomato (**H'-H'''**) and eGFP (**N'-N'''**) expression is primarily in SCs. Scale bar in C: 25 µm and in H' and N': 6.25 µm.

Figure 2. *Fgfr3-iCreER$^{T2/+}$::ROSA26$^{tdTomato/+}$* is robustly expressed in OHCs in an apical to basal gradient. A, For quantification with the *ROSA26$^{tdTomato/+}$* reporter line, the entire cochlea was imaged, measured, and divided into six equal sections. A, apex and B, base. **B,** Quantification of reporter-positive OHCs in *Fgfr3-iCreER$^{T2/+}$::ROSA26$^{tdTomato/+}$* mice given different doses of tamoxifen (TAM). All cells were quantified in each of the six segments, averaged, and expressed as a percentage of labeled cells compared to total cells. (N =3-4). Representative confocal images of tdTomato (red) expression in *Fgfr3-iCreER$^{T2/+}$::ROSA26$^{tdTomato/+}$* controls without tamoxifen (**C-E**) and those given various doses of tamoxifen (**F-K**). **L-M**, higher magnification images from regions marked in H and K respectively. Scale bars: 25 µm.

Figure 3. *Fgfr3-iCreER$^{T2/+}$::CAG-eGFP$^{+/loxP}$* labels fewer HCs except for the apical tip of the cochlea. A, For quantification with the *CAG-eGFP* reporter line, the cochlea was divided into two sections: the apical most 250 µm and the rest of the cochlea. A,

apex and B, base. **B,** Quantification of reporter-positive OHCs in *Fgfr3-iCreER$^{T2/+}$::CAG-eGFP$^{+/loxP}$* mice given different doses of tamoxifen (TAM). All cells were quantified in the two segments, averaged, and expressed as a percentage of labeled cells compared to total cells. (*P<=0.0101, as determined by a paired Student's t test $t(2)$= 9.867) (N =3-4). Representative confocal images of eGFP (green) expression in *Fgfr3-iCreER$^{T2/+}$::CAG-eGFP$^{+/loxP}$* controls without tamoxifen (**C-E**) and those given various doses of tamoxifen (**F-K**). **L-M**, higher magnification images from regions marked in H and K respectively. Scale bars: 25 μm.

Figure 4. *Sox2^{CreERT2}* is expressed in all SCs within the organ of Corti. A, Quantification of total reporter-positive SCs in *Sox2$^{CreERT2+/-}$::ROSA26^{tdTomato}* mice or *Sox2$^{CreERT2+/-}$::CAG-eGFP$^{+/loxP}$* mice given different doses of tamoxifen (TAM). Cells were quantified in two randomly chosen 200 μm regions per cochlear turn, averaged, and expressed as a percentage of labeled cells compared to total cells. (***P<0.0001 as determined by a two-way ANOVA $F(4,33)$=462.3 with a Tukey's post-hoc test) (N =3-4)**.** Representative confocal images of tdTomato (red) expression in *Sox2$^{CreERT2+/-}$::ROSA26^{tdTomato}* controls without tamoxifen (**B-D**) and those given various doses of tamoxifen (**E-G'''**). Representative confocal images of eGFP (green) expression in *Sox2$^{CreERT2+/-}$::CAG-eGFP$^{+/loxP}$* controls without tamoxifen (**H-J**) and those given various doses of tamoxifen (**K-M'''**). Optical cross sections showing location of tdTomato (**G'-G'''**) and eGFP (**M'-M'''**) expression is in SCs and some HCs. Scale bar in B: 25 μm and in G' and M': 6.25 μm.

Figure 5. *Sox2$^{CreERT2+/-}$::ROSA26^{tdTomato}* is expressed in a large number of HCs in a descending apical to basal gradient. A, Quantification of reporter-positive HCs in *Sox2$^{CreERT2+/-}$::ROSA26^{tdTomato}* mice given different doses of tamoxifen (TAM). All cells were quantified in each of the six segments labeled in Figure 2A, averaged, and expressed as a percentage of labeled cells compared to total cells. (For comparison of OHCs ***P = 0.00005 as determined by a Pearson correlation coefficient r^2 = 0.994, for comparison of IHCs ***P = 0.0003 as determined by a Pearson correlation coefficient r^2 = 0.985) (N =3-4). Representative confocal images of tdTomato (red) expression in *Sox2$^{CreERT2+/-}$::ROSA26^{tdTomato}* controls without tamoxifen (**B-D**) and those given various doses of tamoxifen (**E-J**). **K-L**, higher magnification images from regions marked in G and J respectively. Scale bars: 25 μm.

Figure 6. *Sox2$^{CreERT2+/-}$::CAG-eGFP$^{+/loxP}$* labels fewer HCs except for the most apical tip of the cochlea. A, Quantification of reporter-positive HCs in *Sox2$^{CreERT2+/-}$::CAG-eGFP$^{+/loxP}$* mice given different doses of tamoxifen (TAM). All cells were quantified in the two segments labeled in Figure 3A, averaged, and expressed as a percentage of labeled cells compared to total cells. (N =3-4). Representative confocal images of eGFP (green) expression in *Sox2$^{CreERT2+/-}$::CAG-eGFP$^{+/loxP}$* controls without tamoxifen (**B-D**) and those given various doses of tamoxifen (**E-J**). **K-L**, higher magnification images from regions marked in G and J respectively. Scale bars: 25 μm.

Figure 7. Targeting of *Plp-CreER^{T2}* to IPhCs/BCs increased when the *CAG-eGFP* reporter was used. **A,** Quantification of total reporter-positive SCs in the three cochlear turns of *Plp-CreER$^{T2/+}$::ROSA26^{tdTomato}* or *Plp-CreER$^{T2/+}$::CAG-eGFP$^{+/loxP}$* mice given different doses of tamoxifen (TAM). Cells were quantified in two randomly chosen 200 µm regions per cochlear turn, averaged, and expressed as a percentage of labeled cells compared to total cells. Representative confocal images of tdTomato (red) expression in *Plp-CreER$^{T2/+}$::ROSA26^{tdTomato}* controls without tamoxifen (**B-D**) and those given various doses of tamoxifen (**E-J**). Representative confocal images of eGFP (green) expression in *Plp-CreER$^{T2/+}$::CAG-eGFP$^{+/loxP}$* controls without tamoxifen (**K-M**) and those given various doses of tamoxifen (**N-S**). Scale bar: 25 µm. (For comparison among cochlear turns within the same tamoxifen dosing paradigm: 3 mg/40 g tamoxifen dose at P0 only **P*<0.05 as determined by a one-way ANOVA *F*(1.016, 2.031)=7.435 with a Tukey's post-hoc test, for the 3 mg/40 g tamoxifen dose at P0/P1 ***P*<0.01as determined by a one-way ANOVA *F*(1.346, 4.038)=129.5 with a Tukey's post-hoc test. For comparison across tamoxifen dosing paradigms: **P*<0.05 and ****P*<0.001 as determined by a two-way ANOVA *F*(6,44)=40.9 with a Tukey's post-hoc test.) (N =3-4)**.**

Figure 8. Quantification of reporter expression among SC subtypes in *Plp-CreER^{T2}* mice. SC subtypes that expressed the tdTomato or eGFP reporter were quantified as described in 7A. **A**, IPhCs/BCs (For comparison among cochlear turns within the same tamoxifen dosing paradigm: *ROSA26^{tdTomato}* reporter with tamoxifen at P0/P1 ****P*<0.001 as determined by a one-way ANOVA *F*(1.313, 3.940)=71.03 with a Tukey's post-hoc test; and *CAG-eGFP$^{+/loxP}$* reporter with tamoxifen at P0 **P*<0.05,

***P<0.001 as determined by a one-way ANOVA $F(1.012, 2.025)=268.8$ with a Tukey's post-hoc test. For comparison across tamoxifen dosing paradigms: **P<0.01 and ***P<0.001 as determined by a two-way ANOVA $F(6,45)=97.06$ with a Tukey's post-hoc test). **B**, IPCs (For comparison across tamoxifen dosing paradigms: *P<0.05 as determined by a two-way ANOVA $F(6,45)=6.017$ with a Tukey's post-hoc test). **C**, OPCs (For comparison across tamoxifen dosing paradigms: *P<0.05 as determined by a two-way ANOVA $F(6, 60)=8.524$ with a Tukey's post-hoc test). **D**, DCs (For comparison among cochlear turns within the same tamoxifen dosing paradigm: *ROSA26^{tdTomato}* reporter with tamoxifen at P0/P1 *P<0.05 as determined by a one-way ANOVA $F(1.193, 3.579)=39.29$ with a Tukey's post-hoc test and *ROSA26^{tdTomato}* reporter with tamoxifen at P1 *P<0.05 as determined by a one-way ANOVA $F(2, 6)=5.189$ with a Tukey's post-hoc test. For comparison across tamoxifen dosing paradigms: *P<0.05 and **P<0.01 as determined by a two-way ANOVA $F(6,45)=6.313$ with a Tukey's post-hoc test) (N =3-4).

Figure 9. Targeting of *Prox1^{CreERT2}* to PCs and DCs was not altered with changes in tamoxifen induction paradigm. **A,** Quantification of total reporter-positive SCs in the three cochlear turns of *Prox1$^{CreERT2+/-}$::ROSA26^{tdTomato}* or *Prox1$^{CreERT2+/-}$::CAG-eGFP$^{+/loxP}$* mice given different doses of tamoxifen (TAM). Cells were quantified in two randomly chosen 200 µm regions per cochlear turn, averaged, and expressed as a percentage of labeled cells compared to total cells. (For comparison among cochlear turns within the same tamoxifen dosing paradigm: 3 mg/40g tamoxifen dose at P0/P1 *P<0.05 as determined by a one-way ANOVA $F(1.018, 2.036)=37.08$ with a Tukey's post-hoc test; and 5 mg/40g tamoxifen dose *P<0.05 and **P<0.01 as determined by a

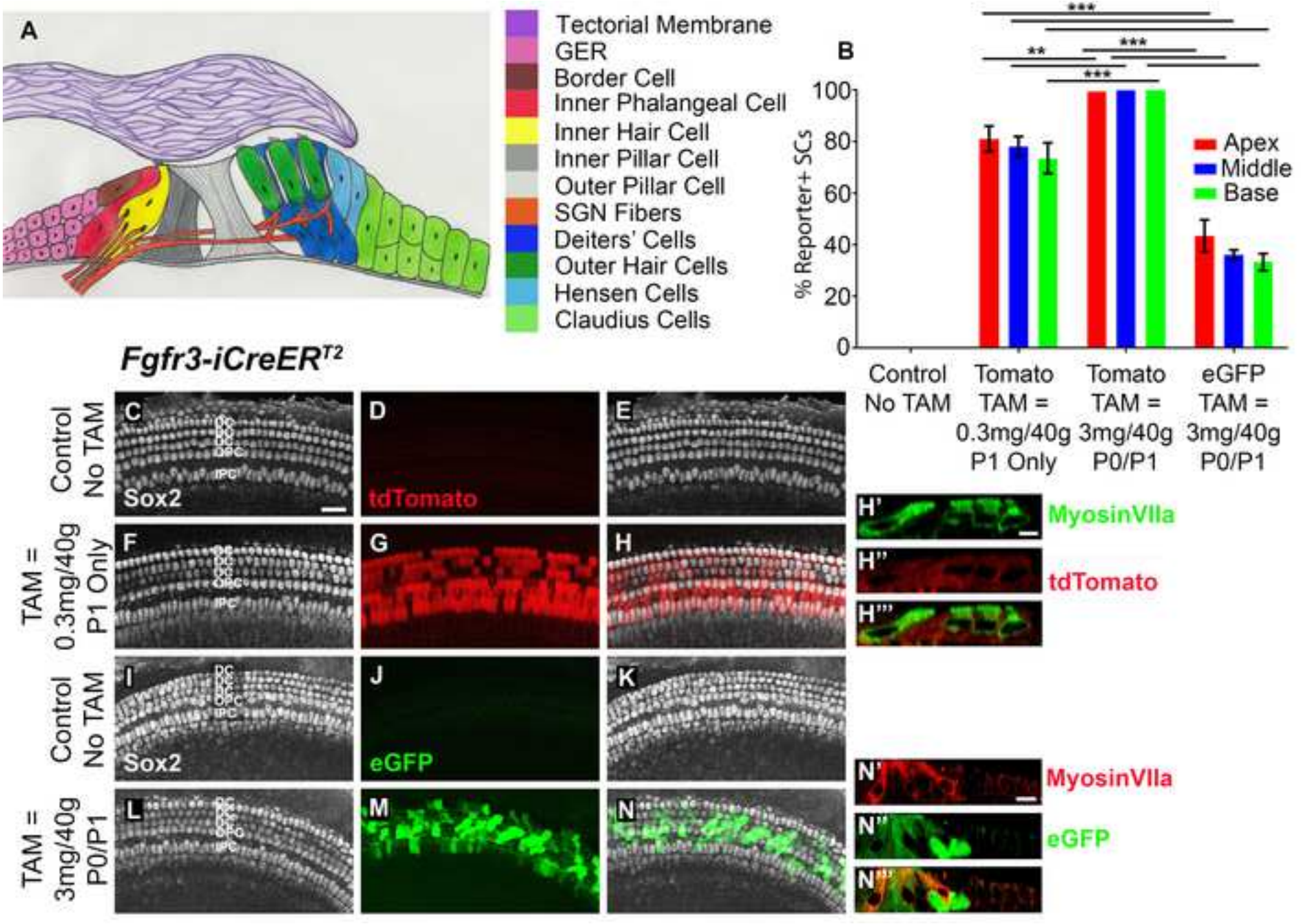
one-way ANOVA $F(1,2)=144.1$ with a Tukey's post-hoc test. For comparison across tamoxifen dosing paradigms: $^{**}P<0.01$ and $^{***}P<0.001$ as determined by a two-way ANOVA $F(4,30)=151.8$ with a Tukey's post-hoc test) (N =3-4). Representative confocal images of tdTomato (red) expression in *Prox1*$^{CreERT2+/-}$*::ROSA26*^{tdTomato} controls without tamoxifen (**B-D**) and those given various doses of tamoxifen (**E-M**). **N-P,** SC subtypes that expressed the tdTomato reporter were quantified as described in A. **N**, IPCs (For comparison among cochlear turns within the same tamoxifen dosing paradigm: 3 mg/40g tamoxifen dose at P0/P1 $^{**}P<0.01$ as determined by a one-way ANOVA $F(1.021, 2.042)=35.11$ with a Tukey's post-hoc test; and 5 mg/40g tamoxifen dose $^{*}P<0.05$ as determined by a one-way ANOVA $F(1.445, 2.890)=47.77$ with a Tukey's post-hoc test. For comparison across tamoxifen dosing paradigms: $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ as determined by a two-way ANOVA $F(4,30)=24.63$ with a Tukey's post-hoc test). **O**, OPCs (For comparison among cochlear turns within the same tamoxifen dosing paradigm: 5 mg/40g tamoxifen dose $^{*}P<0.05$ as determined by a one-way ANOVA $F(1.427, 2.854)=37.35$ with a Tukey's post-hoc test. For comparison across tamoxifen dosing paradigms: $^{**}P<0.01$ and $^{***}P<0.001$ as determined by a two-way ANOVA $F(4,30)=139.5$ with a Tukey's post-hoc test). **P**, DCs (For comparison among cochlear turns within the same tamoxifen dosing paradigm: 5 mg/40g tamoxifen dose $^{*}P<0.05$, $^{**}P<0.01$ as determined by a one-way ANOVA $F(1.156, 2.911)=94.69$ with a Tukey's post-hoc test. For comparison across tamoxifen dosing paradigms: $^{***}P<0.001$ as determined by a two-way ANOVA $F(4,30)=231.2$ with a Tukey's post-hoc test). (N =3-4). **O-Q,** Representative confocal images of a tdTomato+ HC (arrow) in

Prox1$^{CreERT2+/-}$*::ROSA26*^{tdTomato} mice given the highest dose of tamoxifen (5mg/40g, P0/P1). Scale bars: 25 μm.

Table 1: Summary of reporter labeled HC and SC subtypes in *Fgfr3-iCreER*$^{T2/+}$, *Sox2*$^{CreERT2+/-}$, *Plp-CreER*^{T2}, *and Prox1*$^{CreERT2+/-}$ mice. Quantification of tdTomato-positive or eGFP-positive cells was performed as described in Figures 1B, 2A, and 3A.

Mouse Line	Dose	Cell Type	% Labeled Cells						
			Apex		Middle		Base		Whole cochlea
			Segment 1	Segment 2	Segment 3	Segment4	Segment5	Segment 6	
Fgfr3-iCreER$^{T2/+}$ *::ROSA26*$^{tdTomato/+}$	0.3mg/40g P0	SCs	81.2% ± 5.0%		78.3% ± 3.8%		73.6% ± 5.9%		77.7% ± 4.9%
		OHCs	14.6% ± 10.6%	2.9% ± 2.5%	0.3% ± 0.3%	0.3% ± 0.2%	0.0% ± 0.0%	0.2% ± 0.2%	3.9% ± 2.2%
	3mg/40g P0-P1	SCs	97.3% ± 2.3%		92.3% ± 4.3%		93.43% ± 6.6%		94.4% ± 5.5%
		OHCs	98.5% ± 0.7%	77.3% ± 3.8%	50.5% ± 5.1%	19.8% ± 3.5%	7.6% ± 0.9%	13.2% ± 3.1%	43.8% ± 2.9%
Sox2$^{CreERT2+/-}$ *::ROSA26*$^{tdTomato/+}$	3mg/40g P0	SCs	99.9% ± 0.1%		99.9% ± 0%		100% ± 0%		99.9% ± 0.0%
		HCs	97.2% ± 1.6%	68.3% ± 11.0%	25.1 ± 10.0%	7.6% ± 2.2%	2.6% ± 1.8%	6.0% ± 2.7	34.5% ± 4.7%
	3mg/40g P0-P1	SCs	100% ± 0%		100% ± 0%		100% ± 0%		100.0% ± 0.0%
		HCs	97.2% ± 1.7%	66.9% ± 10.2%	31.7% ± 11.2%	10.2% ± 3.8%	3.2% ± 1.2%	2.7% ± 1.2%	35.3% ± 4.8%
Plp-CreER$^{T2/+}$ *::ROSA26*$^{tdTomato/+}$	3mg/40g P0	IPhCs/BCs	46.5% ± 4.7%		80.3% ± 4.6%		91.5% ± 2.6%		72.8% ± 5.4%
		IPCs	6.7% ± 2.0%		6.6% ± 3.3%		8.5% ± 2.4%		7.3% ± 0.7%
		OPCs	9.3% ± 2.8%		13.9% ± 2.2%		3.5% ± 2.4%		8.9% ± 1.3%
		DCs	5.5% ± 1.1%		13.6% ± 4.9%		13.7% ± 5.0%		10.9% ± 2.8%
	3mg/40g P1	IPhCs/BCs	56.8% ± 11.7%		71.0% ± 13.7%		87.1% ± 2.6%		71.7% ± 4.3%
		IPCs	11.0% ± 4.1%		11.4% ± 4.7%		7.2% ± 4.4%		9.9% ± 2.6%
		OPCs	16.4% ± 5.2%		5.9% ± 2.3%		2.0% ± 1.1%		8.1% ± 2.8%
		DCs	4.6% ± 1.3%		7.3% ± 3.2%		14.5% ± 1.7%		8.8% ± 2.0%
	3m/40g P0-P1	IPhCs/BCs	48.2% ± 4.7%		79.9% ± 2.5%		89.2% ± 3.7%		72.4% ± 3.1%
		IPCs	15.6% ± 5.5%		6.7% ± 3.8%		18.2% ± 5.2%		13.5% ± 4.6%
		OPCs	12.3% ± 3.7%		15.7% ± 3.4%		3.9% ± 1.0%		10.6% ± 2.0%
		DCs	5.8% ± 1.9%		9.8% ± 2.8%		29.3% ± 4.7%		15% ± 2.9%
Prox1$^{CreERT2+/-}$ *::ROSA26*$^{tdTomato/+}$	0.75mg/40g P0	IPCs	17.2% ± 2.8%		30% ± 11.3%		3.2% ± 1.7%		16.8% ± 4.0%
		OPCs	46.3% ± 4.0%		32.5% ± 13.3%		29.5% ± 4.7%		37.9% ± 2.5%
		DCs	51.6% ± 2.5%		27.4% ± 10.9%		28.0% ± 8.7%		41.5% ± 2.4%
	3mg/40g P0	IPCs	41.9% ± 5.5%		33.4% ± 6.8%		28.3% ± 5.1%		33.4% ± 2.4%
		OPCs	84.3% ± 1.3%		76.2% ± 5.4%		72.7% ± 5.6%		77.7% ± 3.4%
		DCs	88.5% ± 2.2%		81.4% ± 3.9%		75.5% ± 1.7%		77.9% ± 4.2%
	3mg/40g P0-P1	IPCs	45.9% ± 1.6%		22.2% ± 6.5%		7.4% ± 1.2%		25.1% ± 2.9%
		OPCs	77.7% ± 3.3%		69.0% ± 4.4%		63.8% ± 4.8%		70.1% ± 2.6%
		DCs	89.6% ± 3.0%		80.7% ± 0.6%		61.7% ± 4.0%		76.1% ± 0.1%
	5mg/40g P0-P1	IPCs	47.9% ± 3.5%		14.5% ± 2.6%		11.1% ± 0.7%		24.5% ± 0.9%
		OPCs	88.4% ± 1.8%		69.0% ± 2.9%		56.8% ± 2.6%		71.4% ± 1.3%
		DCs	93.6% ± 0.8%		75.4% ± 0.8%		69.3% ± 1.6%		79.5% ± 0.4%

Mouse Line	Dose	Cell Type	% Labeled Cells				
			Apex		Middle	Base	
			Apical Tip	Rest of Cochlea			Whole cochlea
Fgfr3-iCreER$^{T2/+}$ *::CAG-eGFP*$^{+/loxP}$	3mg/40g P0-P1	SCs	43.6% ± 6.1%		36.3% ± 1.8%	33.4% ± 3.3%	37.8% ± 3.4%
		OHCs	40.5% ± 6.1%	4.4% ± 2.5%			5.8% ± 2.6%
Sox2$^{CreERT2+/-}$ *::CAG-eGFP*$^{+/loxP}$	3mg/40g P1	SCs	74.0% ± 4.9%		65.7% ± 9.0%	58.4% ± 5.7%	66.0% ± 5.9%
		HCs	44.8% ± 10.8%	1.6% ± 0.5%			3.2% ± 0.4%
Plp-CreER$^{T2/+}$ *::CAG-eGFP*$^{+/loxP}$	3mg/40g P0	IPhCs/BCs	10.1% ± 4.5%		24.3% ± 5.9%	43.8% ± 3.9%	26.0% ± 4.8%
		IPCs	0.0% ± 0.0%		0.9% ± 0.9%	0.0% ± 0.0%	0.3% ± 0.3%
		OPCs	0.8% ± 0.8%		0.0% ± 0.0%	0.0% ± 0.0%	0.3% ± 0.3%
		DCs	0.9% ± 0.9%		1.1% ± 0.8%	5.9% ± 4.8%	2.6% ± 2.1%
	3mg/40g P0-P1	IPhCs/BCs	21.8% ± 10.9%		28.2% ± 2.1%	38.1% ± 3.9%	29.4% ± 3.7%
		IPCs	8.8% ± 7.9%		9.1% ± 7.5%	7.3% ± 5.8%	8.4% ± 7.1%
		OPCs	10.2% ± 8.9%		8.4% ± 7.5%	5.8% ± 5.8%	8.1% ± 7.4%
		DCs	5.1% ± 5.1%		12.0% ± 11.4%	9.8% ± 9.4%	9.0% ± 8.6%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Tectorial Membrane
GER
Border Cell
Inner Phalangeal Cell
Inner Hair Cell
Inner Pillar Cell
Outer Pillar Cell
SGN Fibers
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Outer Hair Cells
Hensen Cells
Claudius Cells
B
% Reporter+ SCs
Apex
Middle
Base
Control No TAM
Tomato TAM = 0.3mg/40g P1 Only
Tomato TAM = 3mg/40g P0/P1
eGFP TAM = 3mg/40g P0/P1
Fgfr3-iCreER^T2
Control No TAM
TAM = 0.3mg/40g P1 Only
Control No TAM
TAM = 3mg/40g P0/P1
Sox2
tdTomato
eGFP
MyosinVIIa
tdTomato
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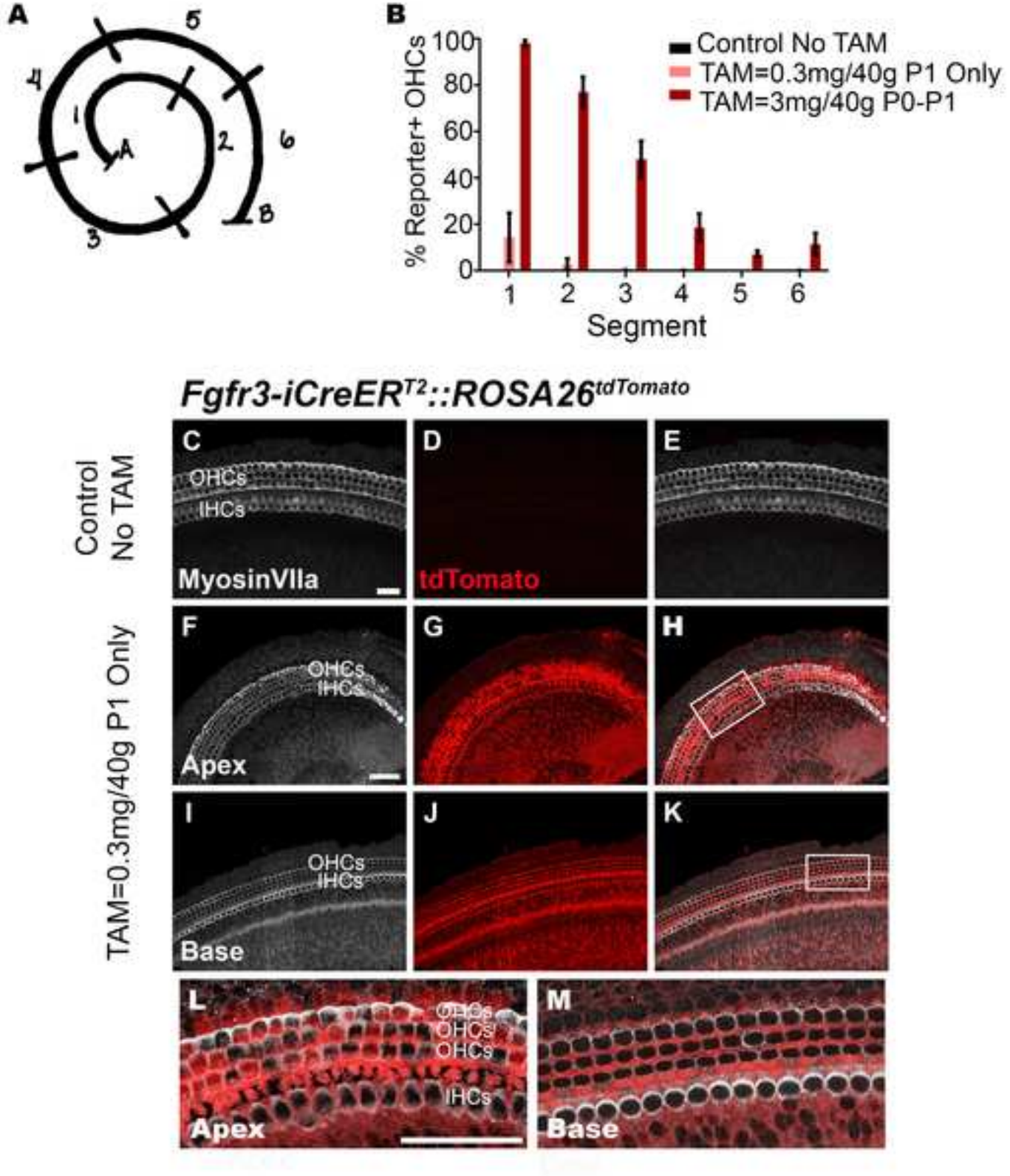
eGFP


A
5
4
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% Reporter+ OHCs
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Segment
Control No TAM
TAM=0.3mg/40g P1 Only
TAM=3mg/40g P0-P1
Fgfr3-iCreER^T2::ROSA26^tdTomato
Control No TAM
TAM=0.3mg/40g P1 Only
C
OHCs
IHCs
MyosinVIIa
D
tdTomato
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OHCs
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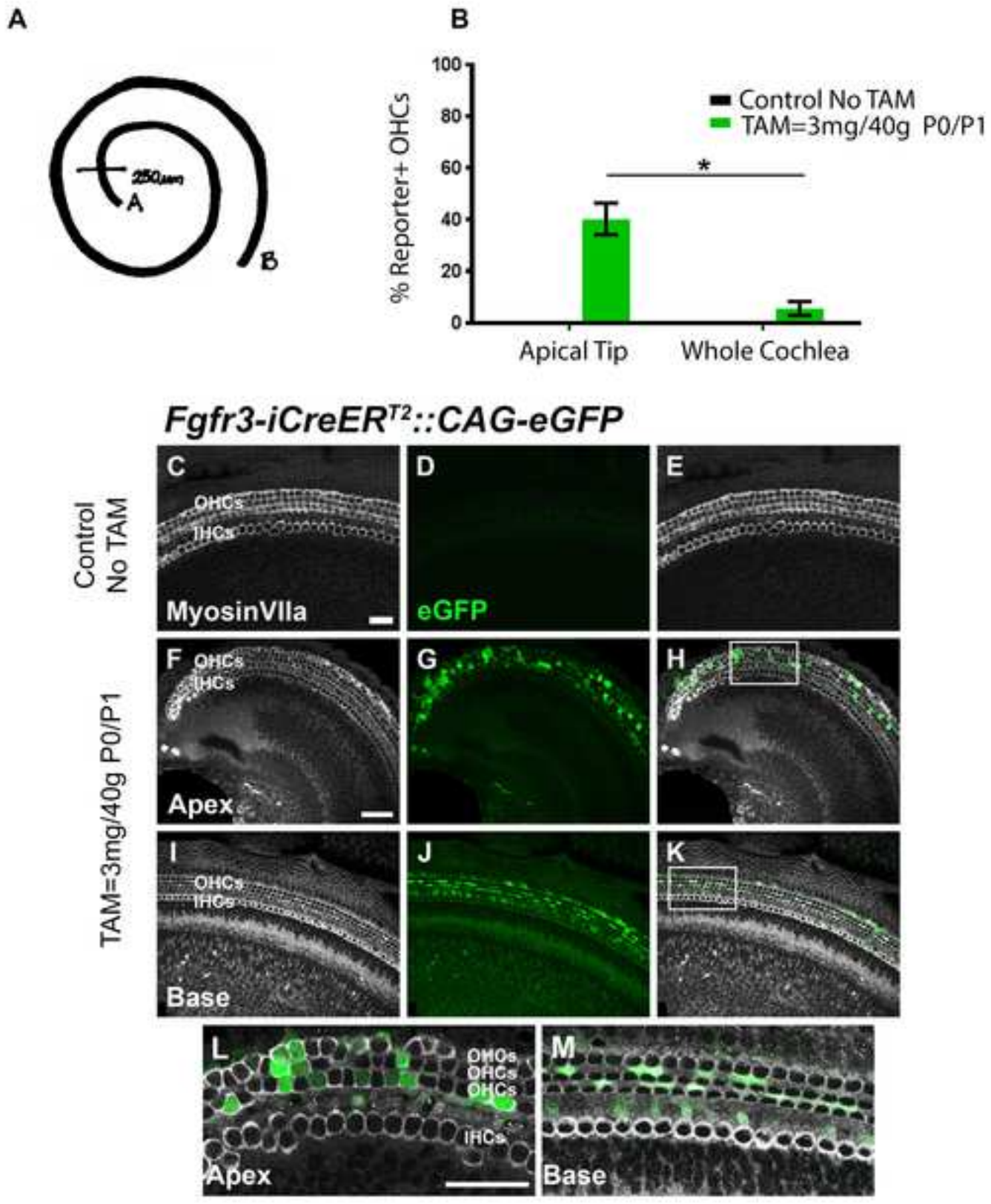
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100
80
60
40
20
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% Reporter+ OHCs
Control No TAM
TAM=3mg/40g P0/P1
*
Apical Tip
Whole Cochlea
Fgfr3-iCreER^T2::CAG-eGFP
Control No TAM
TAM=3mg/40g P0/P1
C
OHCs
IHCs
MyosinVIIa
D
eGFP
E
F
OHCs
IHCs
Apex
G
H
I
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OHCs
OHCs
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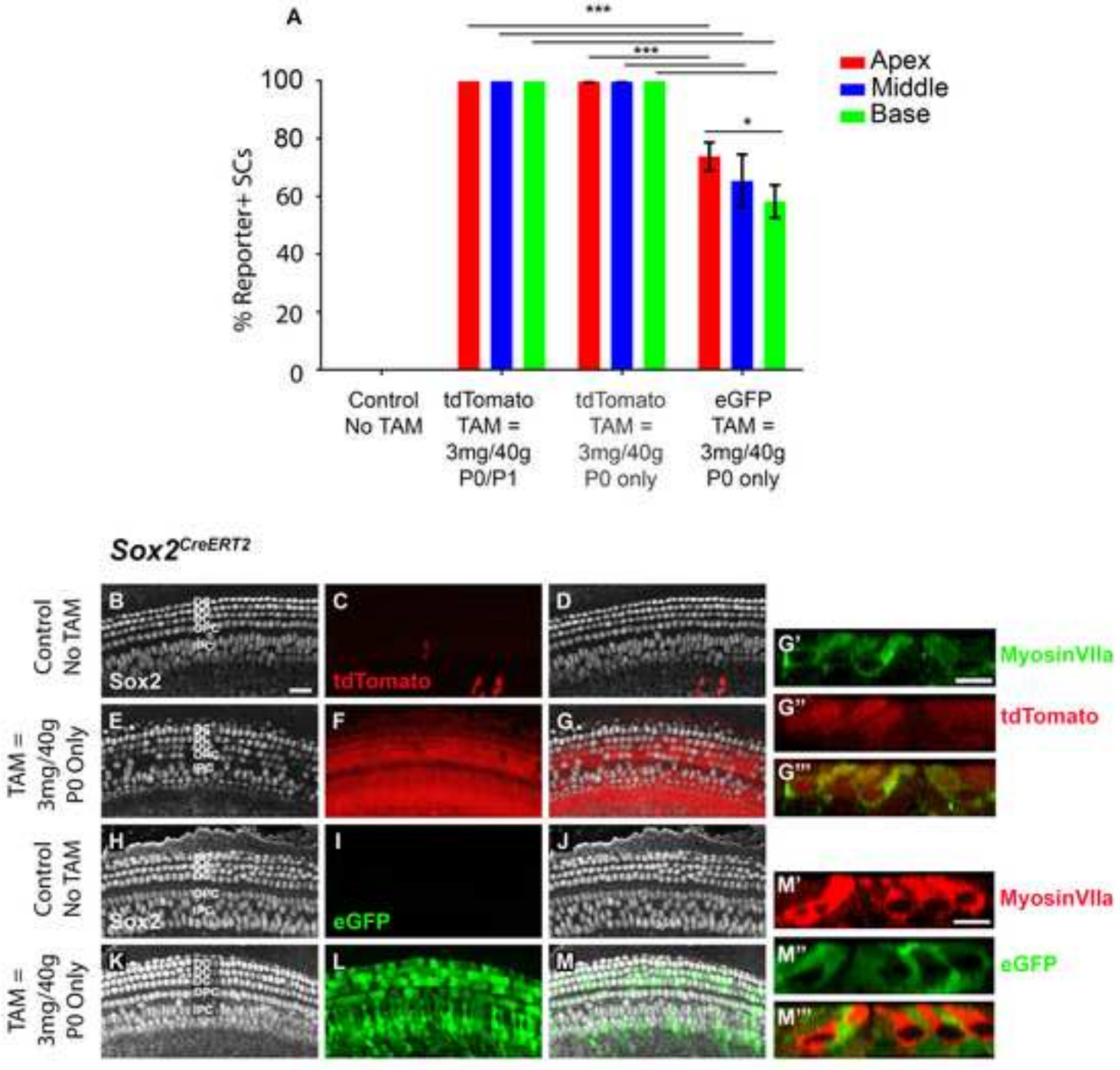
A
***
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Control
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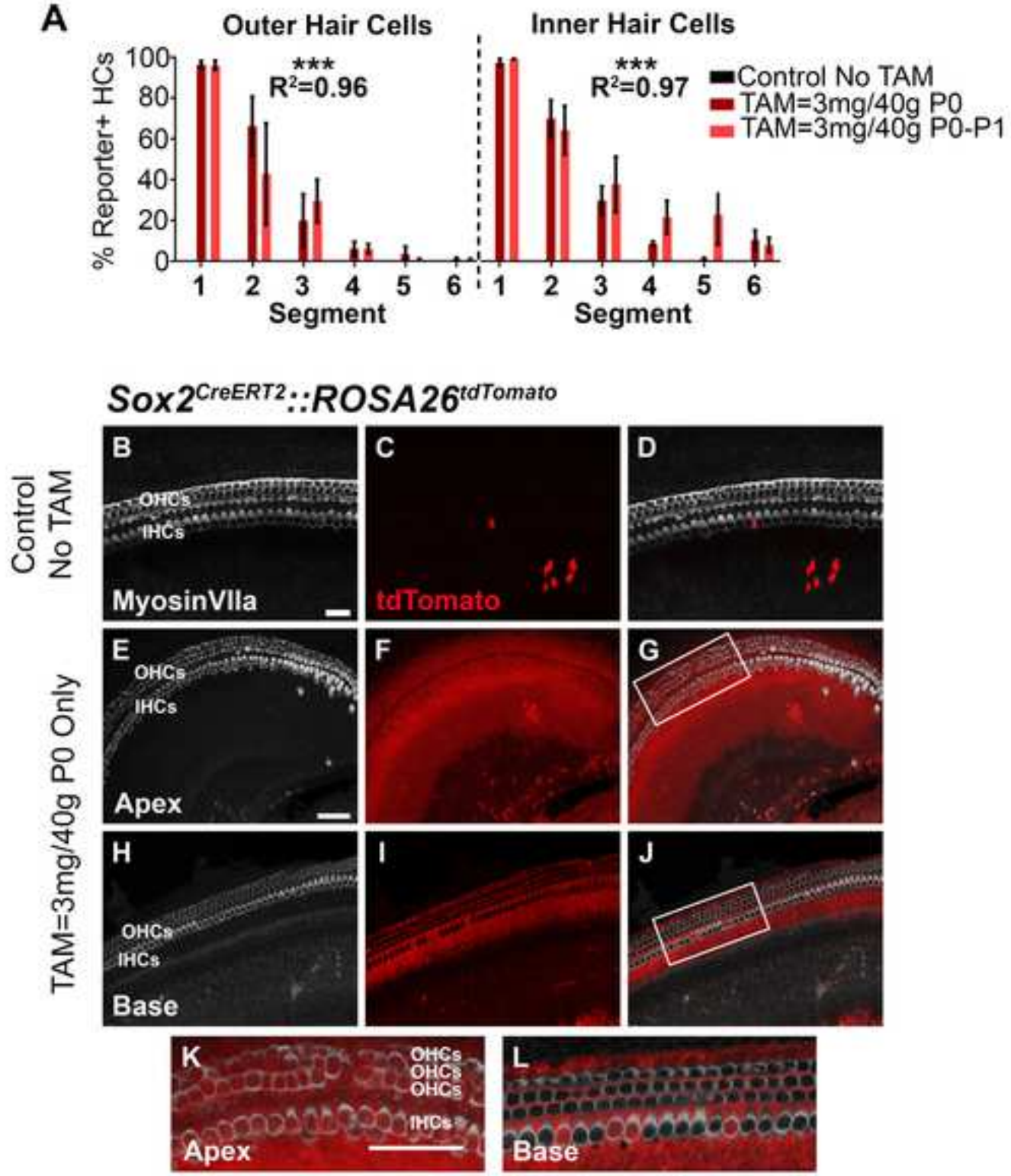
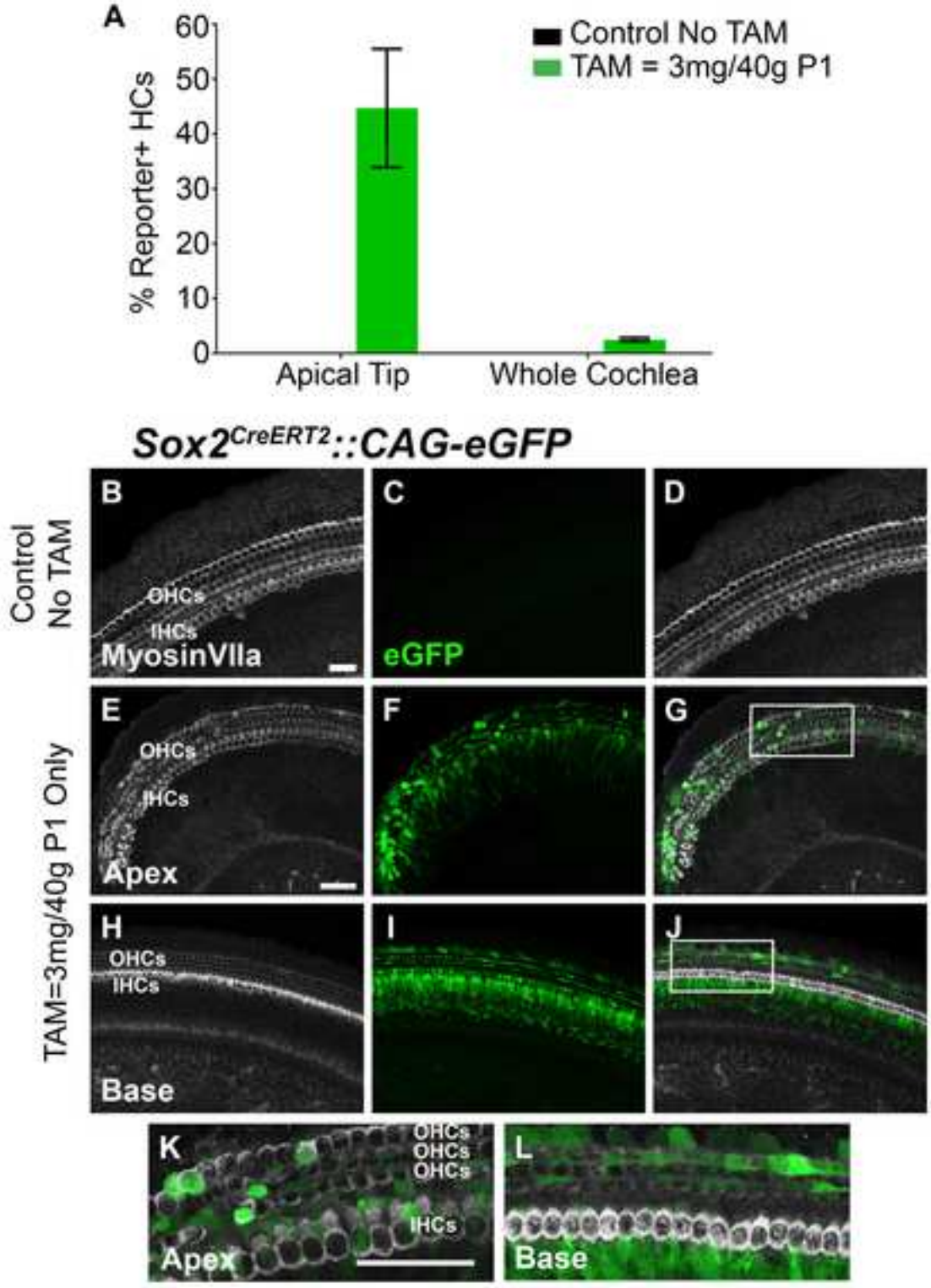
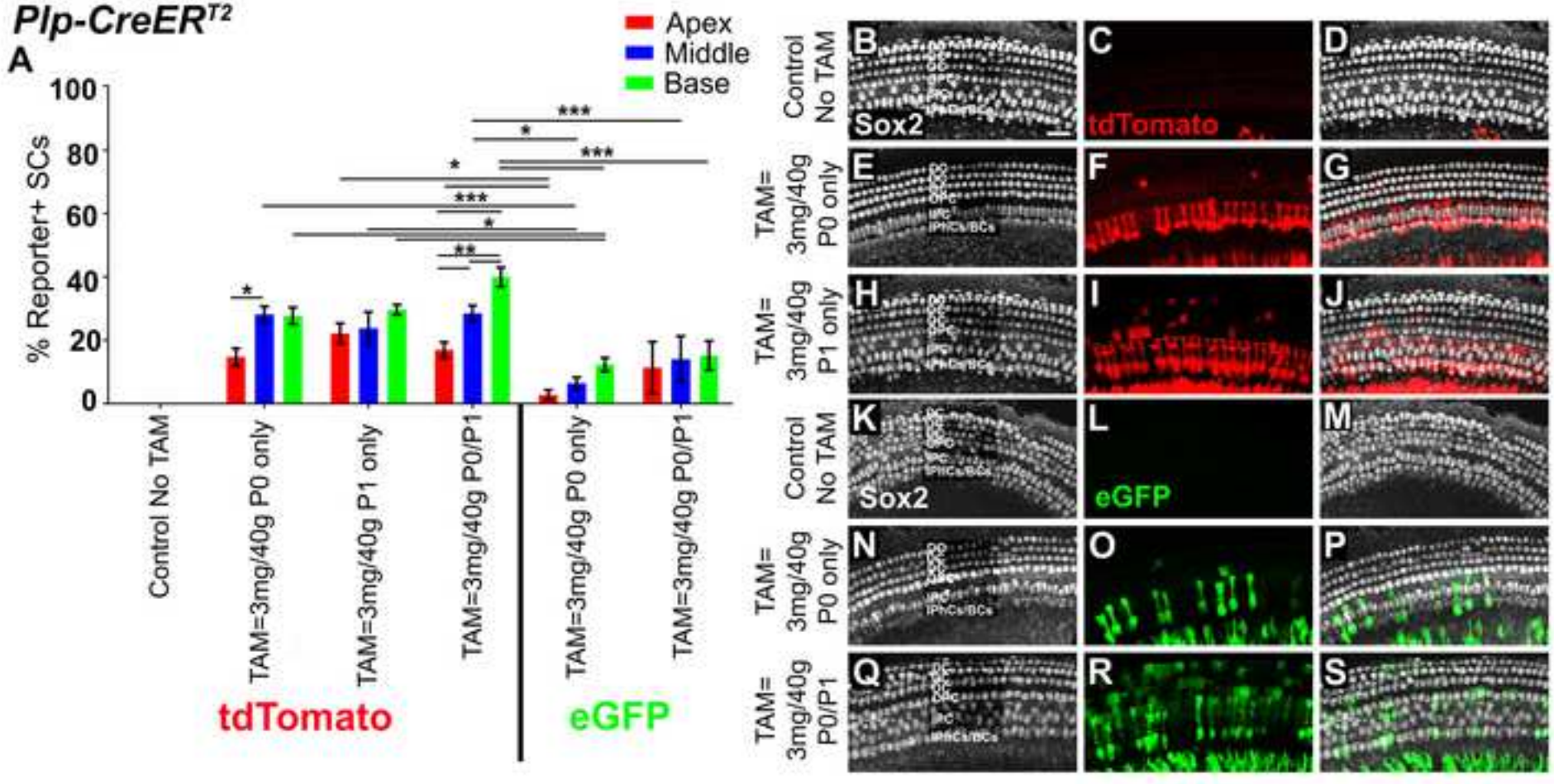
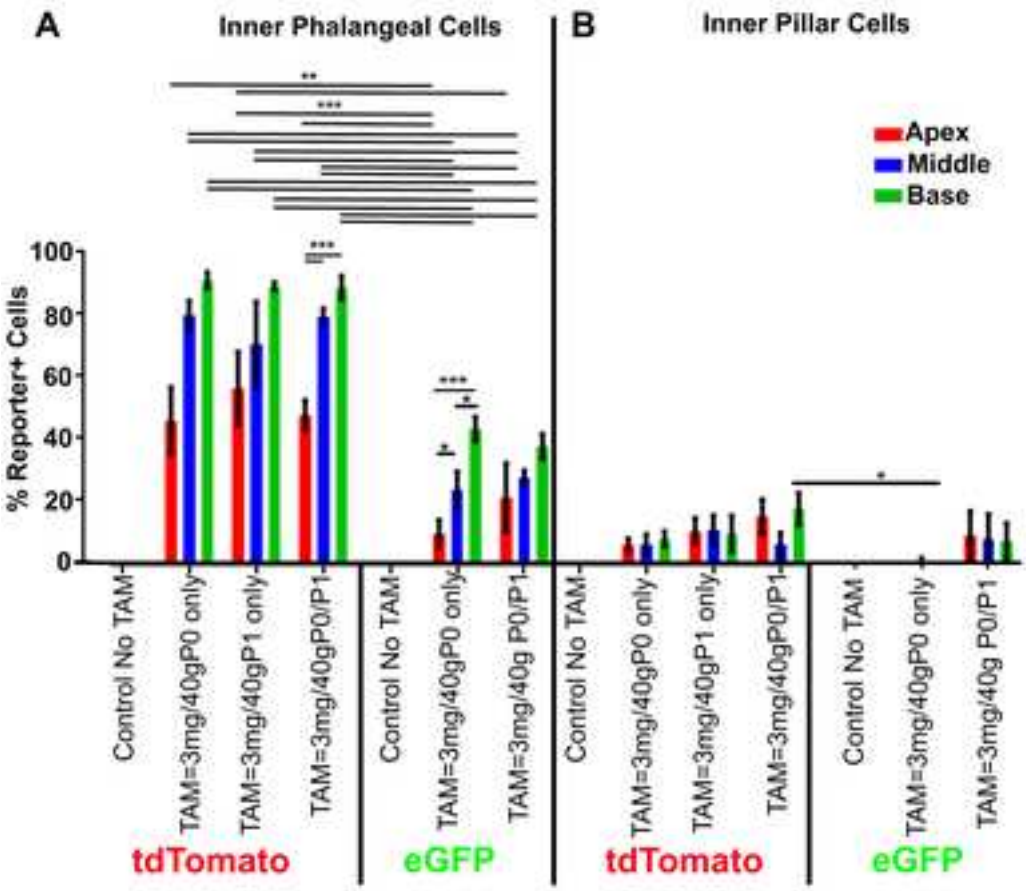
TAM =
3mg/40g
P0/P1
tdTomato
TAM =
3mg/40g
P0 only
eGFP
TAM =
3mg/40g
P0 only
Sox2CreERT2
Control
No TAM
TAM =
3mg/40g
P0 Only
Control
No TAM
TAM =
3mg/40g
P0 Only
B
C
D
E
F
G
H
I
J
K
L
M
Sox2
tdTomato
eGFP
G'
G''
G'''
M'
M''
M'''
MyosinVIIa
tdTomato
MyosinVIIa
eGFP

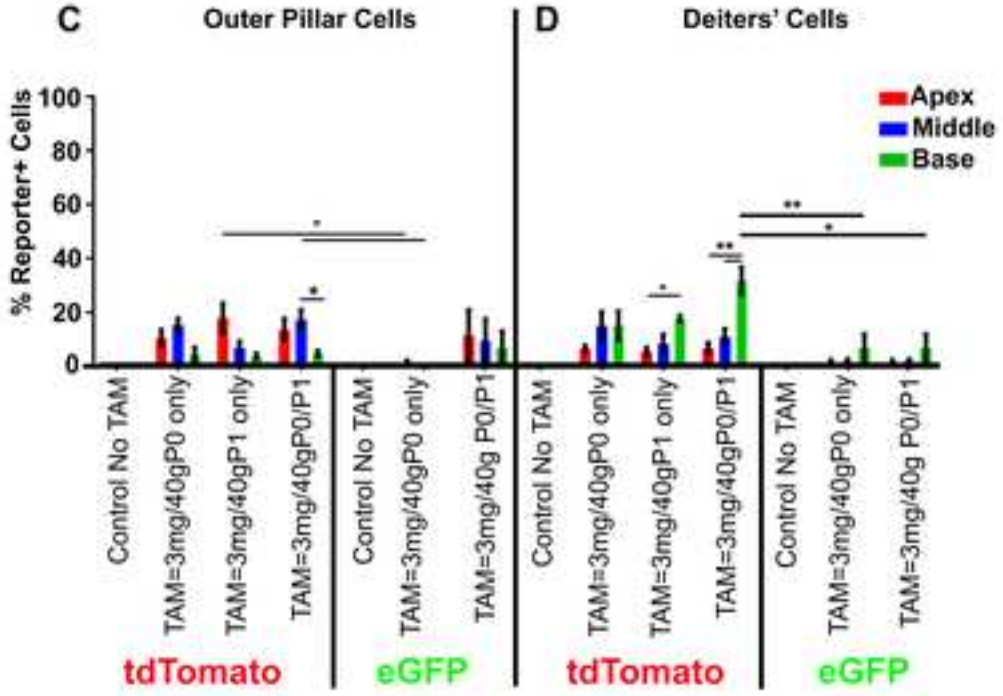

A
Outer Hair Cells
Inner Hair Cells
% Reporter+ HCs
100
80
60
40
20
0
***
R²=0.96
***
R²=0.97
Control No TAM
TAM=3mg/40g P0
TAM=3mg/40g P0-P1
1 2 3 4 5 6
Segment
1 2 3 4 5 6
Segment
Sox2CreERT2::ROSA26tdTomato
Control No TAM
TAM=3mg/40g P0 Only
B
OHCs
IHCs
MyosinVIIa
C
tdTomato
D
E
OHCs
IHCs
Apex
F
G
H
OHCs
IHCs
Base
I
J
K
OHCs
OHCs
OHCs
IHCs
Apex
L
Base

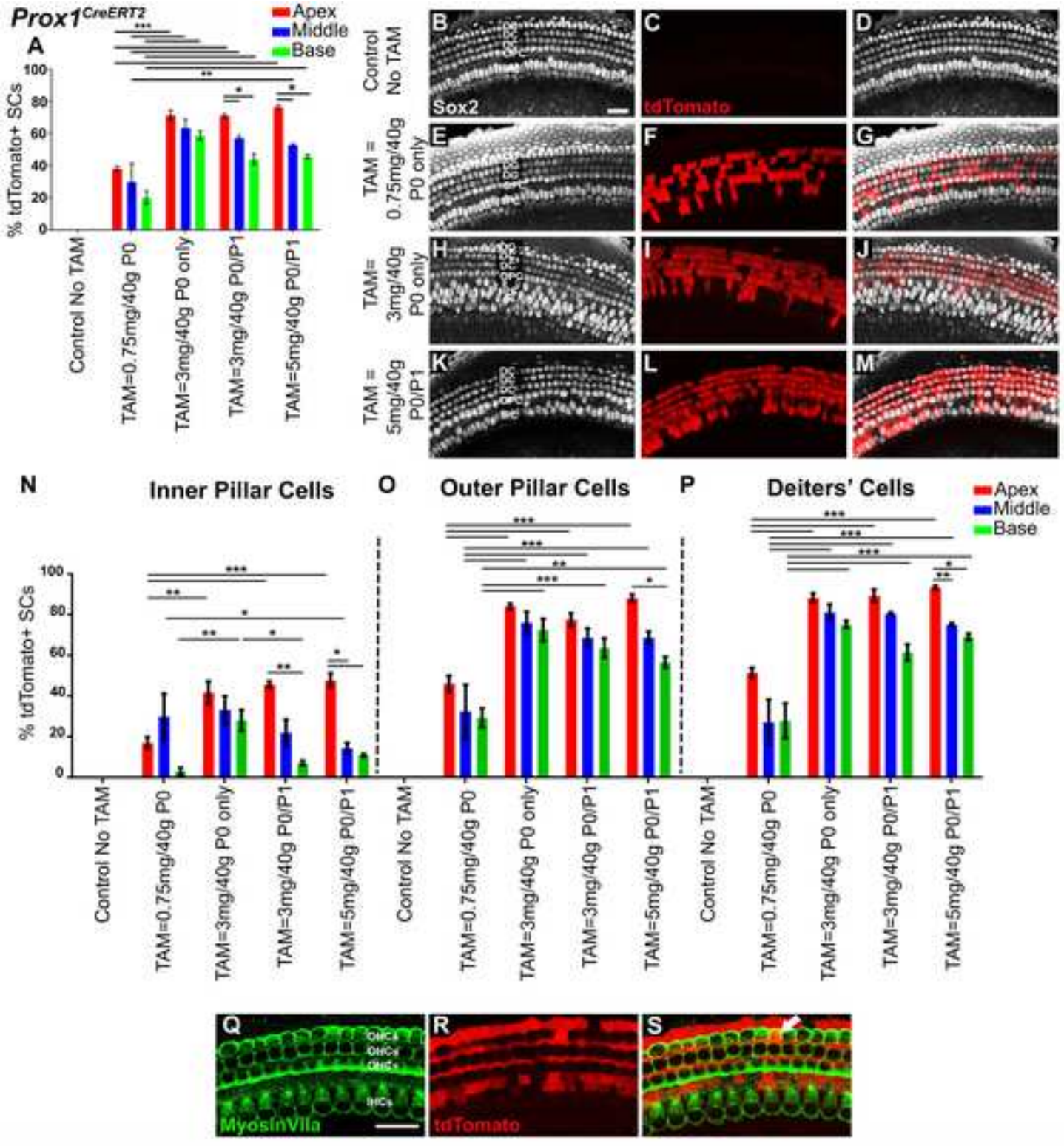

A
60
50
40
30
20
10
0
% Reporter+ HCs
Control No TAM
TAM = 3mg/40g P1
Apical Tip
Whole Cochlea
Sox2CreERT2::CAG-eGFP
Control No TAM
TAM=3mg/40g P1 Only
B
C
D
OHCs
IHCs
MyosinVIIa
eGFP
E
F
G
OHCs
IHCs
Apex
H
I
J
OHCs
IHCs
Base
K
OHCs
OHCs
OHCs
IHCs
Apex
L
Base


Plp-CreER^T2
A
Apex
Middle
Base
% Reporter+ SCs
100
80
60
40
20
0
Control No TAM
TAM=3mg/40g P0 only
TAM=3mg/40g P1 only
TAM=3mg/40g P0/P1
TAM=3mg/40g P0 only
TAM=3mg/40g P0/P1
tdTomato
eGFP
Control No TAM
TAM= 3mg/40g P0 only
TAM= 3mg/40g P1 only
TAM= 3mg/40g P0/P1
Sox2
tdTomato
eGFP
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S

Plp-CreER^{T2}


A
Inner Phalangeal Cells
B
Inner Pillar Cells
Apex
Middle
Base
% Reporter+ Cells
100
80
60
40
20
0
Control No TAM
TAM=3mg/40gP0 only
TAM=3mg/40gP1 only
TAM=3mg/40gP0/P1
TAM=3mg/40g P0/P1
tdTomato
eGFP


C
Outer Pillar Cells
D
Deiters' Cells
Apex
Middle
Base
% Reporter+ Cells
100
80
60
40
20
0
Control No TAM
TAM=3mg/40gP0 only
TAM=3mg/40gP1 only
TAM=3mg/40gP0/P1
TAM=3mg/40g P0/P1
tdTomato
eGFP


Prox1CreERT2
A
Apex
Middle
Base
% tdTomato+ SCs
Control No TAM
TAM=0.75mg/40g P0
TAM=3mg/40g P0 only
TAM=3mg/40g P0/P1
TAM=5mg/40g P0/P1
B
C
D
Control No TAM
Sox2
tdTomato
E
F
G
TAM = 0.75mg/40g P0 only
H
I
J
TAM= 3mg/40g P0 only
K
L
M
TAM = 5mg/40g P0/P1
N
Inner Pillar Cells
O
Outer Pillar Cells
P
Deiters' Cells
Q
R
S
MyosinVIIa
tdTomato

Authors do not have any professional or financial affiliations that may be perceived as a conflict of interest.