HHS Public Access

Author manuscript

Hear Res. Author manuscript; available in PMC 2024 February 01.

Published in final edited form as:

Hear Res. 2023 February ; 428: 108686. doi:10.1016/j.heares.2022.108686.

Fbxo2^{CreERT2}: A new model for targeting cells in the neonatal and mature inner ear

Melissa M. McGovern[a], **Byron Hartman**[b], **Ankita Thawani**[a], **Helen Maunsell**[c], **Hongyuan Zhang**[a], **Rizwan Yousaf**[a], **Stefan Heller**[b], **Jennifer Stone**[d], **Andrew K. Groves**[a,c,e]

[a.]Department of Neuroscience, Baylor College of Medicine, 1 Baylor Plaza, Houston, Texas, 77030

[b.]Department of Otolaryngology, Stanford University, Stanford, California, 94305

[c.]Program in Development, Disease Models & Therapeutics, Baylor College of Medicine, 1 Baylor Plaza, Houston, Texas, 77030

[d.]Department of Otolaryngology-Head and Neck Surgery, Virginia Merrill Bloedel Hearing Research Center, University of Washington School of Medicine, Seattle, WA 98195

[e.]Department of Molecular and Human Genetics, Baylor College of Medicine, 1 Baylor Plaza, Houston, Texas, 77030

Abstract

The mammalian inner ear contains six sensory patches that allow detection of auditory stimuli as well as movement and balance. Much research has focused on the organ of Corti, the sensory organ of the cochlea that detects sound. Unfortunately, these cells are difficult to access in vivo, especially in the mature animal, but the development of genetically modified mouse models, including Cre/Lox mice, has improved the ability to label, purify or manipulate these cells. Here, we describe a new tamoxifen-inducible CreER mouse line, the *Fbxo2*^{CreERT2} mouse, that can be used to specifically manipulate cells throughout the cochlear duct of the neonatal and mature cochlear epithelium. In vestibular sensory epithelia, *Fbxo2*^{CreERT2}-mediated recombination occurs

Corresponding Author: Melissa M. McGovern melissa.mcgovern@bcm.edu.

CRediT Author Statement

Melissa M. McGovern: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Writing – Original Draft, Visualization, Project Administration
Byron Hartman: Conceptualization, Methodology, Formal Analysis, Investigation, Writing – Original Draft
Ankita Thawani: Conceptualization, Methodology, Formal Analysis, Investigation, Writing – Original Draft
Helen Maunsell: Conceptualization, Methodology, Investigation, Writing – Original Draft
Hongyuan Zhang: Investigation, Methodology
Rizwan Yousaf: Methodology
Stefan Heller: Writing – Editing & Review, Formal Analysis, Resources, Supervision, Funding Acquisition
Jennifer Stone: Resources, Writing – Original Draft, Visualization, Supervision, Funding Acquisition
Andrew K. Groves: Conceptualization, Methodology, Writing – Editing & Review, Resources, Supervision, Funding Acquisition

Competing interests

Stefan Heller is a paid consultant for Pipeline Therapeutics and Lineage Therapeutics. No other authors declare significant competing interests.

in many hair cells and more rarely in supporting cells of neonatal and adult mice, with a higher rate of *Fbxo2*^{CreERT2} induction in type 1 versus type 2 hair cells in adults. *Fbxo2*^{CreERT2} mice, therefore, are a new tool for the specific manipulation of epithelial cells of the inner ear and targeted manipulation of vestibular type 1 hair cells.

Keywords

CreER/LoxP; cochlea; vestibular; utricle; Fbxo2; Fbx2

1. Introduction

The adult mammalian inner ear is encased in the hardest bone in the body, limiting access to the delicate structures of the hearing and balance organs. The use of genetic strategies, such as the Cre/loxP system, allows access to these structures to label and isolate cells or manipulate gene expression in the inner ear of living animals. The mammalian inner ear houses six sensory organs that are specialized for hearing and balance. The balance organs are housed in the utricle, saccule, and the lateral, anterior and posterior ampullae, and are located more dorsally where they encode head motion and gravity. The cochlea, which detects auditory stimuli, lies in the ventral half of the inner ear. Because of the extensive cellular heterogeneity of these organs, the development of new mouse lines that express Cre recombinase in the ear will increase the ability to target or purify distinct populations of inner ear cells.

Much of the research in the inner ear has focused on the cells of the organ of Corti, but there are fewer genetic tools to manipulate cells in other cell populations of the inner ear. For example, some cells in the inner sulcus, the interdental cells, anchor the tectorial membrane to the spiral limbus. The tectorial membrane is indispensable to the perception of sound, as it is the structure that deflects the stereocilia on hair cells thus initiating the depolarization of hair cells though the opening of K^+ channels (reviewed in Goodyear and Richardson, 2018). However, there has been comparatively little research into these cells, as well as the cells surrounding them. Additionally, cells in the outer sulcus, Claudius' cells and cells in the lateral wall, are critical for ion homeostasis of the cochlear endolymph. These cells participate in K^+ recycling by moving ions from the organ of Corti back to the stria vascularis, which is responsible for the maintenance of the endocochlear potential (reviewed in Wangemann, 2006). Again, few mouse lines are available to target Cre recombinase expression to these cells.

Vestibular sensory epithelia have two types of hair cells – type 1 and type 2 – and numerous supporting cells that interdigitate between the hair cells. While type 1 and type 2 hair cells have distinct morphologies, molecular markers, and electrophysiological features, less is known about their respective contributions to sensory detection (reviewed by Burns and Stone, 2017; Eatock and Songer, 2011). There are currently few Cre-expressing mouse lines that differentially target type 1 versus type 2 hair cells.

FBX2 is a protein component of the SCF (Skp1, Cullin1, F-box, Rbx1) ubiquitin ligase complex encoded by the *Fbxo2* gene and is expressed in the otocyst as early as E12.5

(Hartman et al., 2018). We developed a new Cre mouse line, the *Fbxo2*^{CreERT2} mouse line, which expresses Cre recombinase fused to an estrogen receptor that has been modified to make it more sensitive to the estrogen analogue tamoxifen. Here, we characterized the expression and activity of *Fbxo2*^{CreERT2} in the cochlea and vestibular system at three key stages: 1) embryonic, 2) neonatal, and 3) adult. We found that at embryonic stages, few cells in the inner ear are labeled by recombination in *Fbxo2*^{CreERT2} mice. However, in neonatal and mature cochleae, *Fbxo2*^{CreERT2} activity is observed in cells of the inner and outer sulcus, the spiral limbus, the stria vascularis, and some cells in the organ of Corti. In adult vestibular epithelia, *Fbxo2*^{CreERT2} activity is enriched in type 1 hair cells and can be detected in a small number of supporting cells. Therefore, *Fbxo2*^{CreERT2} mice provide a new tool to target cells of the hearing and balance systems.

1. Materials and Methods

2.1 Animal Models

Fbxo2^{CreERT2} knock-in mice were generated by targeted homologous recombination using the same strategy described for *Fbxo2*^{VHC} mice (Hartman et al., 2018). The CreERT2 open reading frame was PCR amplified from pCAG-CreERT2 (Matsuda and Cepko, 2007) and inserted into the PGKneoDTA dual selection targeting backbone (Hoch and Soriano, 2006). Homology arms flanking exon 1 of *Fbxo2* (6336 bp of 5` “left arm” and 2985 bp of 3` “right arm”) were PCR amplified from E14tg2a ES cell genomic DNA using high fidelity PCR and cloned into plasmids for sequencing as previously described (Hartman et al., 2018). The Fbxo2CreERT2pgkNeoDTA targeting plasmid was generated by sequentially ligating the right arm and the left arm into the CreERT2pgkNeoDTA plasmid, at the Nhe1/Sal1 and Mlu1/Fse1 sites respectively. All plasmids were fully sequenced and verified at each step. Sequences were designed and analyzed using the LaserGene 12 software suite (DNAstar). Further details on cloning and primer sequences available upon request.

The *Rosa26*$^{loxp\text{-}stop\text{-}loxp\text{-}tdTomato}$ mouse line (*Rosa26*^{tdTomato}; *AI9*; Jax catalog # 007909, Madisen et al., 20105) was obtained from the Jackson Laboratory. Genotyping for all modified alleles was performed by PCR amplification. The protocol and primers for genotyping are listed in Table 1. Cre recombination was induced by the administration of tamoxifen (T5648, Sigma), which was dissolved in peanut oil (P2144, Sigma). Embryonic recombination was induced by administering tamoxifen and progesterone by oral gavage (4mg/40g) to the pregnant dam. Neonatal and adult Cre recombination was induced by injection of tamoxifen (either 3mg/40g or 9mg/40g body weight at postnatal day [P] 7 and either 1 or 2 injections of 9mg/40g body weight at 3-6 weeks, IP). Animals were euthanized CO_2 inhalation followed by decapitation. All animal procedures were conducted at Baylor College of Medicine and approved by its Institutional Animal Care and Use Committee.

2.2 Histology

Embryonic tissue was harvested and fixed overnight in 4% paraformaldehyde (diluted 1:10 from 5016-02, Macron Fine Chemicals), dehydrated in 15% sucrose in PBS and embedded in 7.5% gelatin (300 Bloom, G2500, Sigma) for cryosectioning. 15-18μm sections were collected along the inner ear axis. Neonatal temporal bones were collected at P7 and

adult temporal bones were collected at 5-8 weeks. Neonatal and adult samples were fixed overnight at room temperature with 4% paraformaldehyde (16% stock, 18814-10, Polysciences) diluted in phosphate buffered saline (PBS), pH 7.4 (P3813, Sigma). Neonatal cochleae were decalcified with 0.25M EDTA (E4884, Sigma), pH 8.0 for 1 hour at room temperature following fixation. Cochlea were either dissected via whole mount preparation or embedded in optimal cutting temperature (OCT) compound and cryosectioned in a microtome-cryostat at 14 μM thickness. Adult cochleae were decalcified with 0.25M EDTA pH 8.0 for 2 days at room temperature. Cochlea were either dissected by whole mount dissection or embedded in 5% agarose (815, VWR) and sectioned at 80μm thickness using a Leica V1200S Vibratome. Adult utricles and lateral ampullae were isolated from the temporal bone followed by the removal of the otoconia or cupulae overlying the sensory epithelia by fine dissection or with a water jet.

Tissue was stained following a previously published protocol (McGovern et al., 2017) except that tissue was incubated with primary antibodies overnight at room temperature. Briefly, samples were blocked in PBS containing 1% Triton X-100 (T8787, Sigma), 10% normal horse serum (S-2000, Vector labs), and 1% bovine serum albumin (820451, MP Biochemicals). Primary and secondary antibodies were diluted in a solution of PBS containing 0.1% Triton X-100, 5% normal horse serum, and 1% bovine serum albumin. The following primary antibodies were used: rabbit anti-MYOSIN VIIA (25-6790, Proteus Biosciences), goat anti-SOX2 (AF2018, B&D), and rabbit anti-TUBB3 (MRB-435P, Covance). The following secondary antibodies were used: donkey anti-rabbit 647 (A31573, Invitrogen), donkey anti-rabbit 647 (A10042, Invitrogen) donkey anti-goat 488 (A11055, Invitrogen). Following secondary antibody incubations, samples were incubated with DAPI (1:10,000) to stain nuclei for 10-20 minutes at room temperature prior to a final series of washes and mounting. Samples were viewed and images collected using either a Zeiss Axio Observer epifluorescence microscope with Apotome structured illumination, a Zeiss 880 confocal microscope, or an Olympus FV100 confocal microscope. Digital images were quantified and processed using a combination of Zen lite, Fiji (https://fiji.sc), or Adobe Photoshop.

Neonatal were quantified from images taken of cryosections. Neonatal and adult cochlear cells as well as adult utricle cells were quantified from images taken of surface preparations. Quantification of each organ is reported as the percent of each cell type that is labeled with tdTomato.

2. Results and discussion

3.1 Generation of the *Fbxo2^{CreERT2}* Targeting Construct

Fbx2 is an F-box protein component of the SCF (Skp1, Cullin1, F-box, Rbx1) ubiquitin ligase complex (Nelson et al., 2007). It is expressed in the cochlear duct of the mouse during development and in the adult (Hartman et al., 2018; Nelson et al., 2007). Previously, Hartman et al. created and described the *Fbxo2VHC* mouse line, which expresses a CreER fusion protein downstream from an H2B-Venus fusion fluorescent reporter, and a HygroR resistance cassette (Hartman et al., 2018). This mouse gave only weak staining of inner ear tissue at embryonic and early postnatal ages, raising the possibility that the

long expression cassette containing three coding regions might reduce CreER expression. Here, we used the same targeting construct used to create the *Fbxo2VHC* mouse line but removed the upstream H2B-Venus and the HygroR sequences (Figure 1). Previously, homozygous mutants for Fbxo2 did not exhibit defects in viability or fertility but did show premature degeneration of the inner ear (Nelson et al., 2007). *Fbxo2*^{CreERT2} results in a deletion of the endogenous Fbxo2 protein coding sequence and therefore homozygous *Fbxo2*^{CreERT2} animals do not express Fbxo2. Animals homozygous for *Fbxo2*^{CreERT2} had no obvious negative health impacts, but unlike Fbxo2 knockout mice, did not show gross vestibular dysfunction, or degeneration of the mature cochlear epithelium (data not shown). Fbxo2^{CreERT2} heterozygous mice were used for all histological analyses in this study.

3.2 Activity of the *Fbxo2*^{CreERT2} mouse line in the embryonic ear

We chose 3 stages to examine *Fbxo2*^{CreERT2} mediated lineage tracing in mouse embryos: 1) E10.5, just after the otic cup invaginates, 2) E12.5, when the sensory cochlea is distinguishable, and 3) E15.5, as hair cells are differentiating (Figure 2A). Heads were harvested for analysis at E16.5 following tamoxifen administration at E10.5 or E12.5, and E18.5 for tamoxifen administration at E15.5. Very little tdTomato labeling was observed after Cre activation at E10.5 (data not shown) or E12.5 induction (Figure 2C). It was only following E15.5 Cre induction that we saw notable numbers of tdTomato-positive hair cells and supporting cells in the cochlear duct (Figure 2B, C). At this stage, tdTomato-positive cells were observed in the organ of Corti and sensory domains of the crista and maculae. Some cells of the non-sensory cochlear duct were also labeled in this experimental condition. Additionally, regions of the cerebellum, as well as the sensory olfactory epithelium and the mesenchymal core of the pinna showed robust tdTomato labeling (data not shown), suggesting additional roles for *Fbxo2* in embryonic development. Taken together, these data indicate that, while the *Fbxo2* gene is active during development, the *Fbxo2*^{CreERT2} only sparsely labels cells of the embryonic inner ear.

3.3 Activity of the *Fbxo2*^{CreERT2} mouse line in the neonatal vestibular system

To investigate the activity of the *Fbxo2*^{CreERT2} line at neonatal stages, recombination of tdTomato by *Fbxo2*^{CreERT2} was induced via tamoxifen injection at P0 and P1 (3mg/40g, IP) and tissue was collected one week later at P7-8. Samples were stained with antibodies against MYOSIN VIIA and SOX2. In contrast to the lack of significant Cre recombination in the embryonic inner ear we observed robust labeling of cells in the vestibular organs of the neonatal cochlea. In the utricular maculae tdTomato was detected in 63.8% ± 24.2% of Type I hair cells and 76.7% ± 9.8% of Type II hair cells, only 6.8% ± 3.7% of supporting cells ($p<0.001$ as determined by a 2-way ANOVA with a Tukey's multiple comparison post-hoc test, N=3, Figure 3A, D). In the saccule 67.3% ± 21.0% of Type I hair cells and 64.4% ± 18.6% of Type II hair cells were tdTomato-positive while only 9.5% ± 6.2% of supporting cells expressed tdTomato ($p=0.05$ 70 compared to Type I, and $p<0.05$ compared with Type II, as determined by a 2-way ANOVA with a Tukey's multiple comparison post-hoc test, N=3, Figure 3B, D). In the ampule, 77.9% ± 14.78% of Type I hair cells and 65.8% ± 2.5% of Type II hair cells were labeled, with fewer supporting cells expressing tdTomato (7.2% ± 3.2%, $p<0.0001$, as determined by a 2-way ANOVA with a Tukey's multiple comparison post-hoc test, N=3, Figure 3C, D).

We next investigated whether increased tamoxifen administration would increase the hair cell labeling of the vestibular organs. We injected *Fbxo2*^{CreERT2}*::Rosa26*^{tdTomato} animals with 9mg/40g tamoxifen (IP) on P0 and P1 by increasing the concentration of tamoxifen suspended in oil. Animals were again collected at P7 and analyzed for tdTomato expression. Significantly more Type I hair cells were labeled in the utricle (94.9% ± 4.6%, p=0.0141 as determined by a 2-way ANOVA with a Sidak's post-hoc test, N=3, Figure 3D) and fewer Type II hair cells were labeled in the ampullae (49.3% ± 2.8%, p=0.0050 as determined by a 2-way ANOVA with a Sidak's post-hoc test, N=3, Figure 3D). No differences were observed in supporting cells from any organ, nor was there a difference observed in the saccule. Therefore, *Fbxo2*^{CreERT2} will be a useful tool for specifically targeting both type 1 and type 2 hair cells in the vestibular organs with an enrichment for Type I hair cells at higher doses.

3.4 Activity of the *Fbxo2*^{CreERT2} mouse line in the neonatal cochlea

We next investigated the activity of *Fbxo2*^{CreERT2} in the neonatal cochlea where recombination of tdTomato by *Fbxo2*^{CreERT2} was induced via tamoxifen injection at P0 and P1 (either 3mg/40g or 9mg/40g, IP) and tissue was collected one week later at P7-8. Samples were stained with antibodies against MYOSIN VIIA and SOX2. We found significantly more hair cells were labeled with the higher dose of tamoxifen (34% ± 7.7% compared to 11.6% ± 4.0%, p<0.0001, as determined by a 2-way ANOVA with a Sidak's post-hoc test, N=3, Figure 3E-G), however, no other differences were observed. In addition, we wanted to investigate whether there was a difference in cell labeling across the three cochlear turns (apex, middle, and base). We found that there was a significant decrease in the number of Deiters' cells (10.8% ± 1.3% vs. 4.8% ± 1.4%) and Pillars cells (27.1% ± 3.3% vs. 3.8% ± 3.2%) labeled in the base compared to the apex (p<0.05, as determined by a 2-way ANOVA with a Tukey's multiple comparison post-hoc test, N=3, Figure 3F, H). There was also a trend in the increase of Hensen's cells labeled in the apex (4.9% ± 2.8%) vs. middle (35.4% ± 8.7%) turns (p=0.0557, as determined by a 2-way ANOVA with a Tukey's multiple comparison post-hoc test, N=3, Figure 3F, H). In addition, tdTomato expression was observed in Claudius' cells of the outer sulcus, the lateral wall, the inner sulcus, and the interdental cells of the spiral limbus. Thus, *Fbxo2*^{CreERT2} will be useful to activate recombination in cells of the inner and outer sulcus in the neonatal cochlea.

3.5 Activity of the *Fbxo2*^{CreERT2} mouse line in the adult cochlea

We next investigated the induction of the *Fbxo2*^{CreERT2} mouse line in mature cochlear tissue. Tamoxifen was administered to *Fbxo2*$^{CreERT2+/-}$*::Rosa26*$^{tdTomato+/f}$ animals at 5-6 weeks of age. Samples were collected 1-2 weeks later and stained with antibodies against MYOSIN VIIA and SOX2, and tdTomato labeling was observed by fluorescence of the native protein. tdTomato labeling was detected in the mature cochlear duct and was absent from the modiolus (Figure 4A). TdTomato labeled cells of the stria vascularis (Figure 4A'), the inner sulcus including the spiral limbus (Figure 4A"), the outer sulcus/lateral wall (Figure 4A'"), and the organ of Corti (Figure 4A'").

Fbxo2^{CreERT2} recombination was strongly detected in the medial and lateral non-sensory regions of the cochlea. Therefore, we further examined each region of the cochlear duct

to investigate in which cell types *Fbxo2*^{CreERT2} is active. In the inner sulcus, cells of the spiral limbus (likely interdental cells based on morphology) were labeled with tdTomato, while intermittent cells between the organ of Corti and the spiral limbus were labeled with tdTomato. Cells in the outer sulcus extending from the Hensen's cell and Claudius' cell regions to the spiral prominence expressed tdTomato. Some basal cells and fibrocytes of the stria vascularis were also labeled with tdTomato.

We dissected cochlear tissue from our labeled mice (Figure 4B) and quantified the number of cells that expressed tdTomato in whole mount preparations. Specifically, 6.8% ± 6.8% of hair cells were labeled in the apex, 6.4% ± 1.8% in the middle, and no cells in the base (n=2-3). Labeling was highest in Hensen's cells with 50.6% ± 3.7% labeled in the apex, 95.7% ± 7.4% in the middle, and 80.8% ± 27.2% in the base (n=2-3, Figure 4C). Other supporting cells were labeled at a lower rate, with ~10-23% of pillar cells and 18-41% of Deiters' cells labeled (n=2-3, Figure 4C). Labeling of inner phalangeal and border cells was moderate with ~25-62% cells labeled (n=2-3, Figure 4C). Without tamoxifen administration, Fbxo2^{CreERT2} recombined in very few cells in the cochlear duct (Data not shown).

We tested whether the efficiency of *Fbxo2*^{CreERT2} –mediated recombination would be increased following a second injection of tamoxifen. *Fbxo2*$^{CreERT2+/-}$::*Rosa26*$^{tdTomato+/f}$ animals were injected with tamoxifen (225ug/g, IP) on two consecutive days at 3 weeks of age. Cochleas were collected and analyzed 2 weeks later at 5 weeks of age. We saw no significant increase in the number of supporting cells labeled compared to a single dose of tamoxifen. Two doses of tamoxifen labeled ~80-90% of Hensen's cells, ~20-50% of Deiters' cells, ~30-40% of outer pillar cells, ~20-50% of inner pillar cells, and ~25-55% of Inner phalangeal and border cells (Figure 4). We saw a trend towards greater labeling of hair cells with two doses of tamoxifen: a single dose of tamoxifen labeled 5.1% ± 2.9% of inner and outer hair cells combined, whereas two doses labeled 19.1% ± 5.5%, but this did not reach statistical significance (p=0.1349, as determined by a Two-Way ANOVA with a Sidak's multiple comparison test). However, when labeled inner hair cells were analyzed alone, we saw a significant increase in labeling with 2 injections (37.8% ± 10.5% compared with 5.1% ± 2.9% with a single injection, p=0.0313 as determined by a Two-Way ANOVA with a Sidak's multiple comparison test). Along the axis of the cochlear duct, *Fbxo2*^{CreERT2} targeted 57.1% ± 5.2% of inner hair cells in the apical turn, 37.2% ± 14.6% in the middle turn, and 19.3% ± 15.1% in the basal turn. However, only 0.2-0.5% of outer hair cells were targeted by *Fbxo2*^{CreERT2}. Therefore, a single injection of tamoxifen in adult animals largely labels only supporting cells in the organ of Corti, whereas two doses of tamoxifen increased inner hair cell labeling. These data suggest that the *Fbxo2*^{CreERT2} mouse line will be useful for investigating cells of the mature inner and outer sulcus as well as mosaic labeling of cells in the organ of Corti

3.6 Activity of the *Fbxo2*^{CreERT2} mouse line in the adult vestibular system

Fbxo2^{CreERT}::Rosa26^{tdTomato} mice were injected once with tamoxifen (9mg/40g, IP) as 6 weeks of age and cochleas were analyzed at 8 weeks. We saw abundant tdTomato expression in the hair cells of the mature utricles (Figure 5A-E) and lateral ampullae (Figure G-I). However, we saw little labeling of supporting cells (<20 cells per organ, Figure 5D), cells in

the transitional epithelium (TE) surrounding the organs, or connective tissue cells underlying the sensory epithelia (not shown). We saw only rare labeling in utricles of mice that did not receive tamoxifen (Figure 5A). More extrastriolar Type I hair cells (89.3% ± 8.8%) were tdTomato-positive compared to exrastriolar Type II hair cells (Figure 5A-F)35.3% ± 7.8%, $p<0.001$, as determined by a Two-Way ANOVA with a Tukey's multiple comparison post-hoc test, N=3) or striolar Type I hair cells (54.4% ± 20.8%, $p<0.001$, as determined by a Two-Way ANOVA with a Tukey's multiple comparison post-hoc test, N=3). We investigated whether we could increase the number of Type II hair cells labeled by injecting *Fbxo2*^{CreERT2}::Rosa26^{tdTomato} mice with a second dose of tamoxifen, however there was no change in the number of cells labeled (Figure 5F).

In lateral ampullae, there were more tdTomato-labeled Type I cells (97.7% ± 2.0%) than Type II of cells (42% ± 14.3%, $p<0.001$) in the peripheral zone and more Type I and Type II cells in the peripheral zone compared to the central zone (63.1% ± 9.1% Type I, $p<0.01$, and 43.3% ± 6.0% Type II, $p<0.001$, as determined by a One-Way ANOVA with a Tukey's multiple comparison post-hoc test, N=3, Figure 5G-J). Therefore, *Fbxo2*^{CreERT2} mice label many type 1 hair cells in the peripheral zones of the utricles and lateral ampullae of adult mice. *Fbxo2*^{CreERT2} mice may be suitable for manipulating different types of vestibular hair cells in a region-dependent fashion.

3.7 Summary

We describe a new mouse model that is drives tamoxifen-dependent Cre activity in the neonatal and mature inner ear, but is largely absent from the developing inner ear. In the vestibular apparatus, *Fbxo2*^{CreERT2} is active quite specifically in hair cells and is enriched in Type 1 hair cells. In the cochlear duct, *Fbxo2*^{CreERT2} shows robust activity in non-sensory cells and to a lesser degree in the cells of the organ of Corti. *Fbxo2*^{CreERT2}, therefore, will be useful for targeting vestibular hair cells and non-sensory cells of the cochlea.

Acknowledgements

This work was funded by NIH grants RO1DC014832 (awarded to Andrew K. Groves) and RO1DC013771 (awarded to Jennifer Stone), and a mini grant from the Virginia Merrill Bloedel Hearing Research Center (Jennifer Stone). We thank Alyssa Crowder for assistance with animal husbandry, Jialin Shang and Tot Nguyen for assistance with histology and quantitative analysis, and Kim Miller and Glen MacDonald for help with confocal imaging.

References

Burns JC, Stone JS, 2017. Development and regeneration of vestibular hair cells in mammals Seminars in Cell and Developmental Biology 65, 96–105 10.1016/J.SEMCDB.2016.11.001 [PubMed: 27864084]

Eatock RA, Songer JE, 2011. Vestibular hair cells and afferents: Two channels for head motion signals. Annual Review of Neuroscience 34, 501–534. 10.1146/ANNUREV-NEURO-061010-113710

Goodyear RJ, Richardson GP, 2018. Structure, Function, and Development of the Tectorial Membrane: An Extracellular Matrix Essential for Hearing, in: Current Topics in Developmental Biology. Academic Press Inc., pp. 217–244. 10.1016/bs.ctdb.2018.02.006

Hartman BH, Böscke R, Ellwanger DC, Keymeulen S, Scheibinger M, Heller S, 2018. Fbxo2VHC mouse and embryonic stem cell reporter lines delineate in vitro-generated inner ear sensory epithelia cells and enable otic lineage selection and Cre-recombination. Developmental Biology 443, 64–77. 10.1016/j.ydbio.2018.08.013 [PubMed: 30179592]

Hoch R. v., Soriano P, 2006. Context-specific requirements for Fgfr1 signaling through Frs2 and Frs3 during mouse development. Development 133, 663–673. 10.1242/DEV.02242 [PubMed: 16421190]

Madisen L, Garner AR, Shimaoka D, Chuong AS, Klapoetke NC, Li L, van der Bourg A, Niino Y, Egolf L, Monetti C, Gu H, Mills M, Cheng A, Tasic B, Nguyen TN, Sunkin SM, Benucci A, Nagy A, Miyawaki A, Helmchen F, Empson RM, Knöpfel T, Boyden ES, Reid RC, Carandini M, Zeng H, 2015. Transgenic mice for intersectional targeting of neural sensors and effectors with high specificity and performance. Neuron 85, 942–958. 10.1016/j.neuron.2015.02.022 [PubMed: 25741722]

Matsuda T, Cepko CL, 2007. Controlled expression of transgenes introduced by in vivo electroporation. Proc Natl Acad Sci U S A 104, 1027–1032. 10.1073/PNAS.0610155104 [PubMed: 17209010]

McGovern MM, Brancheck J, Grant AC, Graves KA, Cox BC, 2017. Quantitative Analysis of Supporting Cell Subtype Labeling Among CreER Lines in the Neonatal Mouse Cochlea. Journal of the Association for Research in Otolaryngology 18, 227–245. 10.1007/s10162-016-0598-0 [PubMed: 27873085]

Nelson RF, Glenn KA, Zhang Y, Wen H, Knutson T, Gouvion CM, Robinson BK, Zhou Z, Yang B, Smith RJH, Paulson HL, 2007. Selective cochlear degeneration in mice lacking the F-box protein, Fbx2, a glycoprotein-specific ubiquitin ligase subunit. Journal of Neuroscience 27, 5163–5171. 10.1523/JNEUROSCI.0206-07.2007 [PubMed: 17494702]

Wangemann P, 2006. Supporting sensory transduction: Cochlear fluid homeostasis and the endocochlear potential. Journal of Physiology, 10.1113/jphysiol.2006.112888

Highlights

- *Fbxo2*^{CreERT2} provides a new tool for manipulating gene expression the inner ear.
- Recombination occurs throughout the cochlear duct in the neonatal and adult inner ear.
- Hair cells in the neonatal vestibular end organs are targeted by *Fbxo2*^{CreERT2}.
- *Fbxo2*^{CreERT2} recombination is enriched in Type 1 hair cells in the mature maculae and cristae of the vestibular system

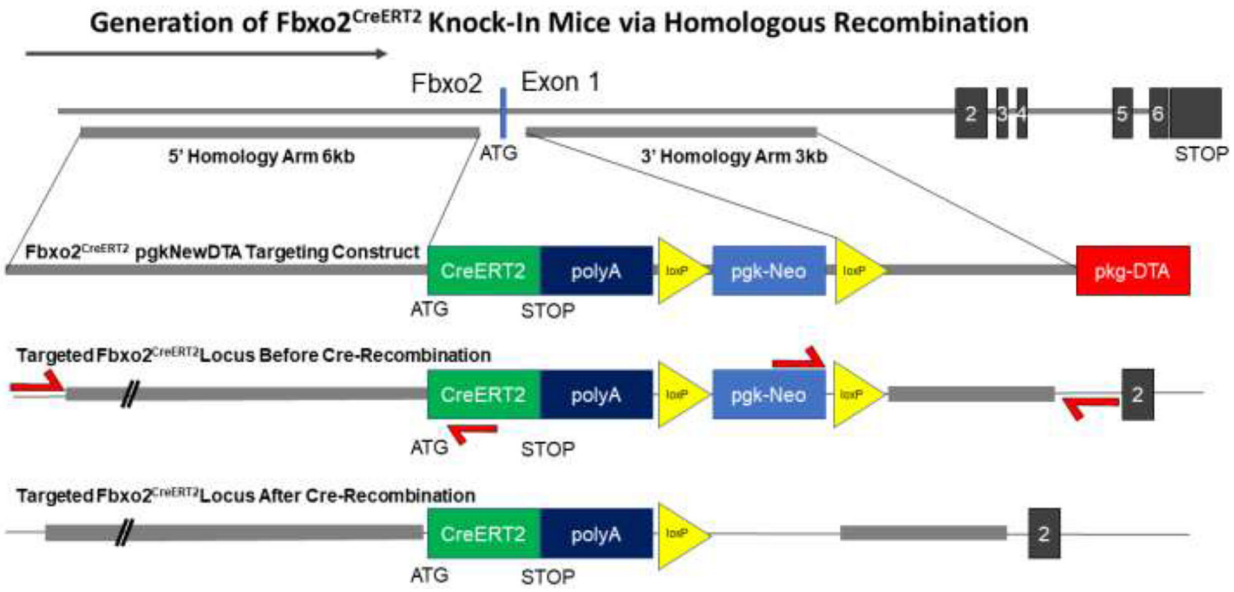

Figure 1: Diagram showing the generation of *Fbxo2*^{CreERT2} mice.

The targeting strategy is modified from the approach used to generate *Fbxo2*^{VHC} mice (Hartman et al., 2018). The wild type *Fbxo2* locus on Chr 4 contains six exons, with the ATG start site for Fbx2 in Exon 1. The Fbxo2VHCpgkNeoDTA targeting vector (Hartman et al., 2018) was modified to remove Venus and Hygromycin resistance coding regions, and then constructed with homology arms from the indicated regions of Chr 4, flanking a cassette containing a CreERT2 coding region followed by floxed pgk-Neo for positive selection. A pgk-DTA cassette was positioned downstream of the 3′-homology arm, for negative selection of off-target genomic insertions. The third line shows a representation of the correctly targeted Fbxo2-CreER locus, replacing Exon 1 of Fbxo2 with the targeting cassette and]pgk-Neo, which was used for selection of ES cell colonies. Red arrows represent the location of primers for PCR screening of ES cell clones. Verified clones were used to make chimeric mice and five clones were transmitted to offspring to produce viable mouse lines. The bottom schematic is the Fbxo2-CreER locus after removal of the pgk-Neo cassette in the germ-line of mice by crossing to CMV-Cre transgenic mice.

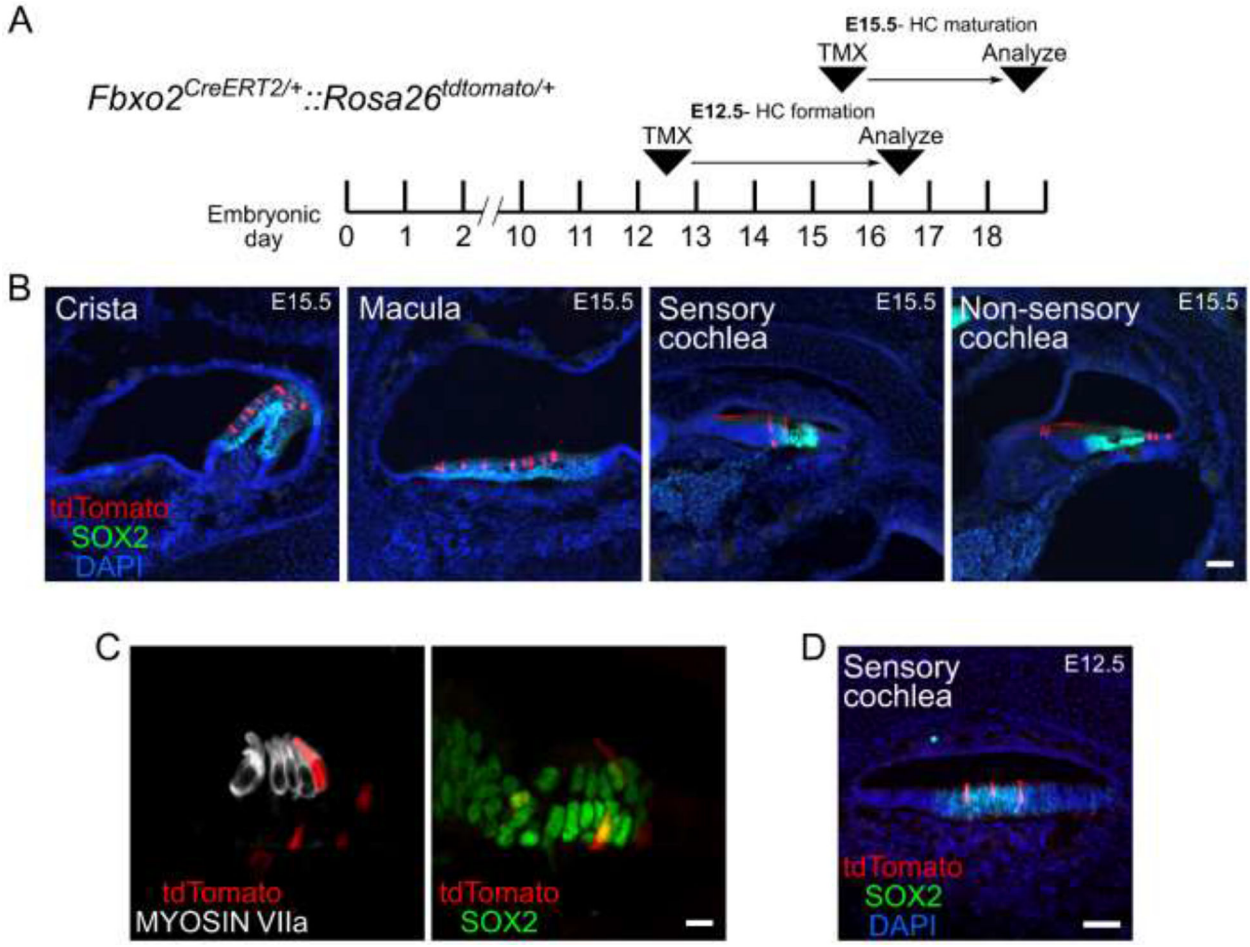

Figure 2. ***Fbxo2*^{CreERT2} lineage tracing is weak at embryonic stages**

A) Experimental paradigm for lineage tracing induction at two embryonic stages- E12.5 and E15.5. **B)** Representative tdTomato (red) lineage tracing labeling in vestibular sensory patches (lateral crista and utricle), and the cochlear duct from E15.5 induction; SOX2 (green), DAPI (blue). **C)** High resolution images of hair cells and supporting cells from an E15.5 induced embryo labeled with MYOSIN VIIa (white), SOX2 (green) to show tdTomato (red) colocalization. **D)** Representative tdTomato (red) lineage tracing labeling in sensory cochlea from E12.5 induction; SOX2 (green), DAPI (blue). Scale bar: B&D = 50μm, C = 10μm.

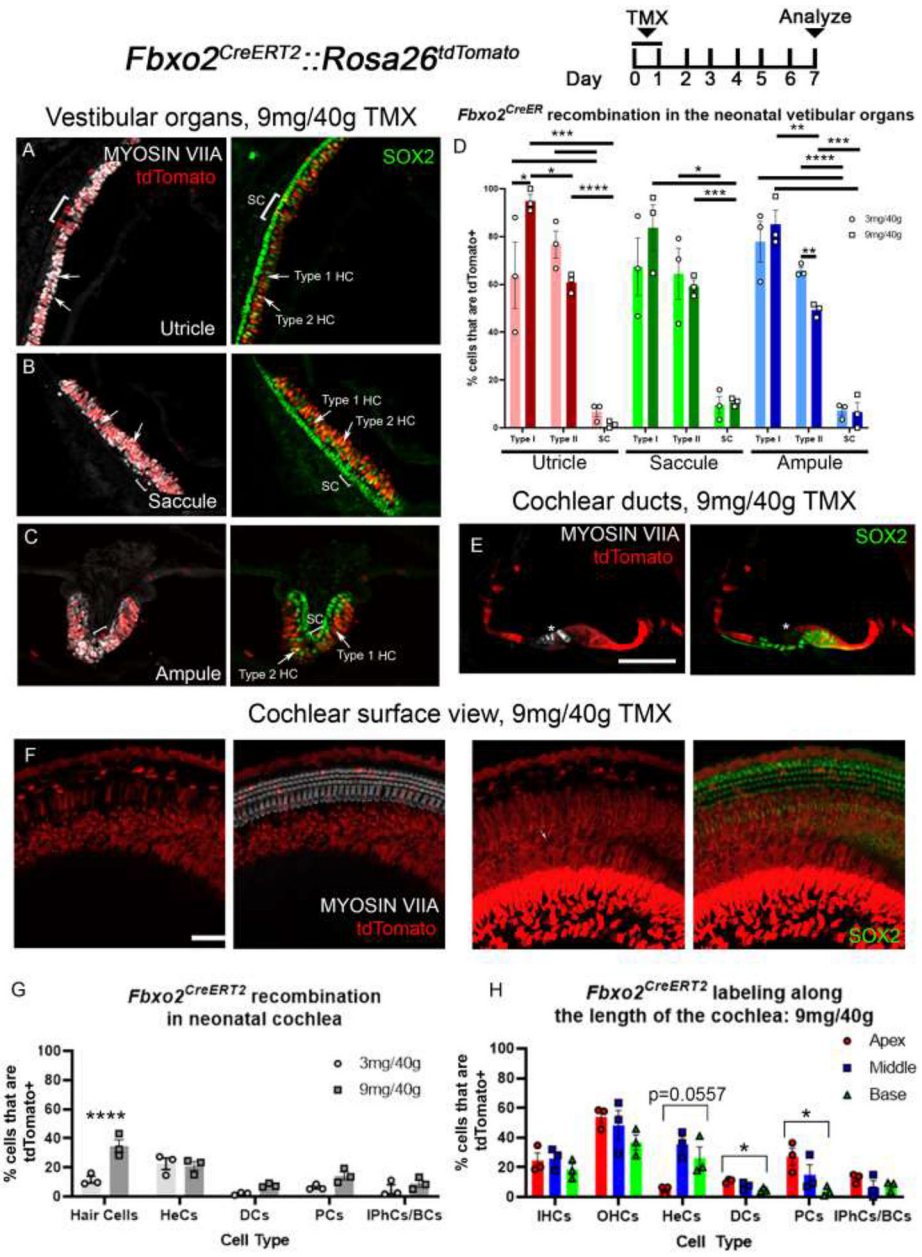

Figure 3: *Fbxo2*^{CreERT2} induction labels many cells in the cochlear duct and vestibular organs of the neonatal mouse.

CreER recombination was induced in the neonatal cochlae by injection of tamoxifen (9mg/40g, IP) at P0 and P1 and was visualized by the expression of the tdTomato reporter (red) at P7. tdtomato expression is detected in the **A)** utricle, **B)** Saccule, and **C)** Ampule of the neonatale vestibular apparatus. **D)** Quantification of the percentage tdTomato-positive Type I hair cells, Type II hair cells, and supporting cells. **E)** tdTomato was observed throughout the non-sensory regions of the cochlear duct **F-H)** tdTomato expression was quantified in the organ of Corti. Arrows in A-C indicate Type I or Type II hair cells, brackets in A-B indicate supporting cells, asterisk in E indicates organ of Corti. Scale bar: A,E = 100 μm, F = 50 μm. Abbreviations in G and H: HeCs = Hensens' cells, DC = Deiters' cells, PC = pillar cells,

IPhCs = inner phalangeal cells, BCs = Border Cells, IHCs = inner hair cells, OHCs = outer hair cells.

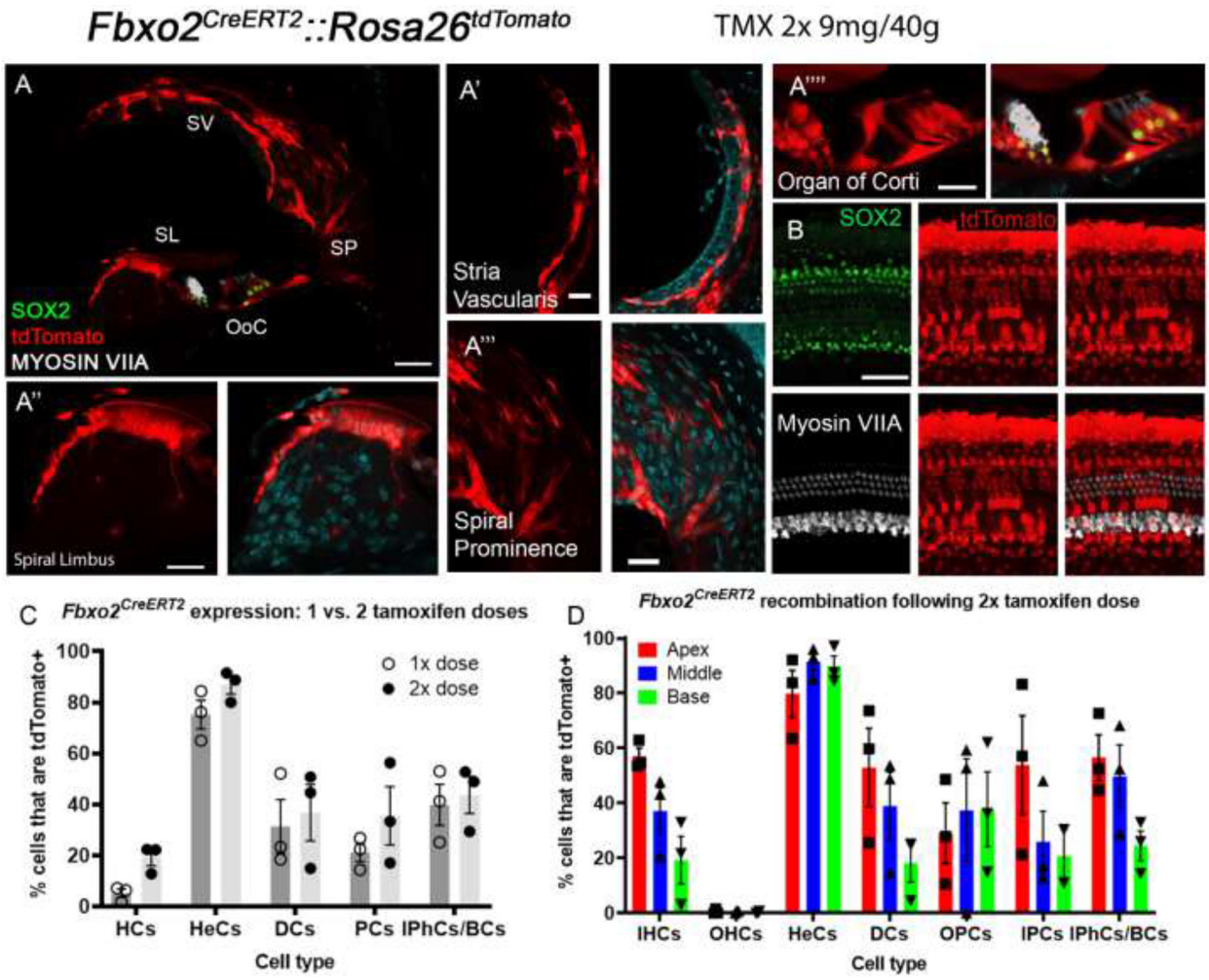

Figure 4: *Fbxo2*^{CreERT2} nduction labels cells In the cochlear duct of mature animals.

CreER recombination was induced at either 3 weeks or 5-6 weeks of age and samples were collected at least one week following tamoxifen (9mg/40g). CreER recombination was visualized using the tdTomato reporter line (Red). **A)** tdTomato is detected throughout the cochlear duct. **A')** tdToamto is observed in the stria vascularis, **A")** the interdental cells of the spiral limbus, and **A'")** in the lateral wall. **A""-B)** Few MYOSIN VIIA-positive cells (White). **C-D)** tdTomato expression was quantified in the hair cells (white) and supporing cells (Green) of the organ of Corti. Scale bar for A, B=50um scale bar for A'-A"" = 25um. Abbreviations in A: SV = stria vascularis, SP = spiral prominence, SL = spiral limbus, OoC = organ of Corti. Abbreviations in C and D: HeCs = Hensens' cells, DC = Deiters' cells, PC = pillar cells, IPhCs = inner phalangeal cells, BCs = Border Cells, IHCs = inner hair cells, OHCs = outer hair cells. OPCs = outer pillar cells, IPCs = inner pillar cells.

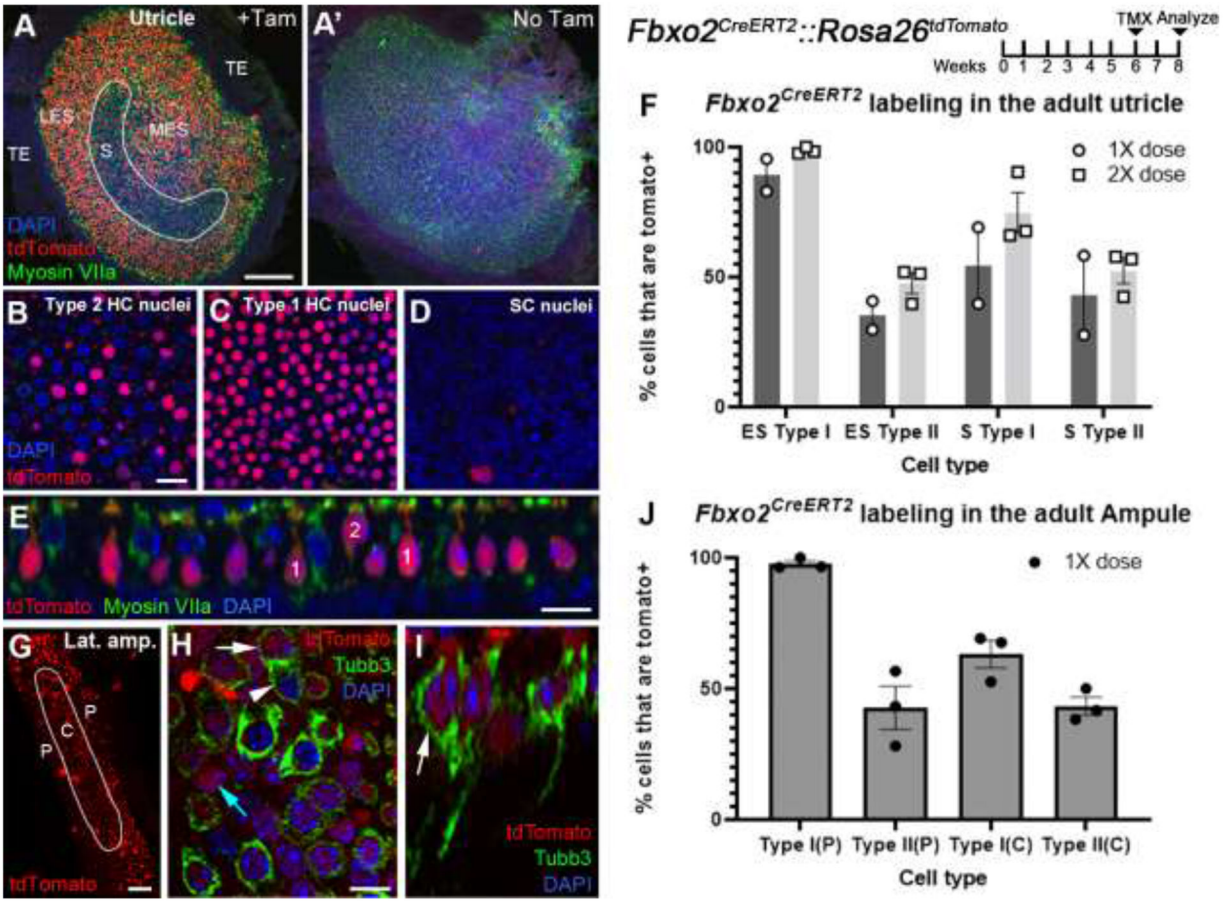

Figure 5: *Fbxo2*^{CreERT2} induction is higher in type 1 hair cells than type 2 hair cells in mature vestibular organs.

All images were generated from Z series confocal slices. Top-down views of whole-mount utricles at 7 days post-tamoxifen (Tam) **(A)** or without tamoxifen treatment **(A').** The sensory epithelium is defined by hair cell labeling with anti-Myo7a antibodies. The three zones of the sensory epithelium (LES, S, MES) are shown in A, as is the location of the transitional epithelium (TE), which surrounds the sensory epithelium. **B-D)** Top-down views (optical slices) of a portion of the lateral extrastriola shows tdTomato-labeled cells in the type 2 cell nuclear layer (B), the type 1 cell nuclear layer (C), and the supporting cell (SC) nuclear layer (D). **E)** Digital reslice (transverse through the utricular sensory epithelium) showing tdTomato labeling in type 1 (1) and type 2 (2) hair cells. **F)** Graph shows percent tdTomato-positive hair cells in each region of the utricular sensory epithelium. Abbreviations: ES = extrastriolar, S = striolar. **G)** Top-down view of a whole-mount lateral ampulla (Lat. Amp) at 7 days post-tamoxifen. P = peripheral; C = central. The approximate position of the central zone is denoted by the white line. **H)** A portion of the central zone is shown in H (top down view). The white arrow points to a tdTomato-labeled type 1 hair cells in a compound calyx labeled with antibodies to ßIII tubulin (Tubb3). The white arrowhead points to a tdTomato-negative cell in the same calyx complex. The blue arrow points to a tdTomato-positive type 2 cell. **I)** A portion of the peripheral zone is shown in I. The white arrow points to a tdTomato-positive type 1 hair cell surrounded by a calyx. **J)** Graph

showing the percent of tdTomato-positive hair cells in each region of the lateral ampulla's sensory epithelium. n=3 mice. Error bars = standard deviations. Scale bars: A = 150 μm (applies to A,A'), B = 10 μm (applies to B-D), E = 10 μm, G = 30 μm, and H = 5 μm (applies to H,I).

Table 1:

Genotyping Primers

Target gene	Forward Primer	Reverse Primer
Fbxo2 Wild Type	5` GTACAGGACTCCCAGCCCTT 3`	5` GGATCACCATCTCCATCCATCGC 3`
Fbxo2^{CreERT2}	5` GCCTGCATTACCGGTCGATGCAACGA 3`	5` GTGGCAGATGGCGCGGCAACACCATT 3`
Rosa26^{tdTomato}	5` CTGTTCCTGTACGGCATGG 3`	5` GGCATTAAAGCAGCGTATCC 3`
Rosa26 Wild Type	5` AAGGGAGCTGCAGTGGAGTA 3`	5` CCGAAAATCTGTGGGAAGTC 3`