

# ***Fgf8* Genetic Labeling Reveals the Early Specification of Vestibular Hair Cell Type in Mouse Utricle**

Evan M. Ratzan<sup>1,2</sup>, Anne M. Moon<sup>3,4</sup>, and Michael R. Deans<sup>1,5,#</sup>

<sup>1</sup>Department of Neurobiology and Anatomy, University of Utah School of Medicine, Salt Lake City, UT 84112, USA

<sup>2</sup>Interdepartmental Program in Neuroscience, University of Utah School of Medicine, Salt Lake City, UT 84112, USA

<sup>3</sup>Departments of Molecular and Functional Genomics and Pediatrics, Weis Center for Research, Geisinger Clinic and Geisinger Commonwealth School of Medicine, Danville, PA 17822, USA

<sup>4</sup>Departments of Pediatrics and Human Genetics, University of Utah, Salt Lake City, UT 84112 USA

<sup>5</sup>Department of Surgery, Division of Otolaryngology, University of Utah School of Medicine, Salt Lake City, UT 84132, USA

#Correspondence should be addressed to MRD at [michael.deans@utah.edu](mailto:michael.deans@utah.edu)

## Abstract

FGF8 signaling plays diverse roles in inner ear development, acting at multiple stages from otic placode induction to cellular differentiation in the organ of Corti. As a secreted morphogen with diverse functions, *Fgf8* expression is likely to be spatially restricted and temporally dynamic throughout inner ear development. We evaluated these characteristics using genetic labeling mediated by *Fgf8*<sup>mcm</sup> gene-targeted mice and determined that *Fgf8* expression is a specific and early marker of Type-I vestibular hair cell identity. *Fgf8*<sup>mcm</sup> expression initiates at E11.5 in the future striolar region of the utricle, labeling hair cells following EdU birthdating, and demonstrates that subtype identity is determined shortly after terminal mitosis. This early fate specification is not apparent using markers or morphological criteria that are not present before birth in the mouse. While analyses of *Fgf8* conditional knockout mice did not reveal developmental phenotypes, the restricted pattern of *Fgf8* expression suggests that functionally redundant FGF ligands may contribute to vestibular hair cell differentiation and supports a developmental model in which Type-I and Type-II hair cells develop in parallel rather than from an intermediate precursor.

**Summary Statement:** The two classes of vestibular hair cell are established during embryogenesis and are readily distinguished from each other by *Fgf8* expression shortly after terminal mitosis.

**Keywords:** FGF8, hair cell, vestibular, inner ear, Cre recombinase

## Introduction

The vestibular sensory organs of the vertebrate inner ear contain mechanosensory receptors called hair cells that enable the detection of linear and rotational accelerations of the head, and gravity. Vestibular hair cells can be broadly divided into two classes based upon morphological and synaptic criteria. Type-I hair cells have a characteristic ampoule-like morphology and an afferent nerve terminal which forms a large calyx surrounding the hair cell. In contrast, Type-II hair cells have a columnar morphology and afferent nerve terminals that resemble synaptic boutons. Type-I hair cells are also physiologically unique with low-voltage activated  $K^+$  currents that enable faster receptor potentials with smaller voltage responses than Type-II hair cells (Rennie et al., 1996, Rusch et al., 1998, Eatock and Songer, 2011).

In the mouse, the terminal mitosis and differentiation of vestibular hair cells begins at embryonic day 11.5 (E11.5) and continues through postnatal development with over half of the hair cells emerging after birth (Burns et al., 2012, Raft et al., 2007, Ruben, 1967). It has been proposed that hair cells appear sequentially with the majority of Type-I hair cells developing at embryonic stages and Type-II hair cells emerging postnatally (McInturff et al., 2018). A noteworthy difference between these cell types is the requirement for postnatal expression of *Sox2* in Type-II hair cells as revealed by the increased number of Type-I hair cells that appear at the expense of the Type-II population after postnatal deletion of *Sox2* (Lu et al., 2019). This has led to the alternative hypothesis that cell identity is determined by *Sox2* and is not established until postnatal development, concurrent with *Sox2* downregulation in the Type-I cells (Warchol et al., 2019, Hume et al., 2007, Oesterle et al., 2008). The commonalities and differences between these alternative etiologies have been difficult to resolve because the molecular, morphological and synaptic characteristics that distinguish hair cell subtypes are also not available until later postnatal stages (P2-P12) (Simmons et al., 2010, Perry et al., 2003, McInturff et al., 2018).

Fibroblast growth factor 8 (FGF8) belongs to a family of secreted morphogens that signal locally to neighboring cells to promote differentiation. For example, in the organ of Corti, FGF8 released from inner hair cells induces neighboring pillar cell differentiation. Fewer inner pillar cells differentiate in

*Fgf8* knockouts whereas overexpression leads to ectopic pillar cell formation (Jacques et al., 2007). FGF8 binds with varying specificity and affinity to FGF receptor tyrosine kinases (FGFR1-3) activating intracellular signaling cascades leading to transcription factor activation (Ornitz et al., 1996, Pirvola et al., 2000, Roehl and Nusslein-Volhard, 2001, Firnberg and Neubuser, 2002). Outside of the ear, *Fgf8* is expressed in multiple important signaling centers during embryogenesis and the complete loss of FGF8 function causes early embryonic lethality by disrupting movements of nascent mesoderm in the primitive streak (Crossley and Martin, 1995, Meyers et al., 1998, Sun et al., 1999, Moon and Capecchi, 2000). Studies using hypomorphic *Fgf8* alleles and Cre/Lox-mediated conditional mutagenesis have elucidated critical roles for FGF8 in left-right axis determination, formation and patterning of the forebrain, olfactory bulb and the cerebellum/midbrain-hindbrain junction, pharyngeal gland development, cardiovascular and craniofacial development, renal development, and limb outgrowth and patterning (Frank et al., 2002, Boulet et al., 2004, Macatee et al., 2003, Reifers et al., 1998, Wright and Mansour, 2003, Lee et al., 1997, Crespo-Enriquez et al., 2012, Griffin et al., 2013, Gao et al., 2018, Lewandoski et al., 2000, Moon and Capecchi, 2000). In the developing neocortex a mutually opposing balance between FGF8 and *Emx2* and related transcription factors contributes to functional domain patterning in a process called arealization (Fukuchi-Shimogori and Grove, 2001, O'Leary and Nakagawa, 2002, Muzio and Mallamaci, 2003). Thus, FGF8 can regulate tissue patterning by signaling between neighboring domains in addition to neighboring cells and is generally necessary for multiple differentiation and patterning events throughout embryonic development.

The diverse roles of FGF8 likely mean that *Fgf8* expression is spatially restricted at different stages of otic development and also highly dynamic as development progresses from one stage to the next. To evaluate this, we examined the spatial and temporal dynamics of *Fgf8* expression during inner ear development using Cre-mediated genetic labeling and *in situ* hybridization (ISH). These analyses were directed towards the vestibular maculae because domains of *Emx2* expression in the utricle and saccule determine hair cell stereociliary bundle orientation (Holley et al., 2010, Jiang et al., 2017) and the reciprocal relationship between FGF8 and *Emx2* determines a boundary of *Emx2* expression in the neocortex (Fukuchi-Shimogori and Grove, 2003, Fukuchi-Shimogori and

Grove, 2001). We found transient *Fgf8* expression in auditory and vestibular neurons, and sustained, specific expression in Type-I hair cells beginning shortly after terminal mitosis at E11.5 and continuing through postnatal development. Genetically labeled vestibular hair cells were distributed throughout the sensory epithelia, exhibited ampoule-like morphology, calyceal afferents, and expressed the molecular marker *Spp1/Osteopontin* all of which are consistent with the Type-I hair cell identity. Despite this unique pattern of expression, *Fgf8* conditional knockout mice showed no changes in hair cell types, stereociliary bundle polarity and organization, or formation of the calyx-synapse suggesting that, as observed in other developmental contexts, there may be functional redundancy with other FGF family members during hair cell development. While the contribution of FGF signaling to vestibular development remains unknown, genetic labeling experiments demonstrate that hair cell sub-type specification occurs earlier than anticipated, happening shortly after terminal mitosis, and establish *Fgf8* expression is the earliest marker distinguishing vestibular hair cell types.

## Materials and Methods

### Design and Construction of the *Fgf8*<sup>mcm</sup> Allele

*Fgf8*<sup>mcm</sup> mice were generated by inserting an IRES sequence (Jang and Wimmer, 1990, Kaminski et al., 1990) upstream of the *mER:Cre:mER* cDNA encoding a fusion protein with two copies of the mouse Estrogen Receptor (mER) flanking Cre recombinase, and a Neomycin-resistance (*NeoR*) selection cassette flanked by Frt recombination sites (Verrou et al., 1999). This assembly was inserted into an *Eag1* site in the *Fgf8* gene located 36 bp 3' of the *Fgf8* stop codon and 5' to the endogenous polyadenylation sequence. The modified genomic fragment was shuttled into a plasmid backbone flanked by thymidine kinase genes. The linearized targeting vector was electroporated into mouse ES cells and subjected to selection with G418 and gancyclovir. Positive clones were identified by digesting genomic DNA, Southern blotting, and hybridizing with a 3' flanking probe, and targeted clones were confirmed to be correct by additional analyses using internal genomic probes and probes within the targeting vector. Following successful gene targeting the *NeoR* selection cassette was removed by Flp-mediated recombination of the flanking Frt sites. Genotyping of the wild type and targeted alleles was performed by PCR using following

primers: *Fgf8* exon 5 sense 5' GAGGTGCACTTCATGAAGCGC; *Fgf8* antisense, 5' ATTCAGGAGAACAGACCAGAG; sense for Frt site in mER:Cre:mER, 5' ATACTATCTAGAGAATAGGAACTTCG.

### Mouse Strains and Husbandry

For tamoxifen inducible genetic labeling, *Fgf8*<sup>mcm/+</sup> males were crossed with ROSA<sup>tdTom(Ai9)/tdTom(Ai9)</sup> reporter mice (Madisen et al., 2010), and CreER was activated with a single intraperitoneal (IP) injection of tamoxifen (37.5-75mg/kg, Sigma #T5648) solubilized in corn oil and delivered to the timed-pregnant dam at embryonic stages ranging from E9.5 to E17.5 as indicated. When embryonic inductions were followed by postnatal collections,  $\beta$ -Estradiol (75ug/kg, Sigma #E8875) was co-injected to help alleviate the anti-estrogen effects of Tamoxifen. In some instances where not a natural birth did not occur on the 19<sup>th</sup> day of gestation pups were delivered by Cesarean-section and cross fostered. Postnatal activation was done with a single IP injection of tamoxifen (75mg/kg) at P2. For *Fgf8* mutant analysis, *Fgf8* CKOs were generated by crossing *Fgf8*<sup>LoxP/LoxP</sup> female mice with *Pax2*-Cre positive or *Atoh1*-Cre positive *Fgf8*<sup>+/-</sup> males. Cre-positive progeny were analyzed with *Fgf8*<sup>LoxP/-</sup> mice serving as CKOs and *Fgf8*<sup>LoxP/wt</sup> as littermate controls. For these studies, *Pax2*-Cre mice (Ohyama and Groves, 2004) were provided by A. Groves (Baylor College of Medicine, Houston, TX, USA), *Atoh1*-Cre mice (Matei et al., 2005) and tdTom(Ai9) reporter mice were purchased from Jackson Labs (Strains #011104 and #007909). *Fgf8* KO and *Fgf8*<sup>LoxP/LoxP</sup> lines have been previously described (Park et al., 2006). For timed breeding and tissue staging, noon on the day in which a vaginal plug was detected was considered E0.5 and the day of birth was considered P0. Mice of either sex were used interchangeably for all experimental analyses. Mouse colonies were maintained at the University of Utah under IACUC approved guidelines, and genotyped using allele-specific PCR reactions.

### Immunofluorescent Labeling and Imaging

Inner ear tissues were fixed using a 4% paraformaldehyde (PFA) solution prepared in 67mM Sorensens' phosphate buffer (pH 7.4) for 2 hours on ice. For whole-mount immunofluorescent labeling, inner ears were micro-dissected to isolate the cochlea or utricular maculae and detergent-

permeabilized using blocking solution (5% normal donkey serum, 1% BSA, in PBS) supplemented with 0.5% Triton-X100. Primary antibodies and Phalloidin Alexa Fluor 488 (Invitrogen, A12379, dil 1:1500) were diluted in blocking solution supplemented with 0.1% Tween-20 and incubated overnight at 4°C. For Osteopontin and Anxa4 immunolabeling, tissue was fixed in 4%PFA prepared in phosphate buffered saline (PBS) for 1hr at room temperature, and 10% donkey serum in PBS was used as a blocking solution as described by (McInturff et al., 2018). Tissue was washed thoroughly using PBS with 0.05% Tween-20 and incubated with blocking solution supplemented with 0.1% Tween-20 and containing species-specific Alexa-conjugated secondary antibodies (Invitrogen A11122, A21202, A31571, Jackson Labs 715-605-150, 705-165-147, 711-605-152, all diluted 1:1500). Tissue was washed thoroughly with PBS/0.05% Tween-20 and mounted for fluorescence microscopy using Prolong Gold Antifade Mountant (ThermoFisher #P10144). For immunolabeling of cryosections, tissue was fixed as described using 4% PFA, cryoprotected by passing through a post-fixation sucrose gradient, and frozen in Neg50 (Richard Allan Scientific) before sectioning at 20 µm using a Leica CM3050 cryostat. Immunolabeling of tissue sections on SuperFrost Plus microscopy slides (Fisherbrand #12-550-15) was completed as described for wholemount tissues except that Tween-20 was excluded from blocking and wash solutions. Fluorescent images were acquired using structured illumination microscopy using a Zeiss Axio Imager M.2 with ApoTome.2 attachment and an AxioCam 506 monochrome camera. Images were processed using Zeiss Zen software followed by figure preparation with Adobe Photoshop and Adobe Illustrator. 3D reconstructions of hair cells were completed using Fluorender software provided by University of Utah Scientific Computing and Imaging Institute. Quantification of hair cells or calyces, and area measurements were facilitated by FIJI (ImageJ2). For measurements of the Lateral Extrastriolar:Striolar Width Ratio the striolar region was defined by the presence of Oncomodulin-positive hair cells and region boundaries were fit using the curvature tool (Adobe Illustrator) to demarcate the smallest area containing all of these cells. The boundary of the utricular sensory epithelia were similarly demarcated based upon Myosin VIIa labeling. Commercial antibodies used in this study were: goat anti-Anxa4 (R&D Systems, AF4146, 1:200), rabbit anti-βIII Tubulin (Covance PRB-435P, 1:500), rabbit anti-dsRed to detect tdTomato (Clontech, 632496, 1:5000), mouse anti-Myosin VIIa (DSHB, 138-1 deposited by DJ Orten, 1:100),

rabbit anti-Myosin VIIa (Proteus Biosciences, 25-6790 1:1000), rabbit anti-NF200 (Millipore, AB1989, 1:1500), goat anti-Oncomodulin (Santa Cruz, sc-7466, 1:250), goat anti-Osteopontin (R&D Systems, AF808, 1:200), and rat anti-tdTomato (Kerafast, EST203, 1:2000).

### EdU Birthdating

For EdU (5-Ethynyl-2-deoxyuridine) labeling of dividing cells, *Fgf8*<sup>mcm/+</sup> males were crossed with ROSA<sup>tdTom(Ai9)/tdTom(Ai9)</sup> reporter mice as previously described and pregnant dams received three intraperitoneal injections of EdU (10ug/kg) at two hours apart on E12.5. Stock solutions of EdU (1ug/ul) were prepared in PBS prior to injection and stored at -20C. At E12.5, the pregnant dam was subsequently injected with 75mg/kg tamoxifen to induce Cre-mediated genetic labeling as previously described. All embryos were collected at E18.5 and fixed in 4% paraformaldehyde solution prepared in 67mM Sorensens' phosphate buffer (pH 7.4) for 2 hours on ice. Following dissection, tissue was permeabilized in PBS containing 0.5% Triton X-100 for 20min, washed with three exchanges of PBS containing 3%BSA, and EdU was detected using the Click-iT cross-linking reaction and Alexa Fluor azide 488 dye as per the manufacturer's specifications (ThermoFisher, C10337), with volumes adapted for a 96-well plate. Tissue was washed twice with two rapid exchanges of PBS containing 3%BSA and processed for wholemount immunofluorescent labeling as previously described.

### In situ hybridization

For wholemount *in situ* hybridizations, tissue was fixed using 4% paraformaldehyde prepared in PBS (pH 7.4) overnight at 4°C and stored in 100% MeOH at -20°C. For wholemount hybridization of inner ear tissues, ear capsules were dissected to expose sensory epithelia which was subsequently rehydrated through a decreasing MeOH gradient (75%, 50%, 25%, and then PBS with 0.05% Tween-20 (PBSt)). Tissue was washed with PBSt, digested with 0.75mg/mL Proteinase K (Ambion #Am2546) for 25 minutes at RT, rinsed with PBSt, and subsequently fixed in 4%PFA/0.25% glutaraldehyde for 25min at RT. Tissue was pre-hybridized for 3hrs in 5X SSC hybridization buffer at 65°C tissue and then hybridized with 150ng/mL digoxigenin-labeled anti-sense probes for 18hrs at 65°C with constant rocking. Tissue received three 50-minute washes in



50% formamide, 2XSSC, 1%SDS solution, followed by 1 hour in ISH blocking solution (1X MABT, 10% Blocking Reagent (Roche #11-096-176-001), 2% and heat inactivated sheep serum). Alkaline phosphatase-conjugated anti-digoxigenin antibodies (Roche #11093274910) were diluted 1:2500 in ISH blocking solution and rocked overnight at 4C. After antibody labeling, tissue was washed six times for 1 hour in Tris buffered saline with Tween-20 (TBST) solution followed by alkaline phosphatase detection using BM Purple substrate (Roche 11442074001). After detection, tissues were post-fixed in 4%PFA/0.25% glutaraldehyde, cleared through an increasing MeOH gradient and mounted onto slides using Sorensens' buffer, 31% glycerol, and 6.25% polyvinyl alcohol for imaging using the Zeiss Axio Imager M.2 and AxioCam 105 color camera. *Fgf8* mRNA distribution in wild-type mice and CKO mice was detected using an anti-sense *Fgf8* probe specific to *Fgf8* exon 5 which is deleted in CKOs, and was provided by S. Mansour (Univ. of Utah).

## Results

### Generation of the *Fgf8<sup>mcm</sup>* allele

Genetic genetic labeling enables heterochronic gene expression studies in which the cells or progeny of cells expressing a gene of interest during a defined period of development can be permanently labeled for evaluation at later stages. To generate a mouse line in which patterns of *Fgf8* gene expression could be evaluated using this approach, cDNA encoding a fusion protein of Cre recombinase flanked by mutated hormone-binding domains of the murine estrogen receptor (mER:Cre:mER) (Verrou et al., 1999) was inserted into the *Fgf8* locus. Commonly called CreER, these modified recombinases localize to the cytoplasm and remain inactive until binding the estrogen analog tamoxifen. Once activated, CreER translocates to the nucleus and catalyzes genetic recombination and excision of DNA located between tandem pairs of 32 base pair LoxP sequences (Zhang et al., 1996). To obtain CreER production in *Fgf8*-expressing tissues without disrupting *Fgf8* function, a cassette containing an IRES sequence (Kaminski et al., 1990, Jang and Wimmer, 1990) followed by the mER:Cre:mER cDNA (Verrou et al., 1999) was inserted into the 3'UTR of the *Fgf8* gene (Fig. 1A). This cassette and strategy have been successfully applied at other loci, and Flp-recombinase/Frt mediated excision of the *NeoR* negative selection gene was

completed to avoid hypomorphic effects of *NeoR* on expression from *Fgf8* or other loci (Frank et al., 2002). To determine whether this novel *Fgf8<sup>mcm</sup>* allele retained intact *Fgf8* expression, phenotypes were examined and *Fgf8* mRNA expression was evaluated in animals heterozygous or homozygous for *Fgf8<sup>mcm</sup>*. Wholemount *in situ* hybridization for *Fgf8* mRNA revealed no detectable difference in hybridization patterns or intensity between wild type and *Fgf8<sup>mcm/mcm</sup>* homozygous embryos (Fig. 1B,C). Furthermore, these embryos develop normally without phenotypes observed in *Fgf8* null, hypomorphic, or conditional mutant embryos. *Fgf8<sup>mcm/mcm</sup>* homozygotes survive to adulthood, are indistinguishable from wild-type animals, and reproduce normally (data not shown). Since even a modest decrease in *Fgf8* function has phenotypic consequences in many systems, these results indicate that the presence of the *IRES:mER:Cre:mER* insertion into the 3'UTR of *Fgf8* does not significantly affect gene function.

*Embryonic Fgf8<sup>mcm</sup> genetic labeling labels a subset of vestibular hair cells.*

To evaluate *Fgf8* expression in the developing vestibular system, *Fgf8<sup>mcm/+</sup>* males were crossed with ROSA<sup>ai9tom/ai9tom</sup> female mice allowing for tamoxifen-dependent, Cre-mediated labeling of *Fgf8* expressing cells by the red fluorescent protein tdTomato. Single injections of tamoxifen at E17.5 resulted in robust tdTomato expression in a subset of vestibular hair cells identified by  $\beta$ II-spectrin labeling (Fig. 2A-E). Following injections, labeled hair cells were present at E18.5 throughout the utricle (Fig. 2A,B), saccule (Fig. 2C,D) and within the semi-circular canal cristae (Fig. 2E). For each sensory organ, *Fgf8<sup>mcm</sup>* activation only labeled a subset of the total vestibular hair cells. This readout of *Fgf8* expression is accurate and is unlikely the result of incomplete recombination because it resembles the distribution of *Fgf8* mRNA visualized by whole mount *in situ* hybridization at P0. *Fgf8* mRNA also appears in a salt-and-pepper pattern within subsets of cells in the utricle (Fig. 2F), saccule (Fig. 2G) and cristae (Fig. 2H). In the cochlea *Fgf8* mRNA is also present in a subset of hair cells, the single row of inner hair cells, as previously described (Fig. 2I, (Jacques et al., 2007)).

Interestingly, early injections of tamoxifen at E11.5 similarly labeled a subset of vestibular hair cells when evaluated in the E18.5 utricular maculae (Fig. 3A,B). Prior birthdating experiments using

tritiated thymidine or EdU incorporation demonstrate that the earliest-born vestibular hair cells undergo terminal mitosis at E11.5 (Ruben, 1967, Jiang et al., 2017) and these cells are enriched in the striolar region of the mature utricle. Similarly, *Fgf8*<sup>mcm</sup> genetic labeling at E11.5 included striolar hair cells based upon their Oncomodulin expression when imaged at E18.5 (Fig. 3A,B) (Simmons et al., 2010, Hoffman et al., 2018). In contrast, induction at later stages (E15.5-E17.5) labeled a broader regional population that included extrastriolar hair cells in the lateral and medial regions (Fig. 2A and S1). Furthermore, newly-born hair cells labeled by EdU at E12.5 are also genetically labeled by *Fgf8*<sup>mcm</sup> when EdU and Tamoxifen are co-administered (Fig. 3C-E) and these cells populate the striolar region at E18.5 (Fig. 3C). Since EdU is only retained in cells that have undergone their last mitosis, and CreER activation by tamoxifen is transient, colocalization indicates *Fgf8* promoter activation shortly after cell division. Together these regional and temporal patterns of *Fgf8*<sup>mcm</sup> activity demonstrate that *Fgf8* expression is initiated in newly-born hair cells, after their specification and during the initial stages of their differentiation, and that expression is maintained throughout embryonic development.

#### *Genetic labeling reveals transient Fgf8 expression in auditory and vestibular neurons.*

While tamoxifen injections prior to E10.5 failed to label hair cells, transient *Fgf8* expression was evident in the spiral ganglion neurons (SGNs) during these early stages of cochlear development. This transient expression follows a development gradient with tamoxifen injections from E10.5 to E13.5 revealing progressive *Fgf8* activity in SGNs from the cochlear base to the apex (Fig. 4A-C), similar to the expression pattern of pro-neural genes such as *neurogenin1* (Koundakjian et al., 2007). Close examination of the peripheral processes of labeled SGNs show that *Fgf8* is expressed by both Type-I and Type-II SGNs based upon the morphology of tdTomato-labeled peripheral axons innervating the organ of Corti (Fig. 4F). Consistent with previous reports (Jacques et al., 2007), *Fgf8*<sup>mcm</sup> genetic labeling also robustly marks inner hair cells with the onset of tdTomato expression also appearing in a developmental gradient initiating in the cochlear base and progressing towards the cochlear apex (Fig. 4D,E). tdTomato-positive neuronal processes were also observed throughout the utricle, saccule and cristae following tamoxifen delivery at E9.5 (Fig. 4G-I) indicating early expression in vestibular neurons as well. More importantly, later stages

of tamoxifen induction (E11.5, E17.5) predominantly labels hair cells and not vestibular neurons demonstrating that, like the SGN, *Fgf8* expression is transient within vestibular neurons and restricted to early stages of neuronal development.

*Embryonic Fgf8<sup>mcm</sup> genetic labeling selectively labels Type-I vestibular hair cells.*

The two different types of vestibular hair cells in the mouse can be distinguished from each other based upon morphologic and synaptic criteria which begin to emerge about the time of birth (19 days post copulation). These features were evaluated in cryosections collected from E18.5 *Fgf8<sup>mcm/+</sup>*; tdTomato-positive embryos following tamoxifen induction at E17.5. At this early stage, many tdTomato-positive hair cells already exhibited an ampoule morphology characteristic of Type-I vestibular hair cells (Fig. 5A). Often, these putative Type-I hair cells were also contacted by a neuronal process that was immunoreactive for Neurofilament that appeared to be nascent calyceal termini although formation of these synaptic structures is not complete until P14 (Fig. 5A'). Other tdTomato-positive hair cells had simpler or possibly immature synaptic contacts and hair cell morphologies that could not be distinguished from Type-II hair cells. Cells with these latter characteristics were more abundant in the periphery of the sensory epithelia.

A subset of Type-I hair cells produce Oncomodulin which is a calcium binding protein found in Type-I hair cells populating the striolar region of the utricle and are contacted by afferent neurons that form a complex calyx encompassing multiple hair cells. tdTomato and endogenous Oncomodulin colocalize in the striolar region following tamoxifen induction at E17.5, consistent with *Fgf8* expression in Type-I hair cells (Fig. 5B). The striolar and extrastriolar regions also contain Type-I hair cells that are contacted by a simple calyx and do not express Oncomodulin, but still develop the characteristic ampoule-like morphology of Type-I hair cells. 3D reconstruction of individual tdTomato-positive, Oncomodulin-negative cells from the striolar or medial and lateral extrastriolar regions revealed this stereotypic Type-I hair cell morphology (Fig. 5C-E). Altogether the morphology and overall distribution of genetically labeled cells is consistent with selective *Fgf8<sup>mcm</sup>* activity in Type-I hair cells. tdTomato-positive cells with similar ampoule-like morphology were also seen in the saccule and semi-circular cristae suggesting that *Fgf8* expression is a general

marker of vestibular hair cell identity (data not shown). The defining characteristic of Type-I hair cells is the calyceal synapse which can be readily seen using neuronal markers such as  $\beta$ III-tubulin at P14. To definitively establish whether embryonic hair cells expressing *Fgf8* differentiated into Type-I hair cells tamoxifen was administered at E11.5 and tdTomato-positive hair cells were evaluated for the presence of a synaptic calyx at P14 (Fig.6A). The majority of these cells were surrounded by a ring of  $\beta$ III-tubulin indicating the presence of a calyx and confirming Type-I hair cell identity.

*Postnatal Fgf8 genetic labeling is restricted to Type-I hair cells.*

Recently it was proposed that hair cell sub-types differentiate sequentially with the Type-II cells predominantly appearing at postnatal stages and conversely, Type-I hair cells differentiating in the embryo (McInturff et al., 2018). In this scenario genes expressed in hair cells at embryonic stages may appear to preferentially label Type-I hair cells in genetic labeling experiments, when in fact they may not be cell type-specific. To determine whether *Fgf8*<sup>mcm</sup> sequentially labeled Type-I and then Type-II hair cells through the course of utricle development, tamoxifen was delivered at E11.5, E17.5, or postnatal day 2 (P2). For each injection paradigm, tdTomato-positive cells were evaluated at P14 when cell type-specific molecular markers are available such as the Type-I hair marker Osteopontin, the Type-II markers *Anxa4* and *Calb2* (McInturff et al., 2018, Sayyid et al., 2019, Perry et al., 2003), or the presence of a calyx. Regardless of the time of tamoxifen induction, tdTomato genetic labeling predominantly labeled Type-I hair cells as evidenced by presence of calyceal synapse (Fig. 6A-C) and at all stages, tamoxifen administration resulted in a comparable frequency of tdTomato-positive cells contacted by a calyx (Fig. 6D).

The postnatal delivery of tamoxifen at P2 also failed to label cells expressing markers associated with Type-II hair cell identity. When evaluated at P14, very few tdTomato-positive hair cells expressed Type-II markers *Anxa4* and *Calb2* (Fig. 7B-E). It is not unexpected that on occasion tdTomato was found in hair cells expressing *Anxa4* or *Calb2*, since this marker is not absolutely restricted to Type-II hair cells and can be detected in a small proportion of cells expressing other Type-I markers (McInturff et al., 2018). In contrast, following tamoxifen delivery at P2, the majority

of labeled hair cells co-localized with the Type-I marker Osteopontin at P14 (Fig. 7A,D). Finally, unlike the transient *Fgf8*<sup>mcm</sup> activity observed in neurons, expression in hair cells appeared sustained across the range of developmental timepoints that tamoxifen was administered (E11.5 through P2).

*Hair cell development and innervation appears normal in Fgf8 CKOs.*

Since FGFs are known secreted signaling molecules, restricted expression of *Fgf8* in Type-I hair cells suggests that FGF8 acts through autocrine and/or paracrine mechanisms during vestibule development. Consistent with a paracrine function in the cochlea, paracrine FGF8 signaling from inner hair cells promotes the differentiation of neighboring pillar cells (Jacques et al., 2007). Despite this, no phenotypic differences in hair cells or cellular organization within the vestibular maculae were identified in *Fgf8* CKOs compared to littermate controls (Fig. 8). CKOs were generated using two Cre lines frequently used for inner ear research, the hair cell restricted *Atoh1*-Cre (Matei et al., 2005), and *Pax2*-Cre which is active in the otic placode and also portions of the CNS and kidney (Ohyama and Groves, 2004). *Atoh1*-Cre mediated gene deletion was incomplete as revealed by in situ hybridization, with some *Fgf8* mRNA persisting in the striolar region of the utricle at P0 (Fig. S3). *Pax2*-Cre on the other hand results in the complete loss of *Fgf8* mRNA throughout the utricle (Fig. S3), however *Pax2*-Cre, *Fgf8* CKO mice die at birth most likely due to kidney agenesis (data not shown). Based upon CKO phenotypes, *Fgf8* is not required for formation of the calyceal synapses that surround Type-I hair cells because these structures remain intact in extrastriolar regions of the utricle lacking *Fgf8* mRNA in *Atoh1*-Cre; *Fgf8* CKOs at P14 (Fig. 8A,B,I). The number and distribution of Type-I hair cells as determined using Oncomodulin to label Type-I hair cells in the striolar region (Fig. 8C,D,J) and Osteopontin to label Type-I hair cells in the extrastriolar regions (Fig. 8E,F,K) is also not changed in *Fgf8* CKOs. Finally, in the developing neocortex *Fgf8* acts in opposition to the transcription factor *Emx2* to regulate cortical patterning (Fukuchi-Shimogori and Grove, 2003, Muzio and Mallamaci, 2003, O'Leary and Nakagawa, 2002, O'Leary and Sahara, 2008). Although *Emx2* also functions in the utricle to regulate stereociliary bundle orientation and formation of the line of polarity reversal {Jiang, 2017

#316}, these features are not disrupted by the loss of *Fgf8* since there are no changes in hair cell polarity (Fig. 8G,H) or the relative position of the striolar region in *Pax2-Cre; Fgf8* CKOs (Fig. 8L).

## Discussion

Using CreER-mediated genetic labeling, we demonstrate temporally regulated, transient expression of *Fgf8* in auditory and vestibular neurons prior to E11.5 and sustained expression in Type I vestibular hair cells at all subsequent developmental stages investigated (E11.5 to P2). We anticipate that the engineered *Fgf8<sup>mcm</sup>* allele will be a valuable experimental resource that provides genetic access to this unique population of hair cells. This should enable further studies of vestibular development and regeneration, and create new approaches for understanding Type-I hair cell function through the application of genetically encoded tools for manipulating their function. *Fgf8<sup>mcm</sup>* may also be applied for studies of cochlear Inner Hair Cell function in which selective *Fgf8* expression has been well documented (Jacques et al., 2007). The *Fgf8<sup>mcm</sup>* allelic design also provides distinct advantages over alternative strategies for placing Cre recombinase at the *Fgf8* locus. First, insertion of the IRES:mER-Cre-mER expression cassette into the 3' untranslated region of *Fgf8* does not perturb its expression. This is significant because FGF8 signaling is dose dependent as evidenced by severe developmental defects and lethality of hypomorphs (Frank et al., 2002, Meyers et al., 1998). Second, the tamoxifen-dependent CreER activity in the *Fgf8<sup>mcm</sup>* line allows recombination to be restricted to hair cells and avoids recombination in those neurons that transiently express *Fgf8*. Finally, for developmental studies, the early onset of *Fgf8* expression, and hence CreER activity, will allow many target genes to be deleted before their transcription is initiated in hair cells.

Cre recombinase activity from the engineered *Fgf8<sup>mcm</sup>* allele is detected in the utricular maculae at the earliest stages of hair cell differentiation, beginning shortly after terminal mitosis, and indicates that cell-type specification occurs earlier than has been documented using other criteria. Given the permanence of genetic labeling, it seems unlikely that *Fgf8* is expressed in Type II hair cells or their precursors during this time. Consistent with this, tomato-positive hair cells at P14 express the Type-I marker Osteopontin, rarely colocalize with the Type-II biased marker *Anxa4*, and are always

contacted by calyceal synaptic endings. Perhaps more striking than this cell specificity is the observation that *Fgf8*<sup>mcm</sup> can be activated in newly-born hair cells at E11.5, a time when the initial cohort of hair cells is completing mitosis and beginning to differentiate. Together these observations suggest that vestibular hair cell identity is defined at the earliest stages of their development and refutes alternative models in which Type-I and Type-II hair cells are derived postnatally from a shared hair cell precursor.

It is more difficult to reconcile the early Type-I restricted expression of *Fgf8* with the potential role that *Sox2* may have in defining vestibular hair cell identity after birth (Lu et al., 2019). The vestibular sensory epithelia are unique because expression of the progenitor cell marker *Sox2* is maintained throughout postnatal development in vestibular supporting cells and Type-II hair cells. While *Sox2* is transiently expressed in Type-I hair cells, it is not downregulated until later stages when these two cell types are readily distinguished using morphological and synaptic criteria. Based upon this correlation, *Sox2* down-regulation after birth in a subset of cells has been suggested to be the mechanism by which the two hair cell types are established. Consistent with this potential mechanism, the postnatal deletion of *Sox2* results in an increase in the ratio of Type-I to Type-II hair cells (Lu et al., 2019). A parsimonious synthesis of the findings from these studies would be that *Fgf8* expression distinguishes between committed and non-committed states of cell fate divergence, and that only *Fgf8* expressing cells are restricted to the Type-I identity. In contrast, *Fgf8*-negative cells may retain the potential to differentiate as either cell type, possibly doing so during postnatal development through a switching mechanism that is *Sox2*-dependent. It remains to be shown whether *Fgf8* expression is activated following *Sox2* gene deletion at postnatal stages or whether the two cohorts of Type-I hair cells that would be generated through these parallel paths might differ from each other.

Loss of function experiments using *Fgf8* CKOs generated with *Pax2*-Cre or *Atoh1*-Cre fail to demonstrate a requirement for FGF8 function during vestibular hair cell development. This is likely due to genetic redundancy with other FGFs that preserve signaling in the absence of FGF8, and consistent with this possibility, *Fgf3* and *Fgf10* are expressed during vestibular development in the



mouse (Hossain and Morest, 2000, Pirvola et al., 2000, Ohuchi et al., 1997, Pauley et al., 2003). Furthermore, genetic redundancy in FGF signaling has been well described in cochlear development (Wright and Mansour, 2003, Mansour et al., 2013). One event where FGF8 functions independently is during pillar cell differentiation where the absence of *Fgf8* expression results in fewer pillar cells. FGF8 signals through FGFR3 to regulate pillar cell development in this context (Jacques et al., 2007). However, FGF8 must signal through different receptors in the utricle because FGFR3 is absent from the developing vestibule based upon genetic labeling experiments using *Fgfr3*-iCre mice (data not shown and (Anttonen et al., 2012). In contrast, *Fgfr1* and *Fgfr2* are expressed in vestibular hair cells of mice and rats, and these receptors are activated by FGF8 (Saffer et al., 1996, Burns et al., 2015, Pirvola et al., 2000). Moreover, the number of vestibular hair cells are reduced in *Fgfr1* CKO mice (Ono et al., 2014), and targeted deletion of *Fgfr2b* results in severe dysgenesis of the cochleovestibular labyrinth (Pirvola et al., 2000), demonstrating their necessity for vestibular morphogenesis and development. Together these findings suggest that multiple FGFs are released by Type-I vestibular hair cells and may be received by FGFR1 and/or FGFR2 rather than FGFR3 as observed for FGF8-dependent pillar cell development in the cochlea.

As a secreted signaling molecule FGF8 is most likely to function through a paracrine pathway to promote local differentiation or through an autologous mechanism that reinforces Type-I differentiation. Based upon FGF8 function in the cochlea, one role for FGF-signaling in vestibular sensory development may be to promote supporting cell differentiation. Unfortunately, the lack of molecular markers and evidence for supporting cell diversity in the utricle makes it difficult to test this hypothesis (Shim et al., 2005, Jacques et al., 2007). An exciting alternative is that FGF signaling directs afferent neuron differentiation and formation of the calyceal synapse that is unique to the Type-1 hair cells. The existence of a hair cell signal that directs post-synaptic specialization is suggested by the morphology of dimorphic afferent neurons which contact both Type-I and Type-II hair cells, but only form calyceal synaptic structures around the Type-I cell. Notably, FGF7 and FGF22 have been shown to promote the differentiation of excitatory and inhibitory synapses as presynaptic organizers (Terauchi et al., 2010) consistent with a potential

role for FGF signaling in vestibular synaptic development. Finally, in the developing neocortex *Fgf8* regulates cortical patterning through a process called arealization in which FGF8 expression in an anterior domain opposes the posterior expression of the transcription factor *Emx2*. We originally hypothesized that a similar process regulated *Emx2* expression in the utricle and thus determined the position of the Line of Polarity Reversal that divides hair cells with opposite stereociliary bundle orientations (Deans, 2013, Jiang et al., 2017). However, the observed *Fgf8* expression throughout the utricle, including the *Emx2* expressing domain in the lateral extrastriolar region are inconsistent with this model. Moreover, the position of the Line of Polarity Reversal is not altered in *Fgf8* mutants.

A remarkable characteristic of utricular hair cells in the mouse is their potential to regenerate at perinatal stages of development. Curiously, the majority of vestibular hair cells regenerated in the mouse are of the Type-II identity {Golub, 2012 #452 {Bucks, 2017 #312}}. Understanding and directing this process to occur at later stages, and generating the correct proportion of hair cell types, is essential if regeneration strategies are to successfully replace lost hair cells. Although the contribution of FGF8 towards identity specification is not known, *Fgf8* expression in Type-I hair cells demonstrates that identity is established earlier than expected and shortly after terminal mitosis. This expression pattern further suggests that FGF or other morphogenic signaling pathways might be manipulated during the course of regeneration to direct cell fate. Thus, the *Fgf8*<sup>mcm</sup> line may catalyze directed regeneration strategies while the distinct cell specific expression makes this line a valuable resource for studying hair cell development and function.

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## **Competing interests**

The authors declare no competing or financial interests.

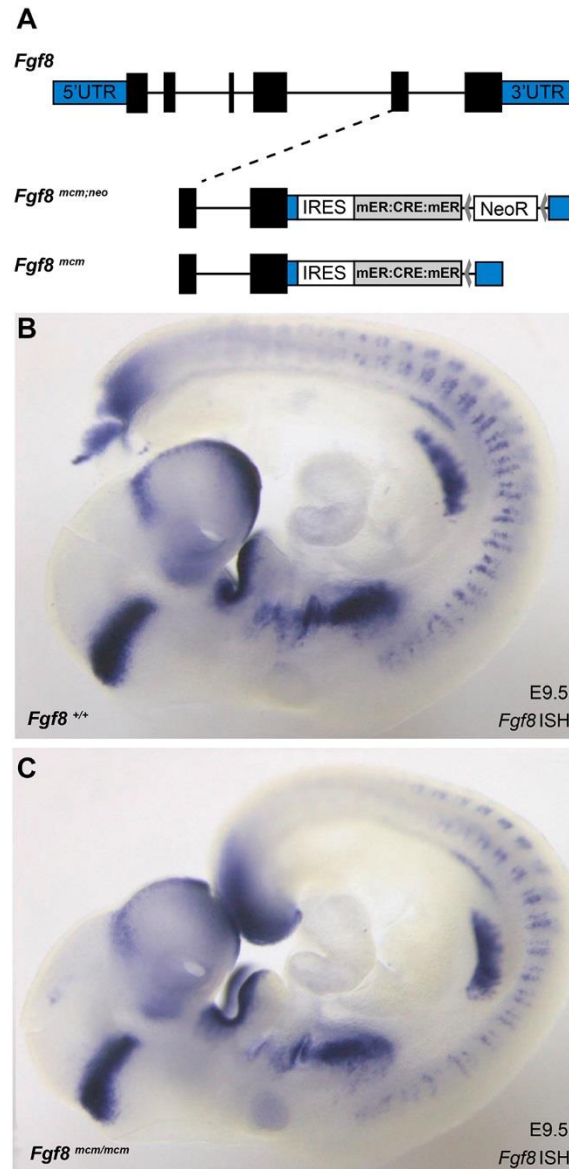
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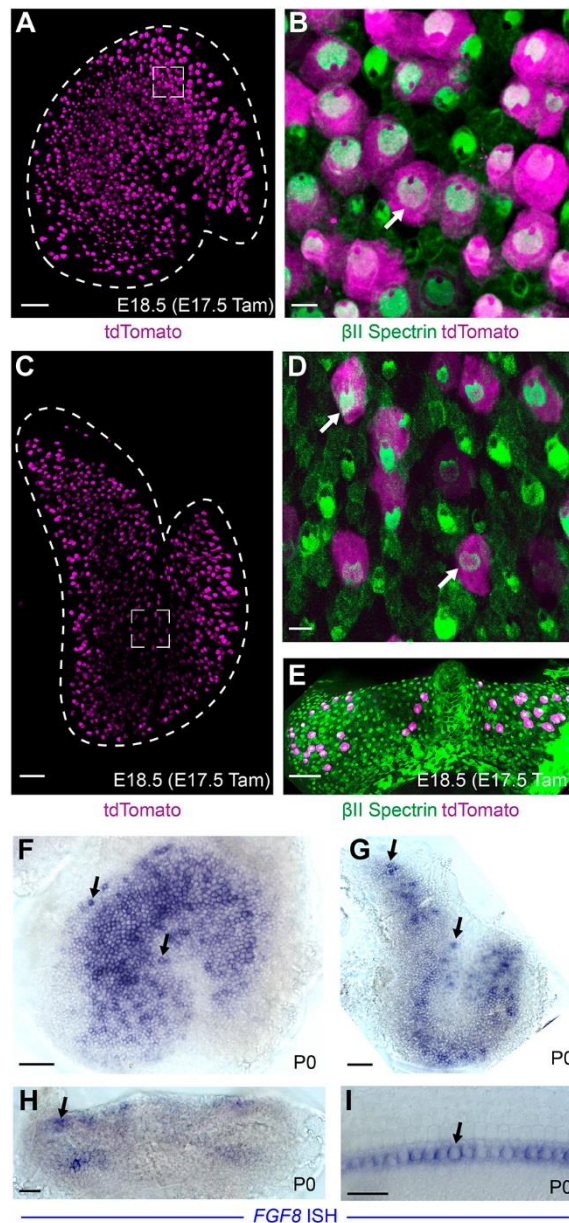
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## Figures



**Figure 1. *Fgf8<sup>mcm</sup>* engineering strategy does not impact gene expression or function**

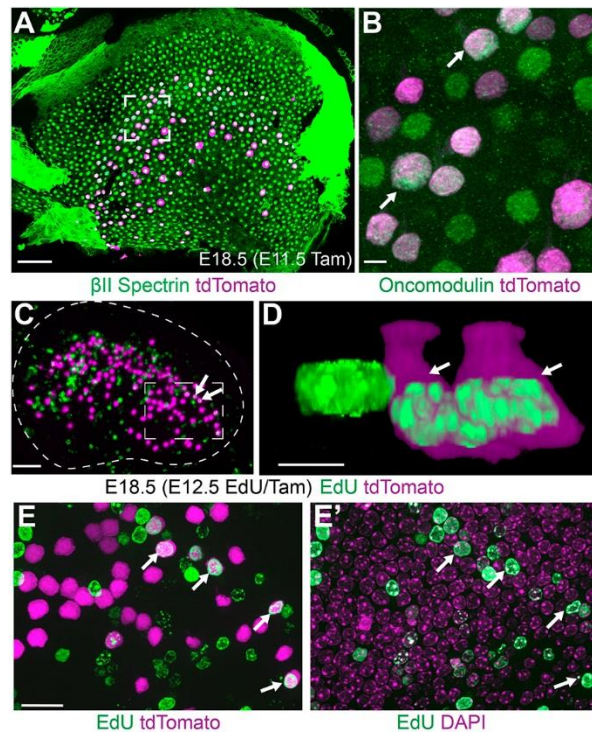
**(A)** The gene targeting strategy used to generate *Fgf8<sup>mcm</sup>* mice inserts an IRES mER:CRE:mER expression cassette into the 3' untranslated region (UTR) of the *Fgf8* gene. 5' and 3' UTRs are marked blue shading while the *Fgf8* coding sequence exons are black. An Frt-flanked Neomycin resistance gene (*NeoR*) used for selection of targeted ES clones was removed using FLP recombinase to produce the final *Fgf8<sup>mcm</sup>* allele. **(B,C)** Wholemount ISH for *Fgf8* reveals no detectable differences in the staining pattern or intensity between wild type (B) and *Fgf8<sup>mcm/mcm</sup>* homozygous (C) embryos at E9.5.



**Figure 2. *Fgf8*<sup>mcm</sup> genetic labeling labels subsets of vestibular hair cells**

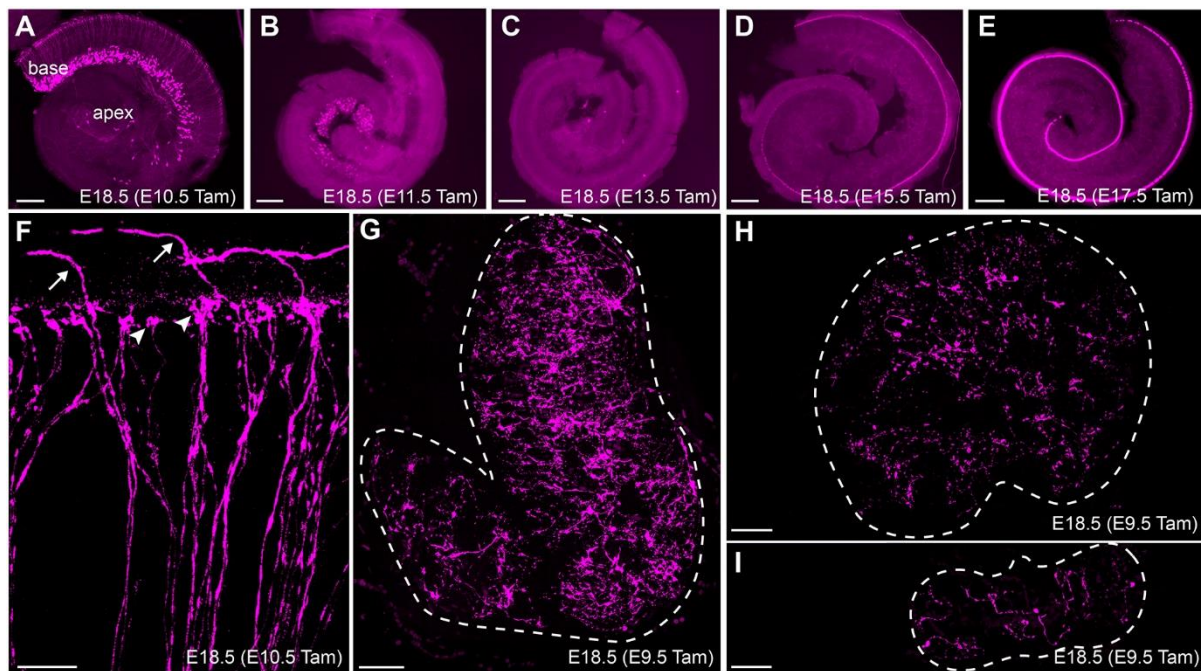
**(A)** tdTomato fluorescence is present throughout the sensory epithelia in an *Fgf8*<sup>mcm/+</sup>; ROSA<sup>tdTom(Ai9)/WT</sup> utricle at E18.5 following tamoxifen induction at E17.5. **(B)** Higher magnification image from the framed region in **(A)** shows tdTomato colocalization with βII Spectrin (example marked by arrow). **(C)** tdTomato is also induced by *Fgf8*<sup>mcm</sup> in vestibular hair cells throughout the saccule, and **(E)** cristae. **(D)** Higher magnification image from the framed region in **(C)** shows tdTomato colocalization with βII Spectrin (examples marked by arrows). **(F-I)** Wholemount ISH for endogenous *Fgf8* in the utricle **(F)**, saccule **(G)**, cristae **(H)**, and cochlea **(I)**. Arrows highlight examples of mRNA expression in individual hair cells. Scale bars: A,C,E,F,G,H (50μm); B,D,I (20μm)





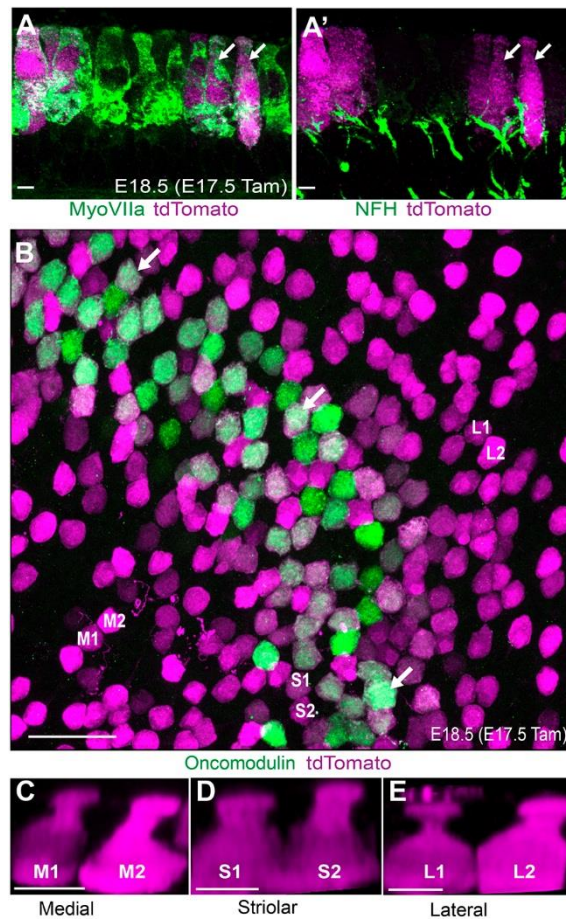
**Figure 3. *Fgf8*<sup>mcm</sup> genetic labeling occurs shortly after hair cell terminal mitosis**

**(A)** Tamoxifen induction at E11.5 induces tdTomato expression in hair cells throughout the central region of the utricle where the striolar region lies. **(B)** Higher magnification image from the framed region in (A) shows tdTomato colocalization with Oncomodulin-positive hair cells (examples marked by arrows). **(C)** Newly divided hair cells labeled by EdU incorporation at E12.5 are genetically labeled by the tdTomato reporter with concurrent tamoxifen induction (examples marked by arrowheads). **(D)** Higher magnification 3D-reconstruction of EdU-labeled hair cells. Arrowheads correspond to the two tdTomato-positive cells annotated in (C). **(E-E')** Higher magnification image corresponding to the framed region in (C) similarly reveals vestibular hair cells containing EdU and labeled by tdTomato. Scale bars: A,C (50 $\mu$ m); B,D (5 $\mu$ m); E (10 $\mu$ m)



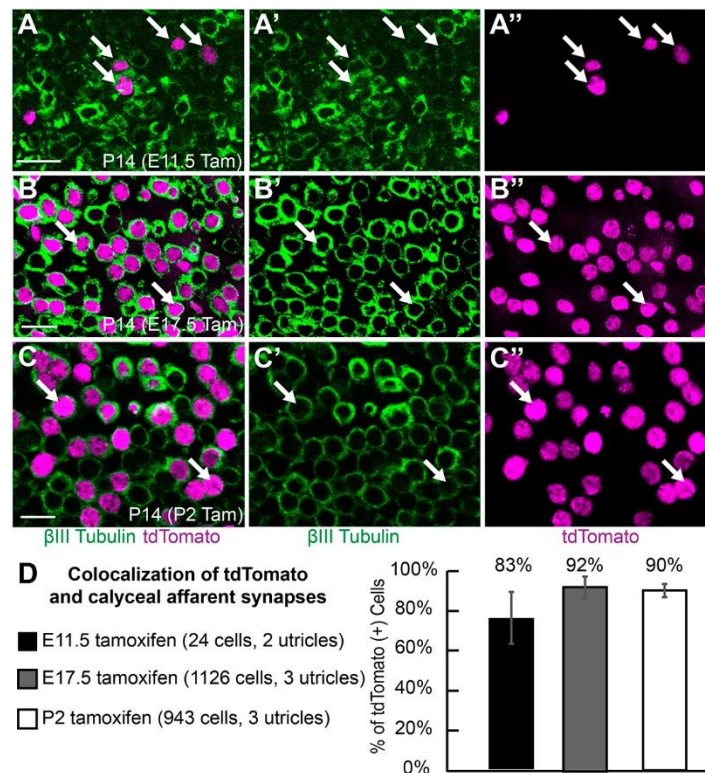
#### Figure 4. *Fgf8* expression in auditory and vestibular neurons is transient

(A-E) *Fgf8*<sup>mcm</sup> genetic labeling in the developing cochlea selectively labels SGNs following tamoxifen induction at E10.5 (A) and E11.5 (B). Little to no labeling is seen with tamoxifen induction at E13.5 (C), while inner hair cells are labeled following tamoxifen induction at E15.5 (D) and E17.5 (E). (F) Higher magnification image of tdTomato labeling in peripheral axons of auditory neurons following tamoxifen induction at E10.5 reveals Type I SGNs (arrowheads) which innervate inner hair cells, and Type II SGNs (arrows) that extend further, turn towards the cochlear base, and innervate outer hair cells. (G-I) Tamoxifen induction at E9.5 labels the peripheral processes of vestibular neurons innervating the saccule (G), utricle (H) and cristae (I). Scale bars: A-E, G-I (200 $\mu$ m); F (20 $\mu$ m)



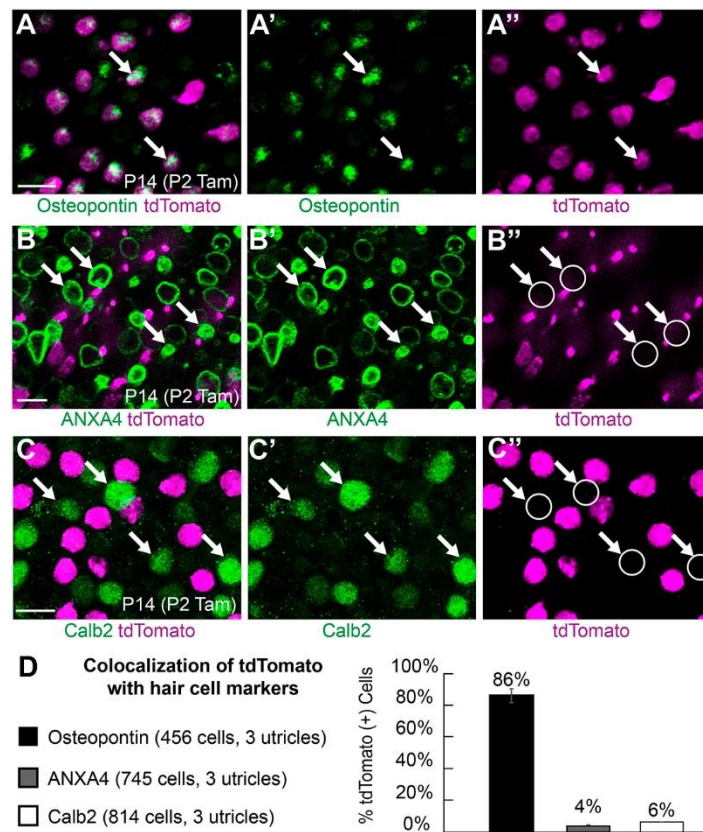
**Figure 5. *Fgf8*<sup>mcm</sup> genetic labeling labels Type-I hair cells at embryonic stages**

**(A)** Cross section through the embryonic utricular sensory epithelia following tamoxifen induction at E17.5 showing tdTomato expression in hair cells with Type-I morphology characterized by a restriction at the apical neck (arrowheads). **(A')** tdTomato labeled hair cells are contacted by presumptive, developing calyceal synapses (arrows). **(B)** Wholemount immunolabeling of *Fgf8*<sup>mcm</sup> utricle reveals colocalization of tdTomato reporter and the striolar Type-I hair cell marker Oncomodulin (examples illustrated by arrows). 3D-reconstructions of tdTomato-positive, Oncomodulin-negative hair cells in **(C)** medial extrastriolar (M1, M2), **(D)** striolar (S1, S2), and **(E)** lateral extrastriolar (L1, L2) regions also reveals ampoule-like Type-I hair cell morphology. Scale bars: A, C-E (5µm); B (20µm)



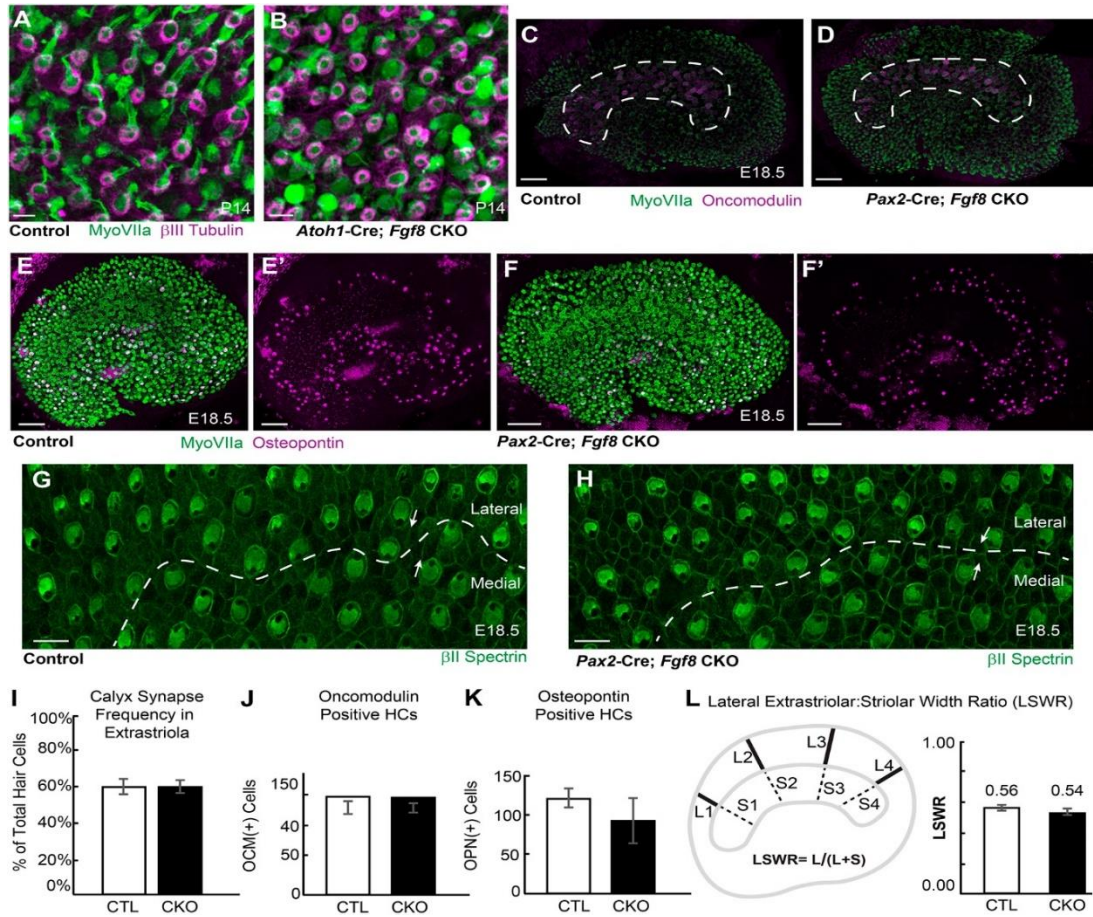
**Figure 6. *Fgf8*<sup>mcm</sup> genetic labeling selectively labels Type-I hair cells contacted by calyces**

**(A)** Following tamoxifen induction at E11.5, tdTomato-positive hair cells are surrounded by βIII-tubulin rich calyces (arrows) when evaluated at P14, consistent with Type-I hair cell identity. **(B)** Following tamoxifen induction at E17.5, tdTomato-positive hair cells are surrounded by βIII-tubulin rich calyces (arrows) when evaluated at P14. **(C)** Following tamoxifen induction at P2, tdTomato-positive hair cells are similarly contacted by calyces (arrows) at P14. **(D)** The frequency of calyx formation about tdTomato-positive hair cells following tamoxifen induction at different developmental stages. Scale bars: 10μm



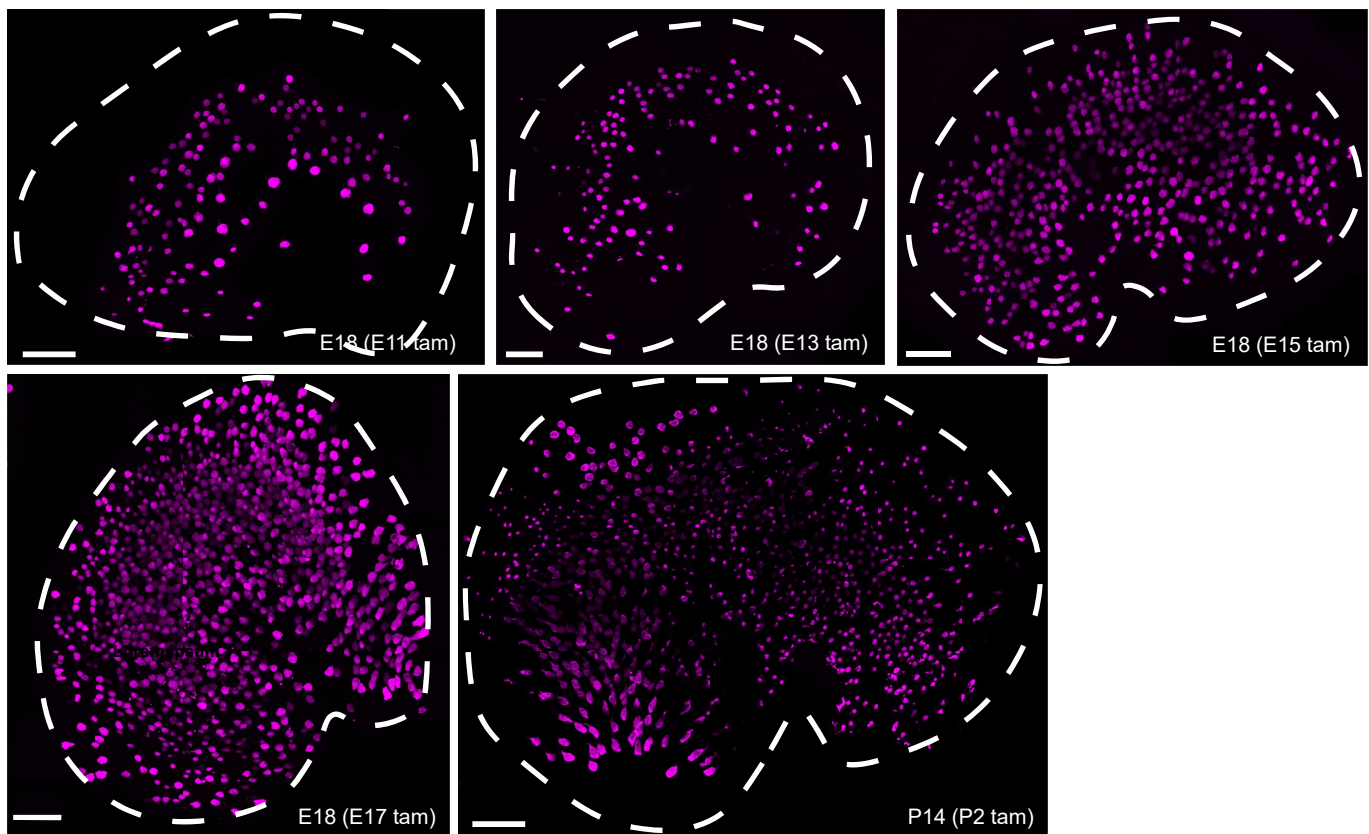
**Figure 7. *Fgf8*<sup>mcm</sup> genetic labeling selectively labels Type-I but not type II hair cells at postnatal stages**

**(A-A'')** tdTomato-positive hair cells labeled by tamoxifen induction at P2 co-localize with the Type-I hair cell marker Osteopontin (examples marked by arrows). **(B-B'')** Hair cells labeled by tamoxifen induction at P2 (arrows) do not co-localize with the Type-II hair cell marker Anxa4. **(C-C'')** Hair cells labeled by tamoxifen induction at P2 do not co-localize with Type-II hair cell marker Calb2. **(D)** The frequency of tdTomato colocalization with cell-type specific markers following tamoxifen induction at P2. Scale bars: 10µm



**Figure 8. *Fgf8* conditional knockout utricles are phenotypically normal**

(A,B) Wholemount immunolabeling using  $\beta$ III-tubulin shows no changes in the average frequency of calyceal synapses in extra-striolar regions of *Atoh1*-Cre; *Fgf8* CKOs and littermate control utricles when evaluated at P14. (C,D) There are no differences in the distribution of Oncomodulin-positive Type I hair cells in the striolar region of *Pax2*-Cre; *Fgf8* CKO compared to littermate control utricles at E18.5. (E,F) There are no differences in the distribution of Osteopontin-positive Type I hair cells in the extrastriolar regions of *Pax2*-Cre; *Fgf8* CKO compared to littermate control utricles at E18.5. (G,H) A Line of Polarity Reversal (dashed line) forms normally and stereociliary bundle orientation (arrows) is undisturbed in *Pax2*-Cre; *Fgf8* CKOs similar to littermate controls. (I) The frequency of calyceal synapses and number Type I hair cells expressing Oncomodulin (J) and Osteopontin (K) at E18.5 is not changed between littermate control and *Fgf8* CKO utricles. (L) The position of the striolar region relative to the lateral border of the utricle is normal in the *Pax2*-Cre; *Fgf8* CKO utricle based upon measures of the Lateral Extrastriolar:Striolar Width Ratio (LSWR). Scale bars: A,B,G,H (10 $\mu$ m); C-F (50 $\mu$ m)



**Figure S1. Timecourse of  $Fgf8^{mcm}$  genetic labeling in the developing mouse utricle.** tdTomato labeling of utricular hair cells following a single dose of tamoxifen delivered at (A) E11.5, (B) E13.5, (C) E15.5 or (D) E17.5 and evaluated at E18.5. (E) tdTomato labeling of utricular hair cells following a single dose of tamoxifen delivered at P2 and evaluated at P14. Panels (A) and (D) are reprinted from primary figures. Scale bars: (50  $\mu$ m)

P14 (E11.5 Tam) from Fig. 6D

Utricle	tdTomato (+)	tdTomato(+) w/ Calyx	% Colocalized	Mean	83%
1	12	9	75%	STDEV	12%
2	12	11	92%	STDERROR	8%
				√N	1.4

P14 (E17.5 Tam) from Fig. 6D

Utricle	tdTomato (+)	tdTomato(+) w/ Calyx	% Colocalized	Mean	92%
1	360	354	98%	STDEV	6%
2	300	277	92%	STDERROR	3%
3	466	403	86%	√N	1.7

P14 (P2 Tam) from Fig. 6D

Utricle	tdTomato (+)	tdTomato(+) w/ Calyx	% Colocalized	Mean	90%
1	34	33	97%	STDEV	7%
2	445	374	84%	STDERROR	4%
3	464	419	90%	√N	1.7

**Figure S2. Raw data used for quantification graphs presented in Figure 6**



P14 (P2 Tam) from Fig. 7D

Utricle	tdTomato (+)	tdTomato (+) w/ OPN	% Colocalized	Mean	86%
1	205	188	92%	STDEV	9%
2	206	187	91%	STDERROR	5%
3	45	34	76%	√N	1.7

P14 (P2 Tam) from Fig. 7D

Utricle	tdTomato (+)	tdTomato (+) w/ Calb2	% Colocalized	Mean	6%
1	28	1	4%	STDEV	5%
2	390	46	12%	STDERROR	3%
3	396	12	3%	√N	1.7

P14 (P2 Tam) from Fig. 7D

Utricle	tdTomato (+)	tdTomato (+) w/ Anxa4	% Colocalized	Mean	4%
1	149	9	6%	STDEV	2%
2	321	10	3%	STDERROR	1%
3	275	10	4%	√N	1.7

**Figure S3. Raw data used for quantification graphs presented in Figure 7**

P14 from Fig. 8I

Utricle	Genotype	MyoVIIa	BIII Tubulin+MyoVIIa	% HCs with Calyx	Mean	61%
1	Littermate Control	778	511	66%	STDEV	4%
2	Littermate Control	845	486	58%	STDERROR	2%
3	Littermate Control	945	566	60%	√N	1.7

Utricle	Genotype	MyoVIIa	BIII Tubulin+MyoVIIa	% HCs with Calyx	Mean	61%
1	<i>Atoh1-Cre;Fgf8</i> CKO	955	525	55%	STDEV	5%
2	<i>Atoh1-Cre;Fgf8</i> CKO	914	575	63%	STDERROR	3%
3	<i>Atoh1-Cre;Fgf8</i> CKO	913	590	65%	√N	1.7

P14 from Fig. 8J

Utricle	Genotype	OPN	Mean	121
1	Littermate Control	138	STDEV	20
2	Littermate Control	99	STDERROR	12
3	Littermate Control	127	√N	1.73

Utricle	Genotype	OPN	Mean	93
1	<i>Pax2-Cre; Fgf8</i> CKO	149	STDEV	51
2	<i>Pax2-Cre; Fgf8</i> CKO	50	STDERROR	29
3	<i>Pax2-Cre; Fgf8</i> CKO	79	√N	1.73

P14 from Fig. 8J

Utricle	Genotype	OCM	Mean	123
1	Littermate Control	134	STDEV	17
2	Littermate Control	104	STDERROR	8
3	Littermate Control	140	√N	2.00
4	Littermate Control	114		

Utricle	Genotype	OCM	Mean	122
1	<i>Pax2-Cre; Fgf8</i> CKO	128	STDEV	10
2	<i>Pax2-Cre; Fgf8</i> CKO	110	STDERROR	6
3	<i>Pax2-Cre; Fgf8</i> CKO	128	√N	1.73

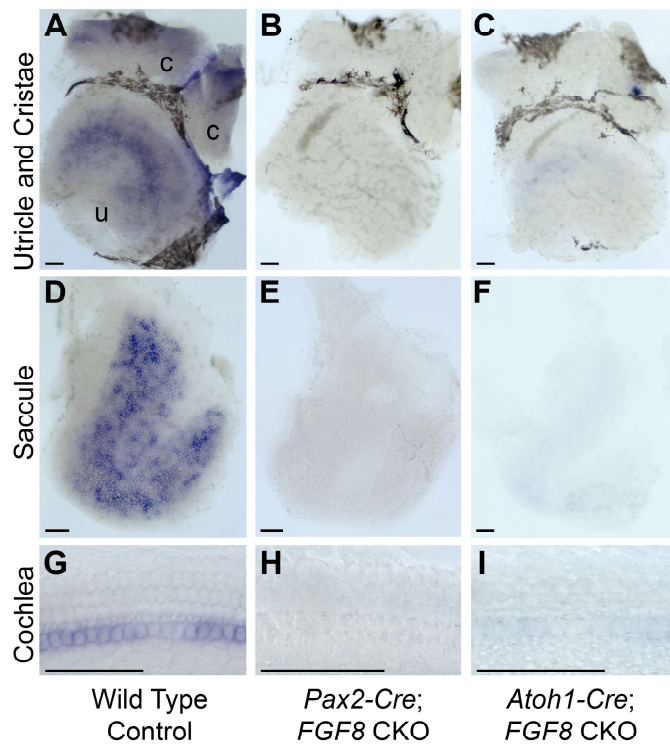
P14 from Fig. 8J

Utricle	Genotype	Mean LWSR	Mean	0.56
1	Littermate Control	0.51	STDEV	0.04
2	Littermate Control	0.57	STDERROR	0.02
3	Littermate Control	0.56	√N	2.45
4	Littermate Control	0.55		
5	Littermate Control	0.55		
6	Littermate Control	0.56		
7	Littermate Control	0.55		
8	Littermate Control	0.66		

Utricle	Genotype	Mean LWSR	Mean	0.54
1	<i>Pax2-Cre; Fgf8</i> CKO	0.56	STDEV	0.08
2	<i>Pax2-Cre; Fgf8</i> CKO	0.45	STDERROR	0.03
3	<i>Pax2-Cre; Fgf8</i> CKO	0.51	√N	2.45
4	<i>Pax2-Cre; Fgf8</i> CKO	0.70		
5	<i>Pax2-Cre; Fgf8</i> CKO	0.56		
6	<i>Pax2-Cre; Fgf8</i> CKO	0.49		
7	<i>Pax2-Cre; Fgf8</i> CKO	0.47		

**Figure S4. Raw data used for quantification graphs presented in Figure 8**

Comparison of E18.5 *Pax2-Cre; Fgf8* CKO utricles to littermate controls for presence of Type I hair cells in the striola Oncomodulin and extrastriola with Osteopontin from Fig. 8J and Lateral extrastriolar width to striolar width ratio (LWSR) compared between E18.5 *Pax2-Cre; Fgf8* CKO utricles corresponding to Fig. 8J.



**Figure S5. *Fgf8* expression is abolished in *Pax2-Cre* and *Atoh1-Cre*; *Fgf8* CKOs**  
 (A) Wholemound ISH for *Fgf8* shows vestibular hair cell expression in littermate control utricle (u) and cristae (c), saccule (D), and inner hair cells (G) of the organ of Corti. (B,E,H) *Fgf8* mRNA is completely lost following gene deletion using *Pax2-Cre* in each of these sensory epithelia. (C,F) *Fgf8* mRNA is lost from the extrastricular regions of the utricle and saccule and the inner hair cells (I) of the organ of Corti following hair cell specific deletion using *Atoh1-Cre*. Brackets indicate the approximate boundaries of the striolar region. Scale bars: (50 $\mu$ m)