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Characterization of Adult Vestibular Organs in 11 CreER Mouse Lines

Jennifer S. Stone,[1] Serena R. Wisner,[1] Stephanie A. Bucks,[1] Marcia M. Mellado Lagarde,[2] and Brandon C. Cox[3]

[1]*Department of Otolaryngology-Head and Neck Surgery, Virginia Merrill Bloedel Hearing Research Center, University of Washington, Seattle, WA, USA*

[2]*Department of Developmental Neurobiology, St. Jude Children's Research Hospital, Memphis, TN, USA*

[3]*Departments of Pharmacology and Surgery, Division of Otolaryngology, Southern Illinois University School of Medicine, Springfield, IL, USA*

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ABSTRACT

Utricles are vestibular sense organs that encode linear head movements. They are composed of a sensory epithelium with type I and type II hair cells and supporting cells, sitting atop connective tissue, through which vestibular nerves project. We characterized utricular Cre expression in 11 murine CreER lines using the *ROSA26*^{tdTomato} reporter line and tamoxifen induction at 6 weeks of age. This characterization included *Calbindin2*^{CreERT2}, *Fgfr3-iCreER*^{T2}, *GFAP-A-CreER*™, *GFAP-B-CreER*™, *GLAST-CreER*^{T2}, *Id2*^{CreERT2}, *Otoferlin*^{CreERT2}, *Parvalbumin*^{CreERT2}, *Prox1*^{CreERT2}, *Sox2*^{CreERT2}, and *Sox9-CreER*^{T2}. *Otoferlin*^{CreERT2} mice had inducible Cre activity specific to hair cells. *GLAST-CreER*^{T2}, *Id2*^{CreERT2}, and *Sox9-CreER*^{T2} had inducible Cre activity specific to supporting cells. *Sox2*^{CreERT2} had inducible Cre activity in supporting cells and most type II hair cells. *Parvalbumin*^{CreERT2} mice had small numbers of labeled vestibular nerve afferents. *Calbindin2*^{CreERT2} mice had labeling of most type II hair cells and some type I hair cells and supporting cells. Only rare (or no) tdTomato-positive cells were detected in utricles of *Fgfr3-iCreER*^{T2}, *GFAP-A-CreER*™, *GFAP-B-CreER*™, and *Prox1*^{CreERT2} mice. No Cre leakiness (tdTomato expression in the absence of tamoxifen) was observed in *Otoferlin*^{CreERT2} mice. A small degree of leakiness was seen in *GLAST-CreER*^{T2}, *Id2*^{CreERT2}, *Sox2*^{CreERT2}, and *Sox9-CreER*^{T2} lines. *Calbindin2*^{CreERT2} mice had similar tdTomato expression with or without tamoxifen, indicating lack of inducible control under the conditions tested. In conclusion, 5 lines—*GLAST-CreER*^{T2}, *Id2*^{CreERT2}, *Otoferlin*^{CreERT2}, *Sox2*^{CreERT2}, and *Sox9-CreER*^{T2}—showed cell-selective, inducible Cre activity with little leakiness, providing new genetic tools for researchers studying the vestibular periphery.

Keywords: CreER/loxP, mouse genetics, utricle, fate-mapping, calbindin, FGFR3, GFAP, GLAST, Id2, otoferlin, parvalbumin, Prox1, Sox2, Sox9

INTRODUCTION

The utricle is a vestibular sense organ located in the inner ear. The sensory epithelium, or macula, of the utricle is composed of two resident cell types—sensory hair cells, which encode linear acceleration of the head, and non-sensory supporting cells, which resemble glia. The macula is surrounded by a thin non-sensory, or transitional, epithelium. A loose connective tissue, called the stroma, supports the macula. The peripheral neurites of the vestibular nerve, whose cell bodies are located in the vestibular ganglion, course through the stroma and into the macula. The stroma also contains Schwann cells, endothelial cells, fibroblasts, immune cells, and other cell types.

Correspondence to: Brandon C. Cox · Departments of Pharmacology and Surgery, Division of Otolaryngology · Southern Illinois University School of Medicine · Springfield, IL, USA. email: bcox@siumed.edu

Although there is a growing understanding of the cellular processes underlying utricular development, maturation, and homeostasis, we have limited tools to define the genes that control these processes. Genetically engineered mice that allow cell type-specific and temporal control of gene expression are very powerful tools to dissect the molecular mechanism of biological pathways, as well as to fate-map cells during development, regeneration, and repair. The Cre-loxP system allows for control of gene expression in select cells by using a cell type-specific promoter or enhancer to drive expression of Cre, a DNA recombinase, and floxed target alleles, which enable deletion of targeted DNA segments and activation or repression of specific genes (Kwan 2002). When a modified estrogen receptor is added to the Cre enzyme, creating a CreER fusion protein (e.g., CreER™, CreER^{T2}, or iCreER^{T2}), temporal control of gene expression is achieved via tamoxifen administration (Feil et al. 1996; Hayashi and McMahon 2002).

More than 30 Cre or CreER alleles have been described for the organ of Corti, the sensory organ for hearing, which like the utricular macula is located in the inner ear and composed of hair cells and supporting cells. In the cochlea, these Cre/CreER lines have been used to manipulate gene expression in different cell types and to fate-map sensory progenitor cells, with studies resulting in significant advances for the auditory field (reviewed in Cox et al. 2012). However, few CreER lines have been characterized for vestibular end organs, including the utricle. Four lines—*Atoh1-CreER*™, *Lgr5$^{eGFP\text{-}CreERT2}$*, *Plp-CreER^{T2}*, and *Sox2^{CreERT2}*—have been used to study hair cell regeneration or postnatal hair cell addition in neonatal utricles (Gómez-Casati et al. 2010; Burns et al. 2012a, b; Wang et al. 2015; Lin et al. 2015; Wu et al. 2016). However, only two CreER lines have been described in the adult vestibular system. After tamoxifen injection at 6 weeks of age, *Plp-CreER^{T2}* mice targeted the majority of supporting cells, as well as a small number of Schwann cells and cells in the transitional epithelium, while *Atoh1-CreER*™ mice targeted ~40 % of type II hair cells. Both CreER lines were recently used to demonstrate hair cell turnover (cell loss and cell addition) in normal adult utricles (Bucks et al. 2017).

Here, we characterized the Cre activity pattern of 11 CreER lines after tamoxifen induction at 6 weeks of age in the mouse utricular macula. We found that five lines—*GLAST-CreER^{T2}*, *Id2^{CreERT2}*, *Otoferlin^{CreERT2}*, *Sox2^{CreERT2}*, and *Sox9-CreER^{T2}*—had inducible Cre activity in utricular hair cells and/or supporting cells, which constitute new genetic tools for investigators to manipulate gene expression or to fate-map cells in the vestibular periphery of adult mice.

MATERIALS AND METHODS

Mouse Models

Calbindin2^{CreERT2} (stock no. 13730, Taniguchi et al. 2011), *GLAST-CreER^{T2}* (stock no. 12586, Wang et al. 2012), *Id2^{CreERT2}* (stock no. 16222, Rawlins et al. 2009), *Parvalbumin^{CreERT2}* (stock no. 10777, Taniguchi et al. 2011), *Sox2^{CreERT2}* (stock no. 17593, Arnold et al. 2011), *Sox9-CreER^{T2}* (stock no. 18829, Kopp et al. 2011), and *ROSA26^{tdTomato}* (also called Ai14, stock no. 7908, Madisen et al. 2010) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). *Fgfr3-iCreER^{T2}* mice (Rivers et al. 2008; Young et al. 2010) were provided by Dr. William Richardson (University College London, UK) and are now available at The Jackson Laboratory (stock no. 25809). *GFAP-A-CreER*™ and *GFAP-B-CreER*™ mice (Chow et al. 2008) were provided by Dr. Suzanne Baker (St. Jude Children's Research Hospital, Memphis, TN). *Otoferlin^{CreERT2}* mice were provided by Dr. Ulrich Müller (The Scripps Research Institute, La Jolla, CA) and are now available from the Mutant Mouse Resource and Research Centers (MMRRC) as stock no. 032782-MU. *Prox1^{CreERT2}* mice (Srinivasan et al. 2007) were provided by Dr. Guillermo Oliver (St. Jude Children's Research Hospital, Memphis, TN) and are now available from The Jackson Laboratory (stock no. 22075). *ROSA26^{tdTomato}* and all CreER lines were used as heterozygotes for all experiments. Genotyping was performed by Transnetyx, Inc. (Cordova, TN) or as described previously (Cox et al. 2014). Both genders were used in all studies. All procedures were conducted in accordance with approved animal protocols from the Institutional Animal Care and Use Committees at St. Jude Children's Research Hospital (Memphis, TN) and Southern Illinois University School of Medicine (Springfield, IL).

Mouse Strain Background

Each CreER line was on a different mixed genetic background based on how the line was generated and bred before it was deposited at The Jackson Laboratory or imported to our mouse colony. Specifically, *Calbindin2^{CreERT2}* mice were on a C57BL/6J:B6(Cg)-Tyr$^{c\text{-}2J}$/J background, *Fgfr3-iCreER^{T2}* mice were on a C57BL/6J:CBA background, *GFAP-A-CreER*™ and *GFAP-B-CreER*™ mice were on a FVB/NJ background, *GLAST-CreER^{T2}* mice were on a C57BL/6J:C57BL/6N:SJL/J background, *Id2^{CreERT2}* mice were on a C57BL/6J:C57BL/6N background, *Otoferlin^{CreERT2}* mice were on a C57BL/6J:NZB:SJL/J background, *Parvalbumin^{CreERT2}* mice were on a C57BL/6J:C57BL/6N background, *Prox1^{CreERT2}* mice were on a NMRI:C57BL/6J:129Sv/Jae background, *Sox2^{CreERT2}*

mice were on a C57BL/6J:129Sv/Jae background, and *Sox9-CreER*^{T2} mice were on a C57BL/6J:BALBc:CD1 background. The *ROSA26*^{tdTomato} reporter line was purchased from The Jackson Laboratory in 2013 on a C57BL/6J background and has been maintained in our colony since then by breeding with these lines as well as other CreER lines not described in this manuscript. Every 8–12 months, new *ROSA26*^{tdTomato} breeders were obtained from Cre-negative pups from different breeding strategies and therefore the strain background of individual *ROSA26*^{tdTomato} breeders varied.

Drug Treatments

Tamoxifen [9 mg/40 g, intraperitoneal injection (IP); Sigma-Aldrich (St. Louis, MO)] was injected once a day on two consecutive days (~ 20–24 h apart) in 6-week-old mice, and samples were collected 1 week after tamoxifen injection, at 7 weeks of age. Controls were age-matched littermates that expressed CreER and *ROSA26*^{tdTomato} alleles but did not receive tamoxifen injection and were housed separately from the tamoxifen-treated experimental mice.

Immunofluorescent Staining

Temporal bones were removed and post-fixed in electron microscopy grade 4 % paraformaldehyde (Polysciences, Inc., Warrington, PA) overnight at room temperature. After fixation, temporal bones were stored in 10 mM phosphate-buffered saline [PBS; Sigma-Aldrich (St. Louis, MO)] until whole utricles were dissected out of temporal bones and placed in 96-well plates for free-floating immunofluorescent labeling. Utricles and, in some cases, ampullae were immunofluorescently stained as described in Bucks et al. (2017). For cell typing, hair cells were labeled with rabbit anti-myosin VIIa primary antibodies (1:300; Proteus Biosciences, Inc.; cat no. 25–6790). The following additional primary antibodies were used: rabbit anti-calbindin2 (1:500; EMD Millipore cat. no. AB5054), rabbit anti-GLAST (1:1000; Abcam cat. no. ab416), chicken anti-neurofilament 200 kD (1:500; Millipore cat. no. AB5539), mouse anti-otoferlin (1:500; Abcam cat. no. AB181781), mouse anti-parvalbumin (1:500; Sigma-Aldrich cat. no. P3088), goat anti-Sox2 (1:500; Santa Cruz Biotechnology cat. no. sc-17320), rabbit anti-Sox9 (1:500; Millipore cat. no. AB5535), and rabbit anti-IBA1 (1:1000; Wako Pure Chemical Industries cat. no. 019-19741). For samples immunostained with anti-GLAST antibodies, an antigen retrieval step was added prior to the blocking/permeabilization step. Here, samples were incubated with a high pH antigen unmasking solution (1:100; Vector labs cat. no. H-3301) at 95 °C for 45 min in a hybridization oven, followed by three PBS washes before proceeding with the rest of the staining procedure. All primary antibodies were detected with Alexa Flour-conjugated secondary antibodies [1:400, Invitrogen (Carlsbad, CA)]. Nuclei were labeled by 4′,6′-diamidine-2-phenylindole (DAPI; Sigma-Aldrich) at 1 µg/mL in 10 mM PBS. Following staining, whole utricles were mounted in Fluoromount-G (Southern Biotech, Birmingham, AL).

Confocal Microscopy and Image Analysis

An Olympus FV-1000 microscope (Olympus, Center Valley, PA) was used to acquire images of fluorescent labeling. Z-series images were taken of whole-mounted utricles or ampullae with either a × 20 air objective or a × 60 oil objective, starting at the hair cell stereocilia and ending a few microns in the stroma. Z steps ranged from 0.5 to 1 µm increments. Images were analyzed using Fiji (http://fiji.sc/). We examined tdTomato labeling in the macula, the transitional epithelium, and the stroma. Images for the publication were generated using Adobe Photoshop CS4. For quantitative analyses, we generated four sets of z-series images—two from the peripheral macula and two from the central macula. The approximate positions of the sampled regions for every animal are indicated in Fig. 1d. The central regions were enriched for striolar epithelium, and the peripheral regions were enriched for lateral extrastriolar epithelium, although some overlap existed. We did not sample much of the medial extrastriola. All images were × 120 (created using a 60× oil objective with × 2 zoom), and when added together, the four images comprised 24 % of the area of sensory epithelium. For several CreER lines, tdTomato-positive cells in each image were counted and classified as either type I or type II hair cells or supporting cells (described in the next section) using the Fiji Cell Counter plug-in. For each set of images, we calculated the number of labeled cells per 10,000 µm^2, or cell density. To estimate the number of tdTomato-positive hair cells or supporting cells per utricle, we did the following. First, we summed cell numbers from the four images. Then, we multiplied this value by 4.16 (or 100 %/24 %), to reflect the proportion of the macula that all images collectively sampled. To estimate the percentage of each cell type labeled per utricle, we divided the estimated number of tdTomato-positive cells per utricle by either 3800 (for hair cells) or 4000 (for supporting cells), based on the assumption that each utricle had 3800 hair cells and 4000 supporting cells (Golub et al. 2012). Averages, standard deviations, and animal numbers, as well as raw data and gender, for each animal in which quantitative analyses were performed are presented in the tables.

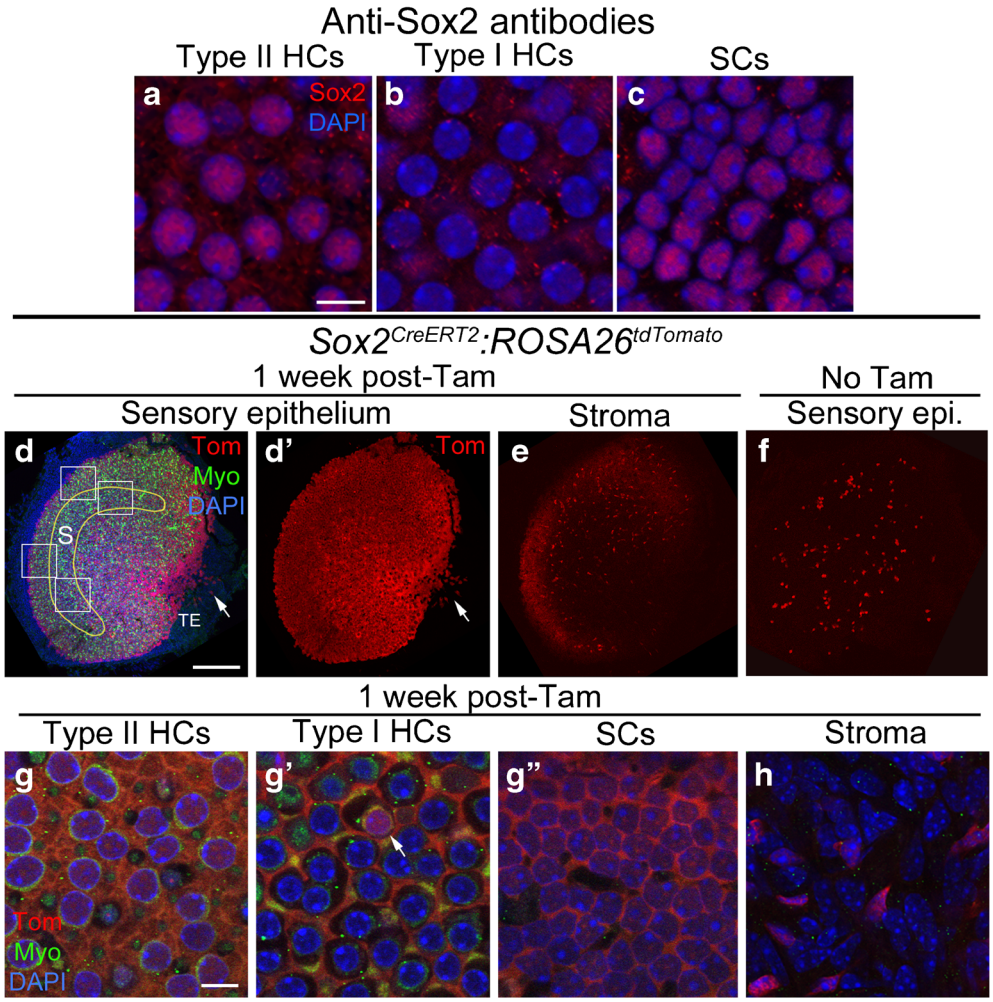

FIG. 1. *Sox2*^{CreERT2} mice have inducible Cre activity in type II hair cells and supporting cells. **a**-**c** Confocal images showing immunolabeling for Sox2 (red) and DAPI nuclear labeling (blue) in slices focused on the nuclei of type II hair cells (HCs) (**a**), type I HCs (**b**), and supporting cells (SCs) (**c**) of the utricular macula. **d**-**e** Confocal images of utricles from *Sox2*^{CreERT2}*:ROSA26*^{tdTomato} mice injected with tamoxifen, focused on either the macula (**d**,**d**) or the stroma (**e**). **d** and **d** show the same view, with labeling for tdTomato (Tom), myosin VIIa (Myo), and DAPI shown in **d** and Tom shown in **d**'. The yellow line in **d** shows the approximate position of the striola (S). The white boxes in **d** show the approximate regions sampled for cell counts for the *Sox2Cre*^{ERT2} line and for all other lines that were quantitatively analyzed. TE = transitional epithelium. The arrow points to the TE near the notch. **e** shows Tom labeling in the stroma. **f** Utricle from a *Sox2*^{CreERT2}*:ROSA26*^{tdTomato} mouse that did not receive tamoxifen, focused on the sensory epithelium. **g**-**h** Confocal slices through the lateral extrastriolar region, with slices through the type II hair cell (HC) nuclear layer (**g**), the type I hair cell (HC) nuclear layer (**g**'), the supporting cell (SC) nuclear layer (**g**"), or the stroma (**h**). The arrow in **g**' points to a Tom-labeled type I HC. Scale bar in **a** = 5 μm and applies to **a**-**c**. Scale bar in **d** = 150 μm and applies to **d**-**f**. Scale bar in **g** = 5 μm and applies to **g**-**h**

Criteria for Cell Typing

Hair cells were classified as type I or II using previously described methods (Rüsch et al. 1998; Eatock and Songer 2011; Pujol et al. 2014; Bucks et al. 2017). Briefly, hair cells were distinguished from supporting cells by myosin VIIa immunoreactivity (Hasson and Mooseker 1997) and the location of their nuclei above the supporting cell nuclear layer. The nuclei of type II hair cells are slightly oblong and larger than type I hair cell nuclei, and they are located in a near-monolayer apical to type I hair cell nuclei. Type II hair cells also have a thick neck and basolateral cytoplasmic processes. Type I hair cells have nuclei that are small and round. They are positioned in a near-monolayer below type II hair cell nuclei and above the supporting cell nuclei, except in the striola, where some type I hair cell nuclei are positioned apical to type II hair cell nuclei. Type I hair cells have a thin neck and lack basolateral cytoplasmic processes. Finally, the cell body of each type I hair cell is encapsulated by a specialized afferent nerve ending called a calyx. By contrast, type II hair cells are contacted by small bouton afferents.

Quantitative Analysis

All statistics were performed using unpaired Student's *t* tests on GraphPad Prism version 6.00 (La Jolla, CA. http://www.graphpad.com). Animal numbers, genders for each mouse, and the parental inheritance of the CreER allele for all of the CreER lines that we analyzed quantitatively are presented in the tables. One utricle per animal was assessed, and the "*n*" value represents the number of mice included in the study.

RESULTS

Structure of the Utricular Macula

The mouse utricle is a disk-shaped organ with a sensory epithelium (macula) sitting atop a layer of loose connective tissue (stroma). The histology of the mammalian utricle is reviewed in Eatock and Songer (2011). The organ is inundated by processes of the vestibular nerve, each of which is surrounded by glial cells located within the stroma, but not within the macula. The macula houses the cell bodies of supporting cells, type I hair cells, and type II hair cells, the nuclei of which are positioned in discrete layers, as described in the "Materials and Methods" section and illustrated in Fig. 2. The macula has two general zones: the central zone, or striola, and the peripheral zone, or extrastriola (Fig. 2a). The striola is a C-shaped region. It is the only location in the macula in which type I hair cells are innervated by pure calyceal vestibular afferent neurons, which encapsulate two or more type I hair cells. The peripheral zone is further broken into the medial and lateral extrastriolar sub-regions, with the lateral extrastriola having a smooth contour and the medial extrastriola marked by a notch. Hair cells in the two extrastriolar regions—their properties and ratios—appear very similar. Type I and II hair cells are distributed in a mosaic across all zones of the macula (Fig. 2b, c). Hair cells are separated by the cell bodies of supporting cells. Only type II hair cells contact each other, and this occurs only at the level of the basolateral processes (Fig. 2c). The transitional epithelium is a monolayer, several cells in width, which lines the periphery of the macula (Fig. 2a).

CreER Lines Targeting Vestibular Hair Cells and Supporting Cells

Each CreER mouse line was bred to the *ROSA26*$^{CAG\text{-}loxP\text{-}stop\text{-}loxP\text{-}tdTomato}$ (*ROSA26*^{tdTomato}) reporter line to identify cells with CreER activity. Mice were injected with tamoxifen (9 mg/40 g, IP) at 6 weeks of age (with two injections given ~20–24 h apart) and analyzed 1 week later (at 7 weeks of age). To determine if there was any CreER activity in the absence of tamoxifen, a phenomenon called Cre leakiness, we examined control samples that expressed both CreER and *ROSA26*^{tdTomato} but did not receive tamoxifen and were housed separately from tamoxifen-injected mice.

Sox2 is a high-mobility group (HMG) box domain transcription factor that is required for hair cell development in the mammalian inner ear (Kiernan

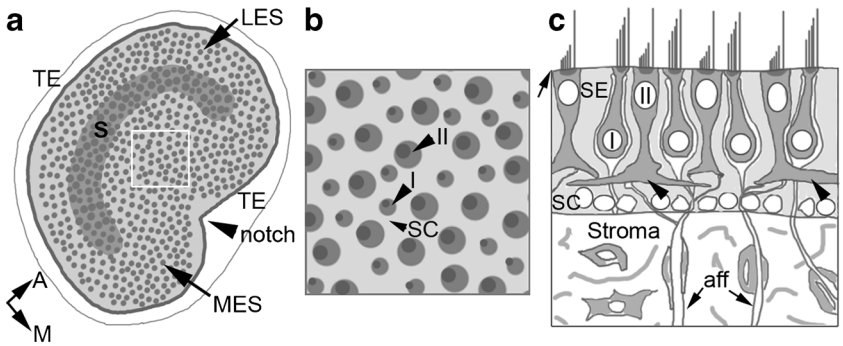

FIG. 2. Histology of the adult mouse utricle. **a** A top-down view of the macula (light gray) including hair cells (dark gray dots) and the transitional epithelium (TE, white border). The gray C-shaped region is the striola (S), and it is surrounded by the lateral extrastriola (LES) and medial extrastriola (MES). The notch in the medial portion of the macula is indicated by an arrowhead. The anatomical orientations (A = anterior and M = medial) apply to **a** and **b**. **b** A higher magnification view of the apical surface of the utricular macula from a region similar to that boxed in **a**. Arrowheads point to a supporting cell (SC), the larger-sized apical surface of a type II (II) hair cell and the smaller-sized apical surface of a type I hair cell (I), each with a stereociliary bundle indicated by a dark gray dot on the lateral side of the cell. **c** A cross-section through the macula (sensory epithelium, or SE) and stroma showing the different cell types in each tissue. Hair cell stereocilia project into the lumenal space from the apical side of the epithelium (arrow). Type II hair cell nuclei lie apical to type I hair cell nuclei. Type I hair cells are surrounded by white nerve calyces of vestibular nerve afferents, whose axons (aff, arrows) travel basally through the stroma to the vestibular ganglion (not shown). Type II hair cells have basolateral processes (arrowheads). The nuclei of supporting cells (SCs) lie along the basal lamina (the thin line at the base of the epithelium, in light gray). In the stroma, several cell types exist, including fibroblasts, macrophages, and myelinating glial cells

et al. 2005; Dvorakova et al. 2016). Prior studies demonstrated that Sox2 immunolabeling is detected in all supporting cells and in most or all type II hair cells in utricles from adult CBA/CaJ mice (Oesterle et al. 2008). Using the same antibody in utricles from mice on a mixed background strain (see the "Materials and Methods" section for more detail), we found a similar cell-selective pattern of Sox2 protein expression (Fig. 1a–c). The *Sox2*^{CreERT2} line is a knock-in allele where *CreER*^{T2} replaced the *Sox2* open reading frame and therefore only one copy of *Sox2* is expressed in these mice (Arnold et al. 2011). We noted no difference in the density or distribution of hair cells or supporting cells, or any change in motor behavior, that would indicate a phenotypic abnormality due to *Sox2* haploinsufficiency. After tamoxifen induction at 6 weeks of age in *Sox2*^{CreERT2}:*ROSA26*^{tdTomato} mice, we estimated that tdTomato was expressed in the cytoplasm and/or nucleus of 5137 ± 337 cells (± 1 standard deviation) per utricle, of which 3945 ± 238 were supporting cells and 1192 ± 99 were hair cells (Fig. 1d, d', g-g"; Table 1). These estimates were made by analyzing about 24 % of the macula, sampling from two peripheral and two central regions, which are shown in Fig. 1d for the *Sox2Cre*^{ERT2} line. This approach was applied for all CreER lines when calculating tdTomato-positive cell counts per utricle. We estimated that Cre was active, on average, in 99 % of supporting cells. We detected no difference in the density of labeled supporting cells in peripheral and central regions (unpaired Student's *t* test, $t(4) = 0.25$, $p = 0.815$). TdTomato was also detected in approximately 32 % of hair cells, 93 % of which were type II hair cells and 7 % of which were type I hair cells (Fig. 1g-g'). Since 93 % of type II hair cells were tdTomato-positive, and type II hair cells comprise a little under 50 % of the utricular hair cell population (Desai et al. 2005), we anticipated we would find that ~ 50 % of hair cells were tdTomato-positive. However, we found that only 32 % of hair cells were tdTomato-positive. This finding is likely due to the fact that we oversampled the striola, which has a smaller proportion of type II hair cells than the extrastriola (Desai et al. 2005). Densities of tdTomato-labeled type II HCs were slightly higher in the peripheral regions than the central regions (unpaired Student's *t* test, $t(4) = 7.53$, $p = 0.002$). There was also a small number of tdTomato-positive cells observed in the transitional epithelium (Fig. 1d, d'). We were unable to attain accurate counts of labeled cells in this region because we could not reliably image it in every utricle. However, we estimated that each utricle had 10–100 tdTomato-labeled transitional epithelial cells, with the highest density in the medial notch. Numerous tdTomato-positive cells were detected in the connective tissue (stroma) (Fig. 1e, h), which lies underneath the utricular macula. In *Sox2*^{CreERT2}:*ROSA26*^{tdTomato} mice that were not treated with tamoxifen, we detected 62.2 ± 38.81 labeled supporting cells and 6.4 ± 5.86 labeled hair cells per utricle (Fig. 1f; Table 1).

Calbindin2 (also called calretinin) is a calcium-binding protein that is detected by immunostaining primarily in type II hair cells and striolar calyceal afferents in utricles from adult CBA/J mice (Dechesne et al. 1991; Desai et al. 2005; Pujol et al. 2014). Desai et al. (2005) found that anti-calbindin2 antibodies labeled the cytoplasm of ~ 64 % of type II hair cells and ~ 5 % of type I hair cells in adult mouse utricles. Our immunolabeling in mixed background strain mice showed similar results, with nuclear and cytoplasmic labeling of many type II hair cells, including their basolateral processes, and little or no labeling of type I hair cells or supporting cells (Fig. 3a–c). *Calbindin2*^{CreERT2} is a knock-in allele, in which *CreER*^{T2} was inserted into the initiation codon of the endogenous *Calbindin2* locus and therefore only one copy of *Calbindin2* is expressed in these mice (Taniguchi et al. 2011). We observed no difference in density or distribution of hair cells or supporting cells, or any change in motor behavior, which would indicate a phenotypic abnormality due to *Calbindin2* haploinsufficiency. Analysis of *Calbindin2*^{CreERT2}:*ROSA26*^{tdTomato} mice showed that there was no apparent difference in the tdTomato expression pattern (numbers or cell types) between samples that were injected with tamoxifen at 6 weeks of age and controls that did not receive tamoxifen (Fig. 3d, d', f, g-g"). Since this finding demonstrates that *Calbindin2*^{CreERT2} is not behaving in an inducible manner in the adult utricle, we did not quantify tdTomato-positive cells. However, qualitative analysis showed in both tamoxifen-injected and control mice that tdTomato was expressed in a large proportion of type II hair cells and in a very small proportion of type I hair cells and supporting cells. No tdTomato expression was detected in neural elements, which was surprising, since antibody labeling has shown calbindin2 to be a selective marker for calyx-only afferents in the striola (e.g., Desai et al. 2005). No tdTomato was detected in the transitional epithelium (Fig. 3d, d'), and a small number of cells in the stroma were tdTomato-positive (Fig. 3e, h).

CreER Lines Targeting Vestibular Hair Cells

Otoferlin is a calcium-binding protein that regulates SNARE-mediated vesicle fusion in cochlear and vestibular hair cells (Roux et al. 2006; Schug et al. 2006). Immunolabeling in rodents has shown that otoferlin is expressed in type I and II vestibular hair cells (Schug et al. 2006; Dulon et al. 2009). Our immunolabeling in utricles from mixed background strain mice showed a similar result, although labeling was not consistent across hair cells (Fig. 4a–e). The strongest labeling was seen in the perinuclear cytoplasm and basolateral processes of type II hair cells, but not all type II hair cells were labeled. Labeling in type I hair cells was usually much weaker or not detected (Fig. 4b, inset; e). *Otoferlin*^{CreERT2} is a knock-in

TABLE 1

Quantification of tdTomato-labeled hair cells and supporting cells in the 5 CreER lines with cell-selective and inducible Cre activity

	Mouse line[c]	*Sox2*^{CreERT2}		*Otoferlin*^{CreERT2}	*GLAST-CreER*^{T2}		*Id2*^{CreERT2}		*Sox9-CreER*^{T2}	
		No. of cells per utricle[d]	*No. of cells per 10,000 μm²*	*No. of cells per utricle*	*No. of cells per utricle*[d]	*No. of cells per 10,000 μm²*	*No. of cells per utricle*[d]	*No. of cells per 10,000 μm²*	*No. of cells per utricle*[d]	*No. of cells per 10,000 μm²*
Tam at 6 weeks	Hair cells (HCs)	1192 ± 99	65.5 ± 5.33	403 ± 41	6.6 ± 10.9	0.41 ± 0.50	13.9 ± 24.0	0.68 ± 1.17	138.3 ± 64.9	6.73 ± 3.16
	% of HCs labeled[a]	32 %		11 %	0.2 %		0.4 %		4 %	
	Type I HCs/ut (% Type I)		4.4 ± 1.32 (7 %)	70 ± 52 (17 %)		4.7 ± 10.5 (0.1 %)		0 ± 0 (0 %)		24.5 ± 31.6 (0.6 %)
	Type II HCs/ut (% Type II)		60.08 ± 4.65 (93 %)	334 ± 38 (83 %)		1.6 ± 3.5 (0 %)		13 ± 22.5 (0.3 %)		109.4 ± 122.9 (0.3 %)
	Supporting cells (SCs)	3945 ± 238		0	2270 ± 1408		1159 ± 416		3483 ± 556	
	% of SCs labeled[a]	99 %		0 %	57 %		29 %		87 %	
	Central SCs		227 ± 13			132 ± 82		48 ± 26		171 ± 48
	Peripheral SCs		199 ± 17			114 ± 71		61 ± 27		205 ± 15
	%tdTom+ cells that are HCs	23 %		100 %	1 %		1 %		4 %	
	%tdTom+ cells that are SCs	77 %		0 %	99 %		99 %		96 %	
	Number of mice analyzed[b]	3		3	5		3		4	
No Tam	Hair cells	6.4 ± 5.86		0	2.8 ± 5.50		0.33 ± 0.58		2.3 ± 0.58	
	Supporting cells	62.2 ± 38.81		0	6.3 ± 10.59		0.67 ± 0.58		1.3 ± 1.53	
	Number of mice analyzed[b]	5		4	4		3		3	

Data represent mean ±1 standard deviation (except for %)

[a]Estimate of the % of all utricular HCs or SCs labeled with tdTomato, assuming 4000 SCs/utricle and 3800 HCs/utricle

[b]One utricle per animal was assessed

[c]Sox2, Otoferlin, and Id2 CreER lines are knock-ins and designated by the following nomenclature: XXX^{CreERT2}. GLAST and Sox9 CreER lines are transgenics and designated by the following nomenclature: XXX-CreER^{T2}

[d]Estimate of numbers of tdTomato-labeled cells per utricle, based on sampling 24 % of the utricle

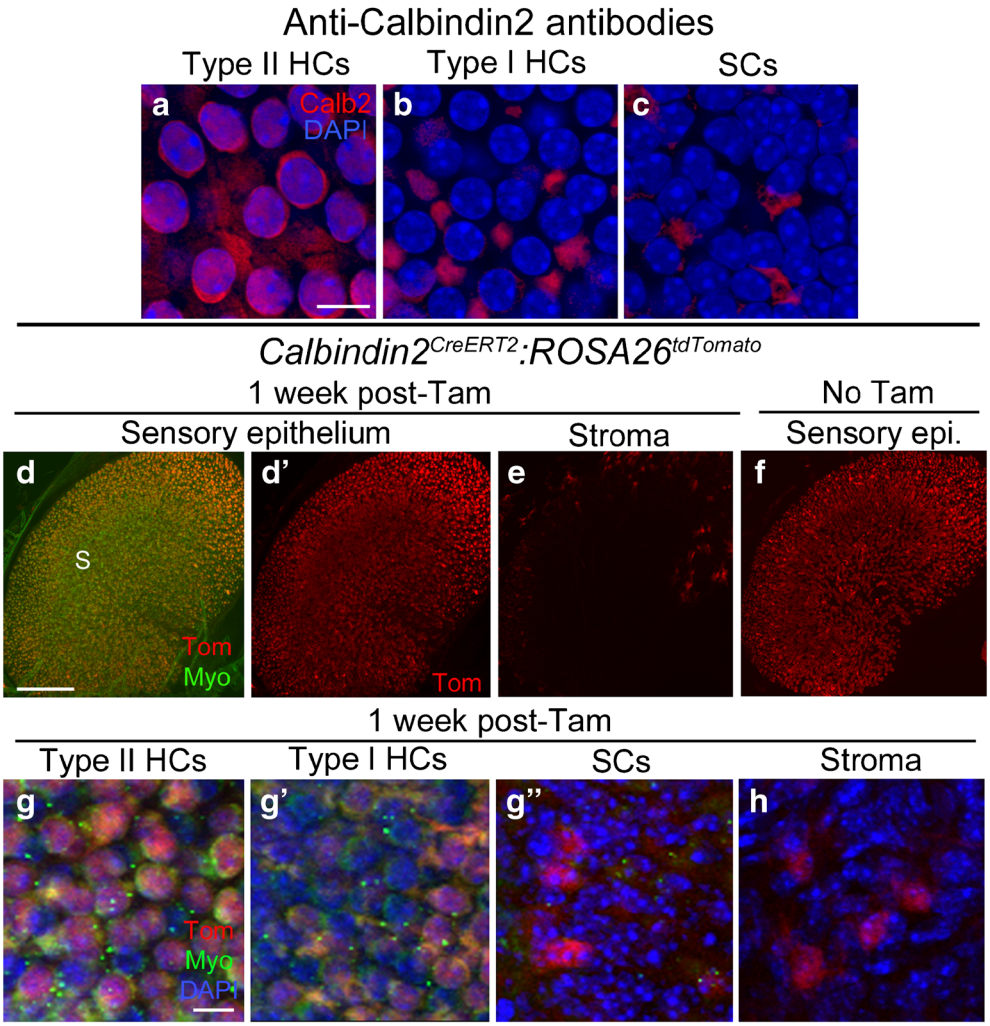

FIG. 3. *Calbindin2*^{CreERT2} mice have Cre activity in type II hair cells and supporting cells. **a**-**c** Confocal images showing immunolabeling for calbindin2 (Calb2, red) and DAPI nuclear labeling (blue) in slices focused on the nuclei of type II hair cells (HCs) (**a**), type I HCs (**b**), and supporting cells (SCs) (**c**) of the utricular macula. **d**-**e** Confocal images of utricles from *Calbindin2*^{CreERT2}*:ROSA26*^{tdTomato} mice injected with tamoxifen, focused on either the macula (**d**,**d**') or the stroma (**e**). **d** and **d**' show the same view, with labeling for tdTomato (Tom) and myosin VIIa (Myo) shown in **d** and Tom only shown in **d**'. In **d**, S = approximate position of the striola. **e** shows Tom labeling in the stroma. **f** Utricle from a *Calbindin2*^{CreERT2}*:ROSA26*^{tdTomato} mouse that did not receive tamoxifen, focused on the sensory epithelium. **g**-**h** Confocal slices through the lateral extrastriolar region, with slices through the type II hair cell (HC) nuclear layer (**g**), the type I hair cell (HC) nuclear layer (**g**'), the supporting cell (SC) nuclear layer (**g**''), or the stroma (**h**). Scale bar in **a** = 5 µm and applies to **a**-**c**. Scale bar in **d** = 150 µm and applies to **d**-**f**. Scale bar in **g** = 5 µm and applies to **g**-**h**

allele where *CreER*^{T2} was inserted into the endogenous *Otoferlin* locus and therefore only one copy of *Otoferlin* is expressed. It was generated as part of the NIH Neuroscience Blueprint Cre-Driver Network (http://www.credrivermice.org/index). We observed no difference in density or distribution of hair cells or supporting cells, or any change in motor behavior, which would indicate a phenotypic abnormality due to *Otoferlin* haploinsufficiency. After tamoxifen induction at 6 weeks of age in *Otoferlin*^{CreERT2}*:ROSA26*^{tdTomato} mice, tdTomato was expressed in 403 ± 41 hair cells per utricle, which is roughly 11 % of all hair cells (Fig. 4g, h, k, l; Table 1). Of labeled hair cells, 83 % were type II and 17 % were type I (Table 1). Although labeling was seen throughout the utricle, some samples appeared to have a higher density of tdTomato-positive hair cells in peripheral regions compared to central regions, although this was not quantified. No expression of tdTomato was observed in supporting cells (Fig. 4m), transitional epithelial cells (Fig. 4g, h), or cells in the stroma (Fig. 4i, n). In control samples that did not receive tamoxifen, no tdTomato-positive sensory epithelial cells were observed (Fig. 4j; Table 1).

CreER Lines Targeting Vestibular Supporting Cells

Glutamate-aspartate transporter (GLAST or SLC1A3) is a high-affinity reuptake pump for glutamate and aspartate often expressed in glia (reviewed in Takayasu et al. 2009).

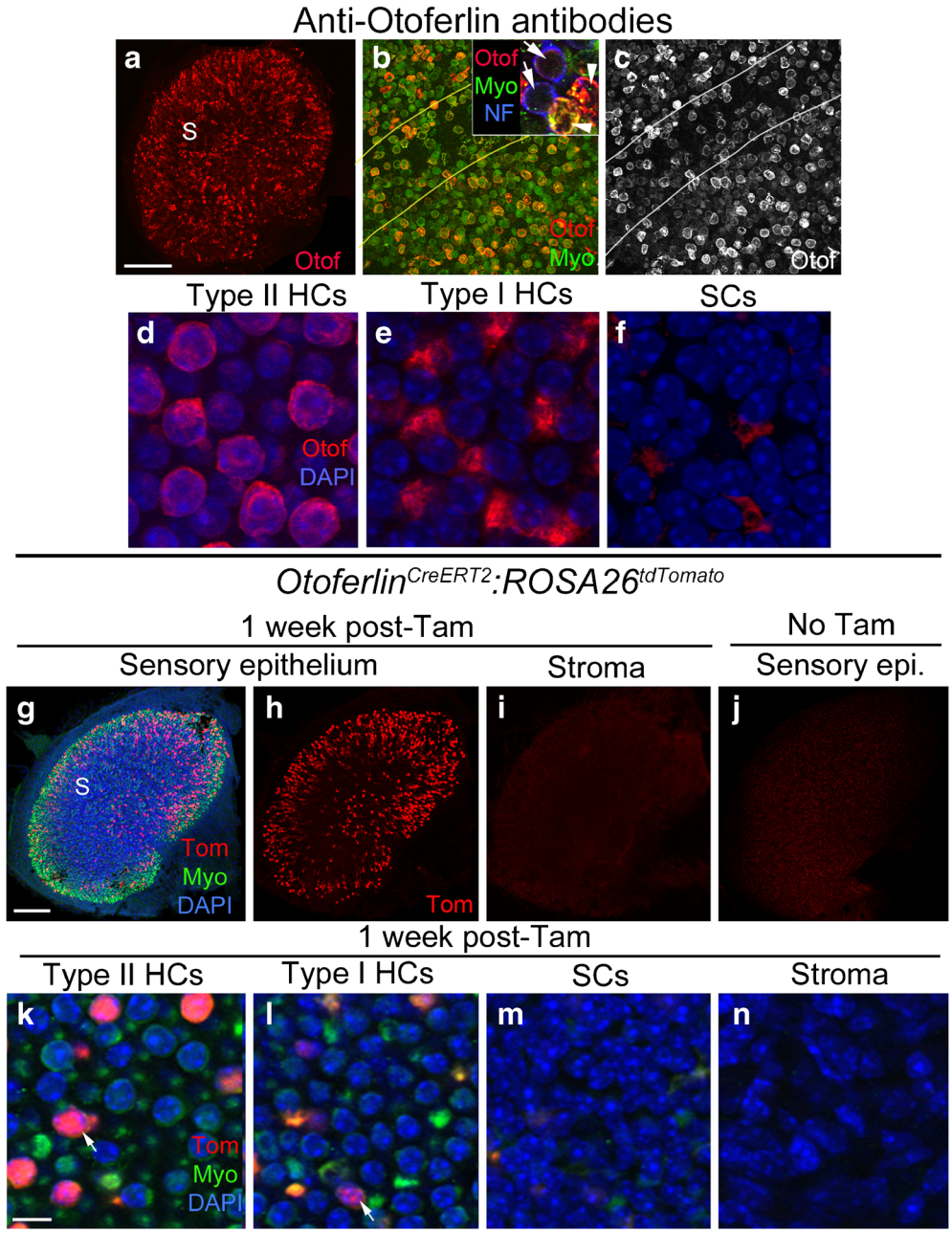

FIG. 4. *Otoferlin*^{CreERT2} mice have inducible Cre activity in many type II hair cells and some type I hair cells. **a**–**f** Confocal images showing immunolabeling for otoferlin (Otof, red), myosin VIIa (Myo, green), and/or 200 kD neurofilament (NF, blue) in utricular hair cells. **a** Whole-utricle view of otoferlin immunolabeling. S = the approximate position of the striola. **b**, **c** Magnified view of the region spanning the striola (demarcated by lines), with otoferlin labeling only shown in **c**. Arrows in the inset in **b** point to two type I hair cells with a light ring of otoferlin immunoreactivity in the cytoplasm (red) and NF labeling of the nerve calyx (blue). Arrowheads point to two strongly labeled type II hair cells. **d**–**f** Confocal images showing otoferlin immunolabeling (red) and DAPI nuclear labeling (blue) in slices focused on the nuclei of type II hair cells (HCs) (**d**), type I HCs (**e**), and supporting cells (SCs) (**f**) of the utricular macula. **g**–**i** Confocal images of utricles from *Otoferlin*^{CreERT2}*:ROSA26*^{tdTomato} mice injected with tamoxifen, focused on either the macula (**g**, **h**) or the stroma (**i**). **g** and **h** show the same view, with labeling for tdTomato (Tom, red), myosin VIIa (Myo, green), and DAPI (blue) shown in **g** and Tom only shown in **h**. In **g**, S = the approximate position of the striola, and TE = transitional epithelium. **i** Tom labeling in the stroma. **j** Utricle from an *Otoferlin*^{CreERT2}*:ROSA26*^{tdTomato} mouse that did not receive tamoxifen, focused on the sensory epithelium. **k**–**n** Confocal images through the lateral extrastriolar macula, with slices through the type II hair cell (HC) nuclear layer (**k**), the type I hair cell (HC) nuclear layer (**l**), the supporting cell (SC) nuclear layer (**m**), or the stroma (**n**). Arrows in **k**, **l** point to tdTomato-labeled cells. Scale bar in **a** = 150 μm for **a**, 25 μm for **c**, **d**, and 8 μm for **d**–**f**. Scale bar in **g** = 150 μm and applies to **g**–**j**. Scale bar in **k** = 5 μm and applies to **k**–**n**

In the vestibular system, GLAST is expressed in the membranes of supporting cells (Takumi et al. 1997; Hartman et al. 2010). With antibodies, we localized GLAST to the membranes of supporting cells (Fig. 5a,

b), but given the nature of the labeling, we were unable to determine numbers of supporting cells that were labeled or if GLAST was also expressed in the membranes of hair cells. *GLAST-CreER^{T2}* is a transgenic line that used a bacterial artificial chromosome (BAC) containing the *GLAST* promoter (Wang et al. 2012). After tamoxifen induction at 6 weeks of age in *GLAST-CreER^{T2}:ROSA26^{tdTomato}* mice, tdTomato was expressed in 2276 ± 1418 cells per utricle, of which 2270 ± 1408 were supporting cells and 6.6 ± 10.9 were hair cells, mostly type Is (Fig. 5c, c', f-f"; Table 1). We estimated that on average, Cre was active in ~57 % of supporting cells. The high variability in tdTomato labeling of supporting cells occurred because one of the five samples had a ~10-fold lower number of tdTomato-positive cells. Also, tdTomato-positive hair cells were only detected in two of the five samples. We detected no significant difference in the density of tdTomato-labeled cells in peripheral regions versus central regions (unpaired Student's *t* test, $t(7) = 0.343$, $p = 0.742$). Some cells in the stroma (Fig. 5d, g) and transitional epithelium (Fig. 5c, c') also expressed tdTomato. We observed 6.3 ± 10.59 labeled supporting cells and 2.8 ± 5.50 labeled hair cells per utricle in controls that were not given tamoxifen (Fig. 5e; Table 1).

Inhibitor of DNA binding 2 (Id2) is a basic helix loop helix transcription factor that regulates cell differentiation (reviewed in Ling et al. 2014). Its expression pattern has not been described for the mammalian vestibular system. However, *Id2* transcripts have been detected in cochlear supporting cells in mice (Jones et al. 2006) and in the chicken utricular macula (Ku et al. 2014). We were unable to find working antibodies to detect Id2 in whole-

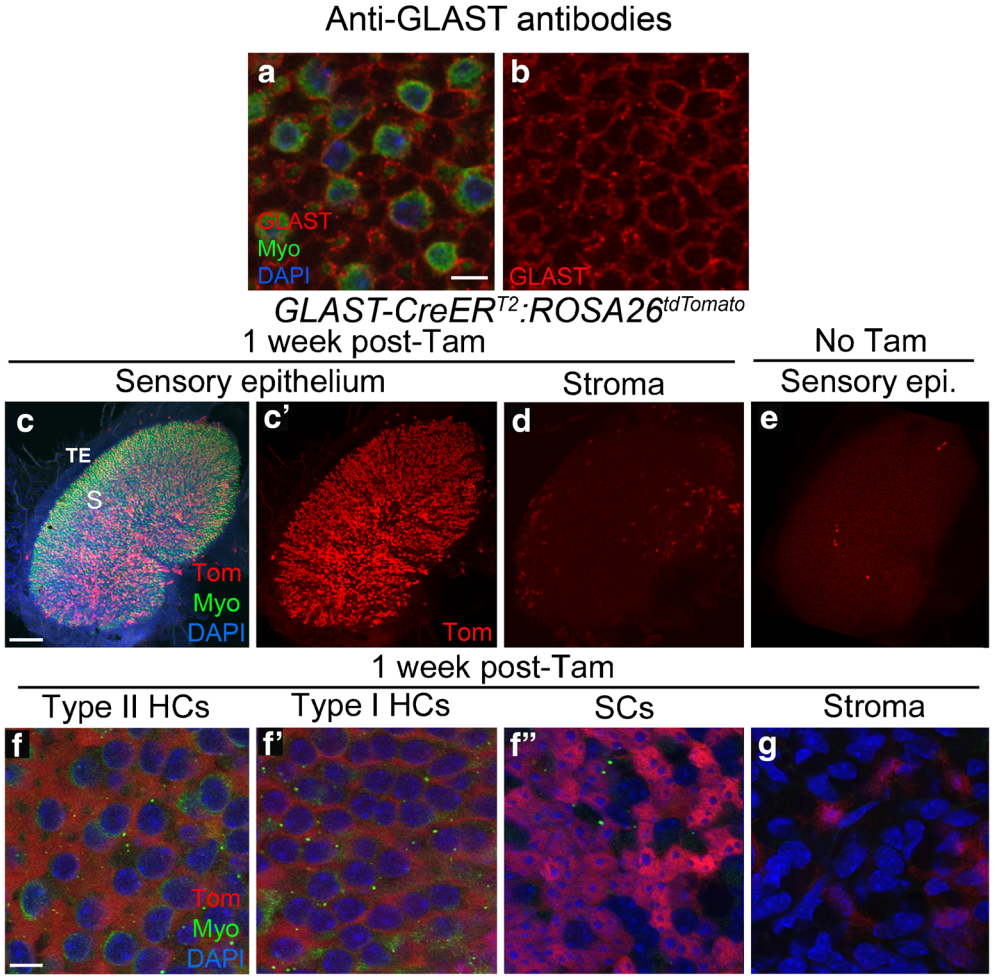

FIG. 5. *GLAST-CreER^{T2}* mice have inducible Cre activity in supporting cells. **a** Confocal images showing immunolabeling for GLAST (red), myosin VIIa (Myo, green), and nuclei (DAPI, blue) in slices focused on the type II hair cell nuclear layer. **b** GLAST labeling only in same field as shown in **a**. **c**-**d** Confocal images of the utricle from *GLAST-CreER^{T2}:ROSA26^{tdTomato}* mice injected with tamoxifen, focused on either the macula (**c**,**c**') or the stroma (**d**). **c** and **c**' show the same view, with labeling for tdTomato (Tom, red), myosin VIIa (Myo, green), and DAPI (blue) shown in **c** and Tom only shown in **c**'.S = approximate position of the striola, TE = transitional epithelium. **e** Utricle from a *GLAST-CreER^{T2}:ROSA26^{tdTomato}* mouse that did not receive tamoxifen, focused on the sensory epithelium. **f**-**g** Confocal slices through the lateral extrastriolar region, with slices through the type II hair cell (HC) nuclear layer **f**), the type I hair cell (HC) nuclear layer (**f**'), the supporting cell (SC) nuclear layer (**f**"), or the stroma (**g**). Scale bar in **a** = 5 µm and applies to **a**,**b**. Scale bar in **c** = 150 µm and applies to **c**-**e**. Scale bar in **f** = 5 µm and applies to **f**-**g**

mount utricles. *Id2*^{CreERT2} is a knock-in allele where *CreER*^{T2} was inserted into the initiation codon of the endogenous *Id2* locus and therefore only one copy of *Id2* is expressed (Rawlins et al. 2009). We observed no difference in the density or distribution of hair cells or supporting cells, or any change in motor behavior, which would indicate a phenotypic abnormality due to *Id2* haploinsufficiency. The expression pattern of *Id2*^{CreERT2}:*ROSA26*^{tdTomato} mice after tamoxifen induction at 6 weeks of age was similar to *GLAST-CreER*^{T2}:*ROSA26*^{tdTomato}, but significantly fewer cells were labeled. In *Id2*^{CreERT2}:*ROSA26*^{tdTomato} mice, tdTomato was expressed in 1172 ± 440 cells per utricle, of which 1159 ± 416 were supporting cells and 13.9 ± 24.0 were type II hair cells (Fig. 6a, a', d–f; Table 1). Approximately 29 % of supporting cells per utricle were tdTomato-positive. We detected no significant difference in the density of tdTomato-labeled cells in central regions compared to peripheral regions (unpaired Student's *t* test, $t(4) = 0.617$, $p = 0.570$). A small number of cells in the stroma (Fig. 6b, g) and the transitional epithelium (Fig. 6a, a') also expressed tdTomato. In controls that were not given tamoxifen, we observed 0.67 ± 0.58 tdTomato-labeled supporting cells and 0.33 ± 0.58 labeled hair cells per utricle (Fig. 6c; Table 1).

Sox9 is a HMG box domain transcription factor that has been immunolocalized to supporting cells in utricles of mature NMRI outbred mice (Loponen et al. 2011). Immunolabeling of utricles from mixed background mice using the same antibody revealed labeling in all supporting cell nuclei, with no significant labeling in hair cells (Fig. 7a–c). *Sox9-CreER*^{T2} is a transgenic line that used a BAC containing the entire mouse *Sox9* sequence with *CreER*^{T2} inserted after the start codon (Kopp et al. 2011). Similar to *GLAST-CreER*^{T2}:*ROSA26*^{tdTomato} and *Id2*^{CreERT2}:*ROSA26*^{tdTomato} mice, the vast majority of tdTomato-positive cells were supporting cells in *Sox9-CreER*^{T2}:*ROSA26*^{tdTomato} mice following tamoxifen injection at 6 weeks of age. In *Sox9-CreER*^{T2}:*ROSA26*^{tdTomato} mice, tdTomato was expressed in 3621 ± 620 cells per utricle, of which 3483 ± 556 were supporting cells and 138.3 ± 64.9 were hair cells, mostly type IIs (Fig. 7d, d', g–g"; Table 1). We estimated that ~87 % of supporting cells per utricle were tdTomato-labeled. Analysis of densities revealed no difference between central versus peripheral regions (unpaired Student's *t* test, $t(6) = 1.485$, $p = 0.188$). A significant number of cells in the transitional epithelium expressed tdTomato (Fig. 7d, d'), but there was no tdTomato expression in stromal

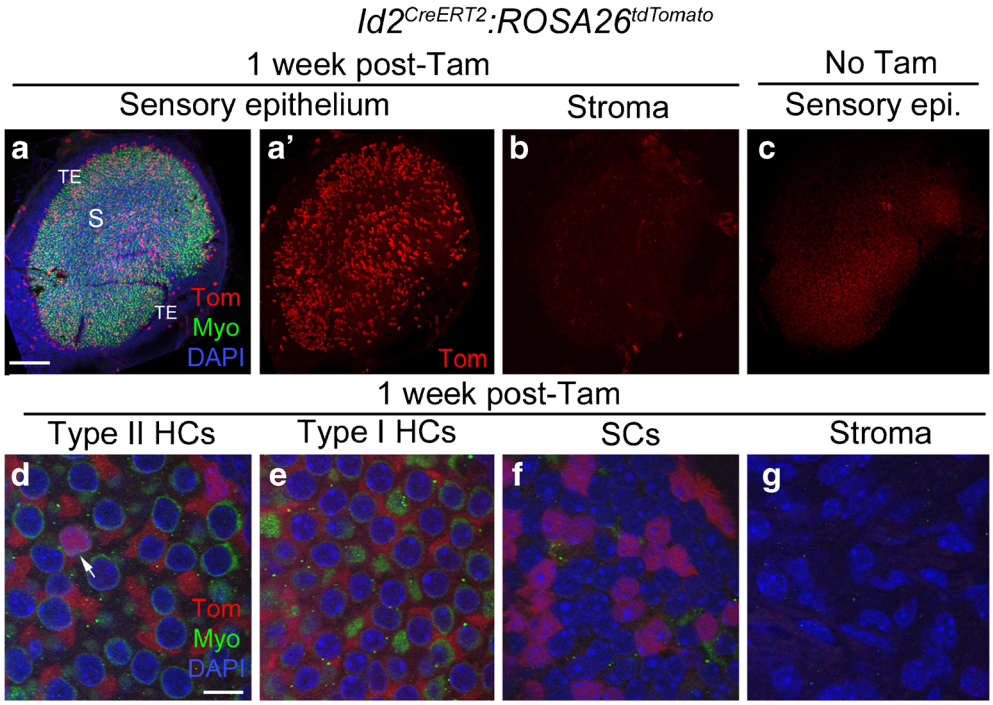

FIG. 6. *Id2*^{CreERT2} mice have inducible Cre activity in supporting cells. **a**-**c** Confocal images of utricles from *Id2*^{CreERT2}:*ROSA26*^{tdTomato} mice injected with tamoxifen, focused on either the macula (**a**,**a**') or stroma (**b**). **a** and **a**' show the same view, with labeling for tdTomato (Tom, red), myosin VIIa (Myo, green), and DAPI (blue) shown in **a** and Tom only shown in **a**'. S = approximate position of the striola, TE = transitional epithelium. **b** shows Tom labeling in the stroma. **c** Utricle from an *Id2*^{CreERT2}:*ROSA26*^{tdTomato} mouse that did not receive tamoxifen, focused on the sensory epithelium. **d**-**g** Confocal slices showing higher magnification views of the lateral extrastriolar region. **d** shows a slice through the type II hair cell (HC) layer, **e** shows a slice through the type I hair cell (HC) layer, **f** shows a slice through the supporting cell (SC) layer, and **g** shows a slice through the stroma. Arrow in **d** indicates a Tomato-positive type II HC. Scale bar in **a** = 150 μm and applies to **a**-**c**. Scale bar in **d** = 5 μm and applies to **d**-**g**

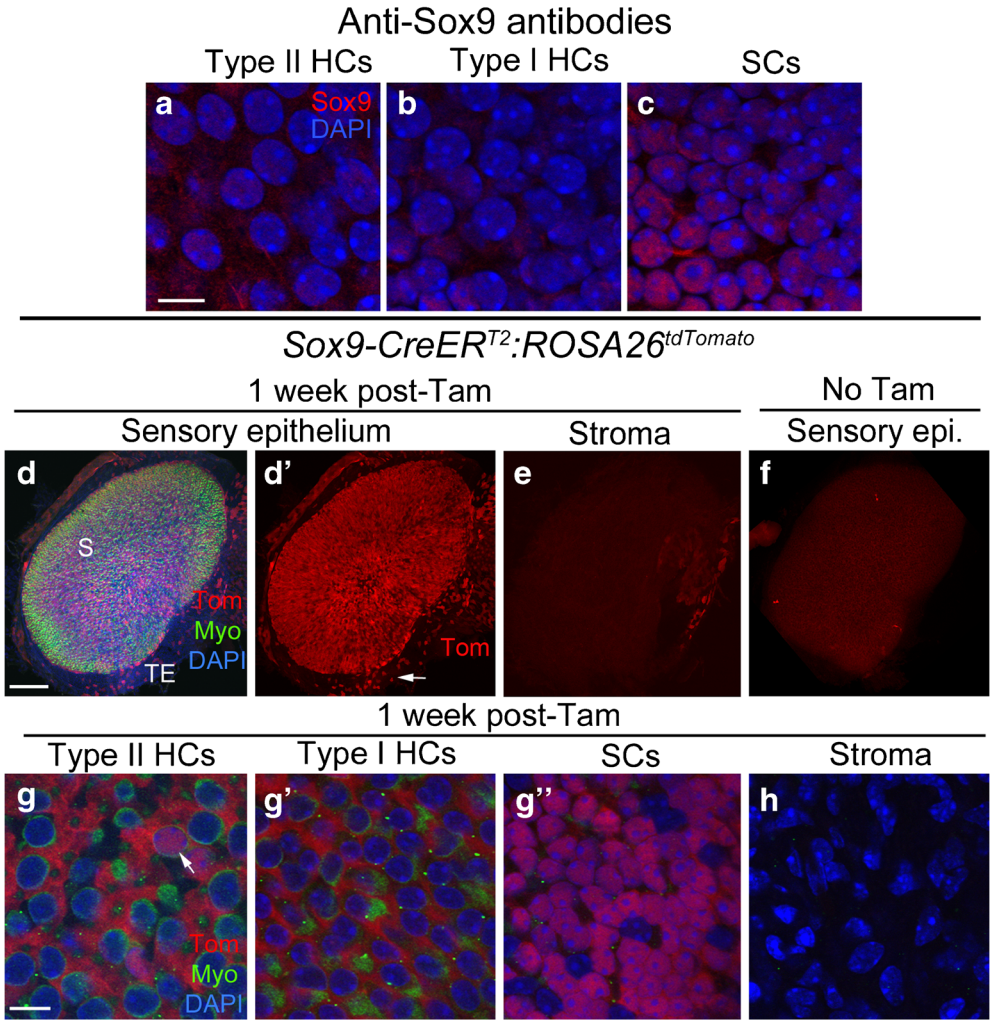

FIG. 7. *Sox9-CreER^{T2}* mice have inducible Cre activity in supporting cells. **a**-**c** Confocal images showing immunolabeling for Sox9 (red) and nuclei (DAPI, blue) in slices focused on the nuclei of type II hair cells (HCs) (**a**), type I hair cells (HCs) (**b**), and supporting cells (SCs) (**c**) of the utricular macula. **d**-**e** Confocal images of the surface of utricle from *Sox9-CreER^{T2}:ROSA26^{tdTomato}* mice injected with tamoxifen, focused on either the sensory epithelium (**d**,**d'**) or stroma (**e**). **d** and **d'** show the same view, with labeling for tdTomato (Tom, red), myosin VIIa (Myo, green), and DAPI (blue) shown in **d** and Tom only shown in **d'**. S = approximate position of the striola, TE = transitional epithelium. Arrow in **d'** points to labeled cells in the TE. **e** shows Tom labeling in the stroma. **f** Utricle from a *Sox9-CreER^{T2}:ROSA26^{tdTomato}* mouse that did not receive tamoxifen, focused on the macula. **g**-**h** Confocal slices through the lateral extrastriolar region, with slices through the type II hair cell (HC) nuclear layer (**g**), the type I hair cell (HC) nuclear layer (**g'**), the supporting cell (SC) nuclear layer (**g''**), or the stroma (**h**). Arrow in **g** indicates a Tomato-positive type II HC. Scale bar in **a** = 5 μm and applies to **a**-**c**. Scale bar in **d** = 150 μm and applies to **d**-**f**. Scale bar in **g** = 5 μm and applies to **g**-**h**

cells (Fig. 7e, h). In controls that were not given tamoxifen, we observed 2.3 ± 0.58 labeled hair cells and 1.3 ± 1.53 labeled supporting cells per utricle (Fig. 7f; Table 1).

Comparisons of CreER Efficiencies in Utricular Hair Cells and Supporting Cells in *GLAST-CreER^{T2}*, *Id2^{CreERT2}*, *Sox9-CreER^{T2}*, *Sox2^{CreERT2}*, and *Otoferlin^{CreERT2}* Mouse Lines

Figure 8 and Table 1 show quantitative data for tdTomato-positive hair cells and supporting cells in the five best-performing CreER lines we examined, which we define as having the highest cell specificity, the fewest non-macular cells with Cre activity, and the smallest level of Cre leakiness. Of the two lines with substantial Cre activity in hair cells (*Sox2^{CreERT2}* and *Otoferlin^{CreERT2}*), *Sox2^{CreERT2}* had the highest efficiency (Fig. 8a), but it also labeled nearly 100 % of supporting cells (Fig. 8b) and had some Cre leakiness (Fig. 1f; Table 1). By contrast, *Otoferlin^{CreERT2}* had no Cre activity in supporting cells (Fig. 8b) and no Cre leakiness (Fig. 4j; Table 1).

All five lines, with the exception of *Otoferlin^{CreERT2}*, showed Cre activity in supporting cells. Cre activity in *GLAST-CreER^{T2}*, *Id2^{CreERT2}*, and *Sox9-CreER^{T2}* was restricted largely to supporting cells (Fig. 8b), with very few Cre-expressing hair cells (Fig. 8a), while in *Sox2^{CreERT2}* mice, ~ 32 % of hair cells (mostly type IIs) and ~ 99 % of

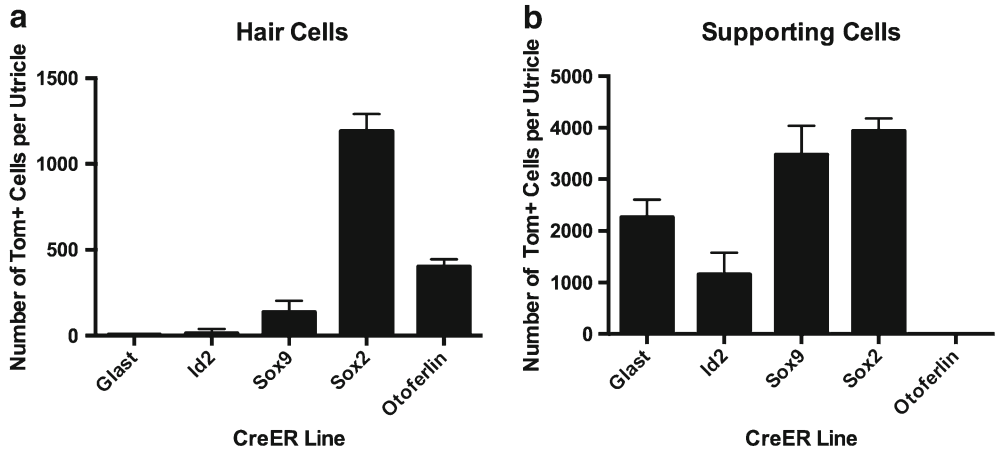

FIG. 8. Graphs comparing Cre efficiencies in hair cells and supporting cells for several lines. Each column represents average number of tdTomato-labeled cells per utricle (+ 1 standard deviation) for hair cells (**a**) and supporting cells (**b**) from each CreER line. TdTomato-positive cells were quantified from two central regions and two peripheral regions, which together comprised 24 % of the total macula's area. Conversion to number of labeled cells per utricle is described in the "Materials and Methods" section

supporting cells had Cre activity (Fig. 8). Furthermore, *GLAST-CreER*^{T2}, *Id2*^{CreERT2}, and *Sox2*^{CreERT2} had some Cre-expressing cells in the stroma (Figs. 1e, h; 5d, g; 6b, g); while *Sox9-CreER*^{T2} had few, if any Cre-positive cells in the stroma (Fig. 7e, h).

CreER efficiencies may vary according to the gender of the parent from which the *CreER* allele was inherited (Lomeli et al. 2000; Hayashi et al. 2003; Cochrane et al. 2007; Gallardo et al. 2007; Heffner et al. 2012). We tracked parental inheritance for all mice upon which we performed quantitative data analysis (Table 2). However, we could not effectively test correlations between inheritance and CreER activity in any of the lines, with or without tamoxifen treatment, because animal numbers were too small. Similarly, although we tracked the gender of each mouse, we could not assess effects of gender upon Cre efficiency, given the small animal numbers.

CreER Lines with Sparse Expression Patterns in the Vestibular Sensory Epithelium

Prospero Homeobox 1 (Prox1) is a homeodomain transcription factor that plays a role in the development of many organs and in tumorigenesis (reviewed in Elsir et al. 2012). Prox1 protein is detected in utricular supporting cells at embryonic but not postnatal ages (Bermingham-McDonogh et al. 2006; Kirjavainen et al. 2008). *Prox1*^{CreERT2} is a knock-in allele where *CreER*^{T2} was inserted after an internal ribosome entry site (IRES) in intron 2 of endogenous *Prox1* (Srinivasan et al. 2007). Therefore, both copies of *Prox1* should be expressed; however, the level of *Prox1* expression may be reduced. We observed no difference in the density or distribution of hair cells or supporting cells, or any change in motor behavior, which would indicate a phenotypic abnormality due to *Prox1* haploinsufficiency. After tamoxifen induction at 6 weeks of age in *Prox1*^{CreERT2}:*ROSA26*^{tdTomato} mice, we observed 1 ± 1 tdTomato-positive hair cell (Fig. 9a). However, we did a quick analysis of the lateral ampulla and found that several tdTomato-positive hair cells and supporting cells were detected in the crista (Fig. 9b). These data were not quantified. In controls that were not given tamoxifen, no tdTomato-positive cells were observed (data not shown).

Parvalbumin is a calcium-binding protein that has been localized by immunostaining to centrally located type I hair cells of the vestibular organs of neonatal rodents, as well as in vestibular ganglion neurons and neurites in developing and mature rodents (Demêmes et al. 1993; Raymond et al. 1993; Pujol et al. 2014). In adult mouse utricles from a mixed background strain, antibodies to parvalbumin labeled afferent fibers and endings, as well as some hair cell nuclei (Fig. 9c–e'; Bucks et al. 2017). *Parvalbumin*^{CreERT2} is a knock-in line where *CreER*^{T2} was inserted into the endogenous *Parvalbumin* locus and therefore only one copy of *Parvalbumin* is expressed (Taniguchi et al. 2011). We observed no difference in the density or distribution of hair cells or supporting cells, or any change in motor behavior, which would indicate a phenotypic abnormality due to *Parvalbumin* haploinsufficiency. After tamoxifen induction at 6 weeks of age in *Parvalbumin*^{CreERT2}:*ROSA26*^{tdTomato} mice, we observed tdTomato expression in 2 to 19 afferent nerve calyces that surrounded type I hair cells in the striola, including some likely multi-calyx afferents (Fig. 9f), and their associated nerve fibers. No tdTomato-positive hair cells or supporting cells were observed and there were a small number of tdTomato-positive cells in the stroma (Fig. 9g). In controls that were not given tamoxifen, no tdTomato-positive cells were observed (data not shown).

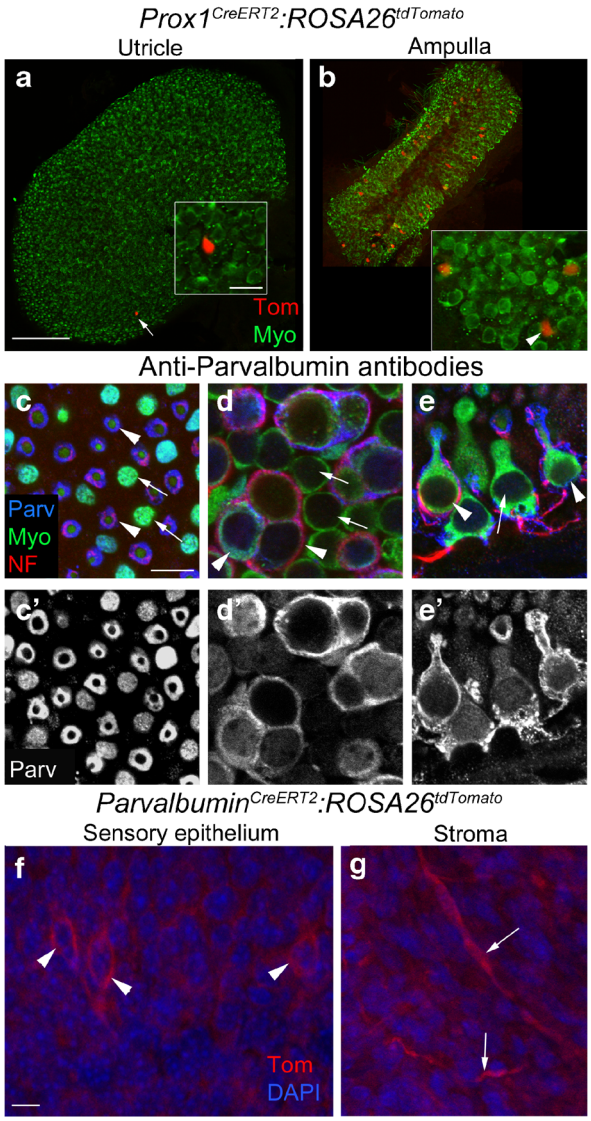

FIG. 9. Cre activity is minimal in the utricular macula in *Prox1*^{CreERT2} and *Parvalbumin*^{CreERT2} lines. **a**,**b** Confocal images of the utricle (**a**) and the horizontal ampullae (**b**) from *Prox1*^{CreERT2}*:ROSA26*^{tdTomato} mice injected with tamoxifen and labeled with tdTomato (Tom, red) and antibodies to myosin VIIA (Myo, green). Arrow in **a** points to one Tom-labeled cell. Inset shows the same cell, which is located in the type II hair cell layer and is Myo-positive, although the strong Tom labeling obscures the Myo labeling. Inset in **b** shows an area from the ampulla at higher magnification, with three labeled cells (2 hair cells and 1 supporting cell—arrowhead). **c**-**e**' Confocal slices showing immunolabeling for parvalbumin (Parv, blue), myosin VIIa (Myo, green), and 200 kD neurofilament (NF, red), focused on the tops of the hair cells (**c**), type I and II hair cell nuclei in the striola (**d**), and the edge of the lateral extrastriola, which affords side views of type I and II hair cells (**e**). Arrowheads in **c**-**e** point to type I hair cells, while arrows point to type II hair cells. **c**'-**e**' show parvalbumin (Parv, white) staining alone. **f**,**g** Confocal slices from utricles of *Parvalbumin*^{CreERT2}*:ROSA26*^{tdTomato} mice injected with tamoxifen, showing tdTomato (Tom, red) labeling in calyces (arrowheads) in the sensory epithelium (**f**) and nerve fibers (arrows) in the stroma (**g**). Scale bar in **a** = 150 μm and applies to **a**,**b**. Scale bar in **a** inset = 10 μm and applies to insets in **a**,**b**. Scale bar in **c**= 5 μm and applies to **c**-**e**'. Scale bar in **f** = 5 μm and applies to **f**,**g**

CreER Lines with No Expression in the Vestibular Sensory Epithelium

Fibroblast growth factor receptor 3 (Fgfr3) is one of five receptors in the FGF family, which plays a key role in morphogenesis and sensorineural differentiation in the developing ear (reviewed in Fritzsch et al. 2006). *Fgfr3* is expressed in supporting cells in the mouse cochlea (e.g., Hayashi et al. 2007). Its expression pattern has not been described for the mammalian vestibular system. However, in the chicken utricle, *Fgfr3* was detected by RNA-Seq (Ku et al. 2014), but not by in situ hybridization (Bermingham-McDonogh et al. 2001). *Fgfr3i-CreER*^{T2} is a transgenic line where an improved version of *CreER* (*iCreER*) was inserted into the first exon of *Fgfr3* in a phage artificial chromosome (PAC) (Rivers et al. 2008; Young et al. 2010). After tamoxifen induction at 6 weeks of age in *Fgfr3-iCreER*^{T2}*:ROSA26*^{tdTomato} mice, no tdTomato-positive cells were observed in the sensory epithelium of the utricle, but numerous star-shaped tdTomato-positive cells were observed in the stroma (data not shown). Considering the possibility that the labeled cells could be macrophages, we labeled utricles with an antibody to ionized calcium-binding adaptor molecule 1 (Iba1), a macrophage marker, but there was no co-labeling with tdTomato. Thus, the identity of these cells is not currently known.

Glial fibrillary acidic protein (GFAP) is an intermediate filament protein that is expressed in astrocytes (reviewed in Pekny and Pekna 2004). It is also expressed in utricular supporting cells in the peripheral, extrastriolar region at postnatal ages (Rio et al. 2002). We investigated the expression pattern of two transgenic CreER lines that use the human *GFAP* promoter to drive CreER expression but have different insertion sites in the genome (*GFAP-A-CreER*™ and *GFAP-B-CreER*™) (Chow et al. 2008). There were no tdTomato-positive cells observed in the utricle of either *GFAP-A-CreER*™*:ROSA26*^{tdTomato} mice or *GFAP-B-CreER*™*:ROSA26*^{tdTomato} mice after tamoxifen induction at 6 weeks of age (data not shown).

DISCUSSION

CreER/loxP is a powerful genetic system that allows dissection of gene functions that may differ across development and in maturity. Deletion or overexpression of specific genes with CreER/loxP occurs in a cell type-specific and time-controlled manner, which allows for further understanding of tissue development and gene function. Fate-mapping of different cells types can also be achieved using CreER/loxP and is important for understanding development, repair, and regeneration processes. The present study char-

TABLE 2

Raw data, gender, and CreER inheritance for each animal in which quantitative analysis was performed

CreER Line[b]	*No Tam*					*Tam at 6 weeks*				
	Sample no.	*Gender*	*Labeled SCs*[a]	*Labeled HCs*[a]	*CreER inheritance*	*Sample no.*	*Gender*	*Labeled SCs*	*Labeled HCs*	*CreER inheritance*
GLAST-CreER^{T2}	3324	F	0	11	Maternal	3480	M	1635	25	Paternal
	3325	F	0	0	Maternal	3483	F	3399	0	Paternal
	3327	F	22	0	Maternal	3305	F	3910	0	Paternal
	3943	M	3	0	Maternal	3942	F	404	8	Maternal
						3945	F	2001	0	Maternal
Id2^{CreERT2}	4345	M	0	1	Paternal	3860	M	1614	0	Maternal
	4347	M	1	0	Paternal	3861	M	799	0	Maternal
	4348	F	1	0	Paternal	3895	M	1065	42	Maternal
Sox9-CreER^{T2}	3435	M	0	3	Maternal	3434	M	3790	220	Maternal
	3441	M	3	2	Maternal	3283	F	3960	158	Maternal
	4113	M	1	2	Maternal	4109	F	2704	100	Maternal
						4112	F	3478	75	Maternal
Sox2^{CreERT2}	4680	F	68	16	Paternal	5438	M	3698	1140	Paternal
	4684	M	120	6	Paternal	5436	F	4172	1131	Paternal
	5439	F	67	5	Paternal	5434	M	3964	1306	Paternal
	5440	F	40	5	Paternal					
	5442	F	16	0	Paternal					
Otoferlin^{CreERT2}	B46	F	0	0	Maternal	B38	M	0	416	Maternal
	B315	F	0	0	Maternal	B346	F	0	436	Maternal
	B516	M	0	0	Maternal	B273	M	0	358	Maternal
	B528	F	0	0	Maternal					Maternal

[a]Estimated total number of labeled cells per utricle

[b]Sox2, Otoferlin, and Id2 CreER lines are knock-ins and designated by the following nomenclature: XXX^{CreERT2}. GLAST and Sox9 CreER lines are transgenics and designated by the following nomenclature: XXX-CreER^{T2}

acterized the Cre expression pattern of 11 CreER alleles in the utricle of adult mice. We found that five lines—*GLAST-CreER^{T2}*, *Id2^{CreERT2}*, *Otoferlin^{CreERT2}*, *Sox2^{CreERT2}*, and *Sox9-CreER^{T2}*—had cell-selective, inducible Cre activity with little leakiness, comprising effective new tools for researchers who wish to employ conditional and inducible genetic manipulation to study the vestibular periphery in adult mammals. We also report that the other 6 CreER lines we studied have little to no CreER activity in the adult utricle or, in the case of *Calbindin2^{CreERT2}* mice, are not inducible in adult mice under the conditions used in the study.

Supporting cells in the adult utricle were targeted by *GLAST-CreER^{T2}*, *Id2^{CreERT2}*, *Sox2^{CreERT2}*, and *Sox9-CreER^{T2}*. However, each CreER line varied in the amount of supporting cells with CreER activity. Specifically, we estimate that *Sox2^{CreERT2}*, *Sox9-CreER^{T2}*, *GLAST-CreER^{T2}*, and *Id2^{CreERT2}* had inducible CreER activity in ~99, ~87, ~57, and ~29 % of supporting cells, respectively. This variability across lines may reflect slightly different expression patterns of these 4 genes and could suggest that not all supporting cells in the utricle are the same. This is most plausible with the two knock-in lines—*Id2^{CreERT2}* and *Sox2^{CreERT2}*—since Cre expression is controlled by endogenous regulatory elements in these lines. However, it is also possible that the tdTomato labeling pattern is not an accurate reflection of native gene expression in *Sox9-CreER^{T2}* and *GLAST-CreER^{T2}* lines, which are transgenics where the promoter used to drive CreER may not contain all regulatory elements used by the endogenous genes. Also, positional effects may have occurred where the transgene insertion site within the genome is near repressor or enhancer elements that affect CreER expression (Jaenisch et al. 1981; Wilson et al. 1990). For *GLAST-CreER^{T2}*, *Sox2^{CreERT2}*, and *Sox9-CreER^{T2}* lines, tdTomato expression was generally consistent with antibody labeling, which supports the notion that Cre expression reflects native gene expression. However, we were unable to make this assessment with *Id2^{CreERT2}*, since we were not able to find antibodies for immunolabeling of Id2.

Very little is known about vestibular supporting cell properties and whether there are subpopulations of supporting cells with discrete functional, molecular, or physiological properties. Indeed, supporting cells across the adult utricle share many common features, including cytoplasmic ultrastructure, nuclear shape and position, chromatin and nucleolar properties. A few markers show non-uniform distribution across the utricle. For instance, a *Hes5* reporter mouse showed higher levels of reporter expression in supporting cells located in the central region of the utricle in maturity (Hartman et al. 2009). It is interesting that hair cells are regenerated mainly in the lateral extrastriolar region in adult mouse utricles after near-complete hair cell destruction (Golub et al.

2012), which suggests that supporting cells in different regions may have variable capacity to serve as hair cell progenitors. Unique regional capacities for regeneration have been reported among supporting cells in other hair cell epithelia, including lateral line neuromasts (Romero-Carvajal et al. 2015) and avian auditory organs (Cafaro et al. 2007).

In mice that received tamoxifen, we detected small numbers of tdTomato-labeled hair cells in *Sox9-CreER*^{T2}, *GLAST-CreER*^{T2}, and *Id2*^{CreERT2} lines. Numbers were greater than those seen in no-tamoxifen controls. These labeled hair cells may reflect supporting cell conversion into hair cells, which occurs normally in adult mouse utricles (Bucks et al. 2017). Because there is some Cre leakiness in each line, in both hair cells and supporting cells, it is possible that labeled hair cells were derived from tdTomato-positive supporting cells over long periods of time, rather than simply during the week after tamoxifen injection. It is also possible that some supporting cells with induced Cre converted into hair cells in the 7-day survival time post-tamoxifen. Finally, small number of hair cells may actually express each gene.

Our observation that only ~11 % of hair cells expressed tdTomato after tamoxifen injection in *Otoferlin*^{CreERT2} mice was surprising. Otoferlin regulates fusion of synaptic vesicles at the ribbon synapse (Moser and Starr 2016). In rodents, mature utricular hair cells—type I and II—have ribbon synapses (e.g., Rüsch and Eatock 1996; Nouvian et al. 2006; Pujol et al. 2014), and prior studies showed that otoferlin is immunolabeled in both hair cell types (Schug et al. 2006; Dulon et al. 2009). Since *Otoferlin*^{CreERT2} is a knock-in allele, we expected many more hair cells to be tdTomato-positive. We also detected otoferlin by immunostaining in hair cells throughout the utricle, but not all hair cells were labeled strongly and some seemed to lack otoferlin labeling. It is possible that the otoferlin protein has a slow turnover rate and the activity of the *Otoferlin* promoter may be low. Since we only injected tamoxifen twice (on two consecutive days) and the half-life of tamoxifen is ~16 h (Robinson et al. 1991), only cells with active O*toferlin* promoters at the time of injection would show CreER activity. It is also possible that some vestibular hair cells use an alternative protein to otoferlin to mediate vesicle fusion. However, this is unlikely, because studies have shown that vesicle exocytosis in hair cells in *Otoferlin*$^{-/-}$ mice have slower kinetics, reduced calcium sensitivity, and nonlinear calcium dependence (Dulon et al. 2009), suggesting otoferlin function is critical in many vestibular hair cells. Similar explanations could be made for the small number of Cre-positive neurons in *Parvalbumin*^{CreERT2} mice, in which we expected to see many afferent neurons labeled, based on immunolabeling here and in prior papers (Demêmes et al. 1993; Pujol et al. 2014; Raymond et al. 1993).

The absence of CreER activity prior to tamoxifen induction is crucial for conditional gene studies because the effects of leakiness can accumulate over time and complicate the interpretation of results. Therefore, our characterization studies also included samples that expressed each CreER allele and the *ROSA26*^{tdTomato} reporter line but did not receive tamoxifen to assess the level of leakiness. While the ligand-binding region of ER fused to Cre has been mutated to decrease its affinity for endogenous estrogen, it still retains a low affinity for estrogen which may cause leakage in some cells. CreER™ contains a single point mutation (G525R), while CreER^{T2} has three point mutations (G400V/M543A/L544A) in this region (Danielian et al. 1998; Indra et al. 1999). Therefore, one might predict that CreER™ alleles are more likely to be leaky. However, our results suggest that leakiness varies among individual CreER lines, because of the nine CreER^{T2} alleles we investigated, there were three different results. No Cre leakiness was observed in *Fgfr3-iCreER*^{T2}, *Otoferlin*^{CreERT2}, *Parvalbumin*^{CreERT2}, and *Prox1*^{CreERT2} mice; a small degree of leakiness was seen in *GLAST-CreER*^{T2}, *Id2*^{CreERT2}, *Sox2*^{CreERT2}, and *Sox9-CreER*^{T2} lines; and the same pattern of tdTomato expression with or without tamoxifen was observed in *Calbindin*^{CreERT2}. These differences may have been caused by the expression level of CreER. Previous studies showed that CreER lines with higher levels of promotor activity and thus high levels of CreER protein are more likely to be leaky (Buelow and Scharenberg 2008). Others have reported evidence of proteolysis, where ER is cleaved from Cre in some cells, as the source of leakiness (Nakamura et al. 2006). There are also reports of differential CreER leakiness based on parental inheritance where Cre recombination occurs spontaneously in the germline of one parent. Several studies have shown this to occur more frequently when the female parent transmits Cre to the offspring (Lomeli et al. 2000; Hayashi et al. 2003; Cochrane et al. 2007; Gallardo et al. 2007; Heffner et al. 2012). However we were not able to assess this in our study due to small sample size (Table 2). Regardless of the cause, our results suggest that *Calbindin*^{CreERT2} is not inducible in the adult utricle and additional controls not injected with tamoxifen are needed for experiments using *GLAST-CreER*^{T2}, *Id2*^{CreERT2}, *Sox2*^{CreERT2}, and *Sox9-CreER*^{T2} lines due to a low level of leakiness.

Four of the CreER lines we investigated—*Id2*^{CreERT2}, *Otoferlin*^{CreERT2}, *Prox1*^{CreERT2}, and *Sox2*^{CreERT2}—are knock-in alleles, where CreER replaced the coding region of the endogenous gene. Since we used heterozygotes for this study, only one copy of the endogenous gene is present and if these lines were bred as homozygotes, they would in essence be a germline knockout. While we did

not detect any obvious differences in utricle histology or motor behavior from these CreER lines, we did not test vestibular function and others have reported haploinsufficient phenotypes in some knock-in Cre lines (Siegenthaler et al. 2008; Atkinson et al. 2018). Therefore, if any of these lines are used to delete or overexpress a gene, Cre-positive controls (either without tamoxifen injection or without the floxed allele) would be important to make proper conclusions of the data.

In summary, our study has identified five CreER lines with cell type-specific and inducible expression of CreER in the adult utricle in supporting cells and/or hair cells. These lines constitute new genetic tools for investigators to manipulate gene expression or to fate-map cells in the vestibular periphery of adult mice.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest Brandon C. Cox is a consultant with Turner Scientific, LLC. No other authors have any professional or financial affiliations that may be perceived as a conflict of interest.

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