

Reviewed Preprint

v1 • April 2, 2026

Not revised

Reviewed Preprint

v2 • January 1, 1970

Revised by authors

✉ For correspondence:

js2ee@virginia.edu

Competing interests:

No competing interests declared

Funding:

See [page 20](#)

Reviewing editor:

Doris K Wu,
National Institutes of Health, United States

© 2026, De Hoyos et al. This article is distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use and redistribution provided that the original author and source are credited.

ATP8B1–TMEM30B Flippase Activity Maintains Stereocilia Lipid Asymmetry Required for Hearing

Henry N De Hoyos^{1,2,6}, Sihan Li¹, Jun-Sub Im¹, Alyssa Luz-Ricca¹, Takesha Foster¹, Betsy Szeto⁵, Rachel Jonas⁵, Emma Kim¹, Nikhil Amin¹, Jung-Bum Shin^{1,2,3,4,5} ✉

¹Departments of Neuroscience, School of Medicine, University of Virginia, Charlottesville, VA 22903, United States •

²Biochemistry and Molecular Genetics, School of Medicine, University of Virginia, Charlottesville, VA 22903, United States

• ³Cell Biology, School of Medicine, University of Virginia, Charlottesville, VA 22903, United States • ⁴Membrane and Cell Physiology, School of Medicine, University of Virginia, Charlottesville, VA 22903, United States • ⁵Otolaryngology-Head and Neck Surgery, School of Medicine, University of Virginia, Charlottesville, VA 22903, United States • ⁶Medical Scientist Training Program, School of Medicine, University of Virginia, Charlottesville, VA 22903, United States

eLife Assessment

Mechanical transduction channels of sensory hair cells possess lipid scramblase activity. Membrane lipid disruption resulting from mechanical transduction is thought to be restored by flippase activities. This **fundamental** study provides **compelling** evidence that ATP8B1, a P4-ATP flippase and its subunit TMEM30B, are key in mediating this restorative function in outer hair cells of the mammalian cochlea.

<https://doi.org/10.7554/eLife.110466.2.sa3>

Abstract

Sensory hair cells convert sound-induced vibrations into electrical signals through a process called mechano-electrical transduction (MET). While the protein components of the MET complex are well studied, increasing evidence indicates that MET channel properties are significantly modulated by the surrounding lipid bilayer. The asymmetric distribution of membrane lipids between the inner and outer membrane leaflets is well established to shape membrane mechanics. The recent discovery that the core MET components TMC1 and TMC2 also act as lipid scramblases suggests a direct role for membrane lipid asymmetry in the dynamic shaping of auditory transduction. Because scramblase activity of TMC1/2 disrupts lipid asymmetry, we hypothesized that an opposing flippase may be required to restore and maintain lipid asymmetry. Here, we identify the P4-ATPase ATP8B1 and its chaperone TMEM30B as selectively expressed in outer hair cells (OHCs), enriched in stereocilia, and upregulated following the onset of MET and hearing. Loss of either protein results in elevated auditory brainstem response (ABR) thresholds, phosphatidylserine (PS) externalization, and rapid hair-cell degeneration, demonstrating that lipid homeostasis is crucial for OHC survival. Together, these findings establish ATP8B1 and TMEM30B as key regulators of membrane lipid asymmetry in sensory hair cells and establish TMEM30B as a novel deafness gene.

Introduction

Hearing depends on the ability of mechanosensory hair cells to convert sound-evoked mechanical forces into electrical signals (Hudspeth 1989 [↗](#); Gillespie and Müller 2009 [↗](#); Fettiplace and Kim 2014 [↗](#)). Sound vibrations transmitted through the middle ear displace the basilar membrane and deflect the stereocilia bundles that crown auditory hair cells. This deflection modulates tension on tip links which are filamentous connectors that couple adjacent stereocilia and directly gates

mechanotransduction (MET) channels. The architecture of the hair bundle, including the graded heights of stereocilia rows and the polarity of the MET machinery, is essential for tuning the sensitivity and dynamic range of auditory signaling. Many of the protein components of the MET complex have now been identified. At the core of this machinery is a force-transmission complex that couples the extracellular tip link to the MET channel complex. Tip links are formed by cadherin 23 and protocadherin 15 (Pickles et al. 1984 [↗](#); Assad et al. 1991 [↗](#); Siemens et al. 2004 [↗](#); Kazmierczak et al. 2007 [↗](#); Ahmed et al. 2006 [↗](#)), which relay mechanical force to the MET channel through a network of associated proteins, including LHFPL5 (Xiong et al. 2012 [↗](#)), CIB2 (Riazuddin et al. 2012 [↗](#); Giese et al. 2017 [↗](#)), and TMIE (Zhao et al. 2014 [↗](#)). TMC1 and its paralog TMC2 are proposed to constitute the ion-conducting components of the MET channel, with TMC1 serving as the dominant pore-forming subunit in mature mammalian hair cells (Kawashima et al. 2011 [↗](#); Pan et al. 2013 [↗](#), 2018 [↗](#); Assad et al. 1991 [↗](#); Pickles et al. 1984 [↗](#)). Intriguingly, TMC1 and TMC2 share structural similarity with TMEM16 family lipid scramblases, of which TMEM16A and B function as ion channels (Le and Yang 2021 [↗](#)). More broadly, TMEM16, TMC, and TMEM63/OSCA are members of a structurally related superfamily of membrane proteins that includes ion channels and lipid scramblases (Medrano-Soto et al. 2018 [↗](#)). Consistent with this relationship, TMC1 and TMC2 were shown to possess lipid-scrambling activity in addition to their proposed ion-conducting function (Ballesteros et al. 2018 [↗](#); Ballesteros and Swartz 2022 [↗](#); Clark et al. 2024 [↗](#); Peineau et al. 2025 [↗](#); George and Ricci 2025 [↗](#)). Cryo-EM structures of *C. elegans* homologues of TMC1 reveal that TMC forms a dimer associated with TMIE and calcium-binding accessory proteins and engages extensively with the surrounding lipid bilayer (Jeong et al. 2022 [↗](#); Clark et al. 2024 [↗](#)). These structures resolve conserved lipid-like densities at defined positions around the complex, including a lipid-filled cavity at the TMIE-TMC interface and sterol-like densities near the dimer interface (Jeong et al. 2022 [↗](#); Clark et al. 2024 [↗](#)). Such lipid-mediated interactions appear to stabilize subunit assembly and may influence conformational changes during channel gating. Together, these findings suggest that the membrane is an active component of the MET complex, with lipid-protein interactions poised to shape MET properties including sensitivity, resting open probability and adaptation.

These observations further imply that MET function may require a specialized lipid environment within stereocilia, whose composition and organization are distinct from the surrounding apical membrane. However, the molecular mechanisms, including the lipid-modifying enzymes, transport pathways, and regulatory factors, that establish, maintain, and dynamically regulate this unique lipid milieu are unknown.

Lipids are not uniformly distributed across the plasma membrane (Bretscher 1972 [↗](#)). Instead, eukaryotic membranes maintain a highly asymmetric distribution of phospholipids between their inner and outer leaflets (Cooper 2000 [↗](#); Lorent et al. 2020 [↗](#)). Phosphatidylserine (PS) and phosphatidylethanolamine (PE) are normally enriched on the cytoplasmic leaflet, whereas phosphatidylcholine and sphingomyelin predominate externally (Cooper 2000 [↗](#); Lorent et al. 2020 [↗](#)). This asymmetry is actively maintained by ATP-dependent flippases and floppases and disrupted by scramblases (Nagata et al. 2020 [↗](#)).

Possibly best studied is the asymmetric distribution of PS. Loss of PS asymmetry is widely recognized as a potent cellular signal, as PS is normally confined to the cytoplasmic leaflet by ATP-dependent flippases (Nagata et al. 2020 [↗](#)). Its externalization is best known as a hallmark of apoptosis, where it functions as an “eat-me” signal that promotes rapid clearance of dying cells (Nagata et al. 2010 [↗](#); Suzuki et al. 2010 [↗](#)). However, PS exposure is not exclusively associated with cell death. In immune cells and platelets, transient PS externalization accompanies physiological activation states and serves signaling or catalytic roles, illustrating that regulated loss of asymmetry can be functionally repurposed (Shin and Takatsu 2020 [↗](#)). PS exposure has also been implicated in cell-cell fusion events, where localized scrambling appears to facilitate membrane remodeling and lower energetic barriers to cell-cell fusion (Whitlock and Chernomordik 2021 [↗](#)).

Beyond signaling, lipid asymmetry has significant biophysical consequences for the membrane. Asymmetric enrichment of charged, unsaturated lipids such as PS and PE in the inner leaflet, opposed by phosphatidylcholine, sphingomyelin- and cholesterol-rich outer leaflets, creates differences in lipid packing, order, and viscosity across the bilayer (Reddy et al. 2012 [↗](#); Hossein and Deserno 2020 [↗](#); Doktorova et al. 2020 [↗](#); Kakuda et al. 2022 [↗](#); X. Li et al. 2024 [↗](#); Wang et al. 2025 [↗](#)). This asymmetry strongly influences membrane tension, curvature stress, and bending rigidity, parameters that govern how membranes deform and how embedded proteins respond to force (Lorent et al. 2020 [↗](#); Hossein and Deserno 2020 [↗](#); Wang et al. 2025 [↗](#)).

In this context, the scramblase activity associated with TMC proteins raises an important question: does TMC-mediated lipid scrambling represent an unintended byproduct of MET, or does it serve a specific functional role? Initial models proposed that scrambling might facilitate adaptive membrane remodeling or ectosome shedding (Ballesteros and Swartz 2022 [↗](#)). Given the exquisite sensitivity of MET channel function to the surrounding lipid environment, it is also hypothesized that regulated scrambling directly modulates MET gating by altering membrane mechanical properties (George and Ricci 2025 [↗](#)). Regardless of its precise physiological benefit, sustained lipid scrambling would be incompatible with cellular homeostasis, as aberrant PS exposure and loss of membrane asymmetry are broadly harmful for the cell (Ballesteros and Swartz 2022 [↗](#); Beurg et al. 2025 [↗](#)). Thus, mechanisms that actively restore lipid asymmetry are likely essential in hair cells.

P4-ATPases, the canonical phospholipid flippases, are prime candidates for maintaining this asymmetry (Segawa et al. 2016 [↗](#)). These enzymes selectively transport PS and other phospholipids from the outer to the inner leaflet and require CDC50 family accessory subunits (also known as TMEM30A, TMEM30B, and TMEM30C) for proper folding, trafficking, and activity (Lenoir et al. 2009 [↗](#); Bryde et al. 2010 [↗](#); van der Velden et al. 2010 [↗](#); Segawa et al. 2018 [↗](#)) (Fig. 1A [↗](#)). While TMEM30A is ubiquitously expressed and supports multiple P4-ATPases, TMEM30B exhibits more restricted tissue distribution and remains poorly characterized (Segawa et al. 2018 [↗](#); T. Li et al. 2021 [↗](#); Xing et al. 2023 [↗](#)). TMEM30C is known to only be expressed in mammalian testis (Osada et al. 2007 [↗](#)). Transcriptomic analyses indicate that TMEM30B is expressed in the inner ear (Liu et al. 2018 [↗](#); Scheffer et al. 2015 [↗](#); Kolla et al. 2020 [↗](#); Orvis et al. 2021 [↗](#)), and a recent hearing loss screen in a cohort of 3006 mouse knockout (KO) strains reported that *Tmem30b* KO mice develop hearing loss (Bowl et al. 2017 [↗](#)). However, the cellular and molecular basis of the mechanism underlying the hearing loss, and the developmental regulation and subcellular localization of TMEM30B in hair cells remained unknown.

Here, we investigate the roles of the flippase ATP8B1 and its accessory subunit TMEM30B in auditory hair cells. We show that both proteins are highly enriched in stereocilia and the apical membrane. Using genetic KO mouse models, we demonstrate that loss of ATP8B1 leads to loss of lipid membrane asymmetry, early-onset hair cell pathology and rapid hearing decline. *Tmem30b* KO mice phenocopy these defects. Together, our findings identify ATP8B1 and TMEM30B as essential regulators of membrane lipid asymmetry in OHCs and reveal a previously underappreciated role for lipid flippase pathways in hair cell function and survival.

Results

ATP8B1 is specifically targeted to outer hair cell stereocilia

Of the 14 P-type ATPase flippases conserved between mice and humans, nine localize to the plasma membrane (Shin and Takatsu 2025 [↗](#)), and transcriptomic analyses identify four as being expressed in hair cells (Mark et al. 2013 [↗](#); Liu et al. 2018 [↗](#); Kolla et al. 2020 [↗](#)). Recent work has implicated several phospholipid flippases, including ATP8B1, ATP8A2, and ATP11A in auditory dysfunction (Coleman et al. 2014 [↗](#); Pater et al. 2022 [↗](#); Stapelbroek et al. 2009 [↗](#)). Because available antibodies lacked sufficient specificity, we generated HA knock-in (KI) mouse lines for ATP8A1, ATP8A2, ATP8B1 and ATP11A (Fig. 1B-C [↗](#)). Importantly, ATP8B1-HA mice exhibited normal hearing thresholds compared to C57BL/6J controls at P30, indicating that insertion of the HA tag did not impair auditory function (Sup. Fig. 1 [↗](#)). Together, these data support the use of the

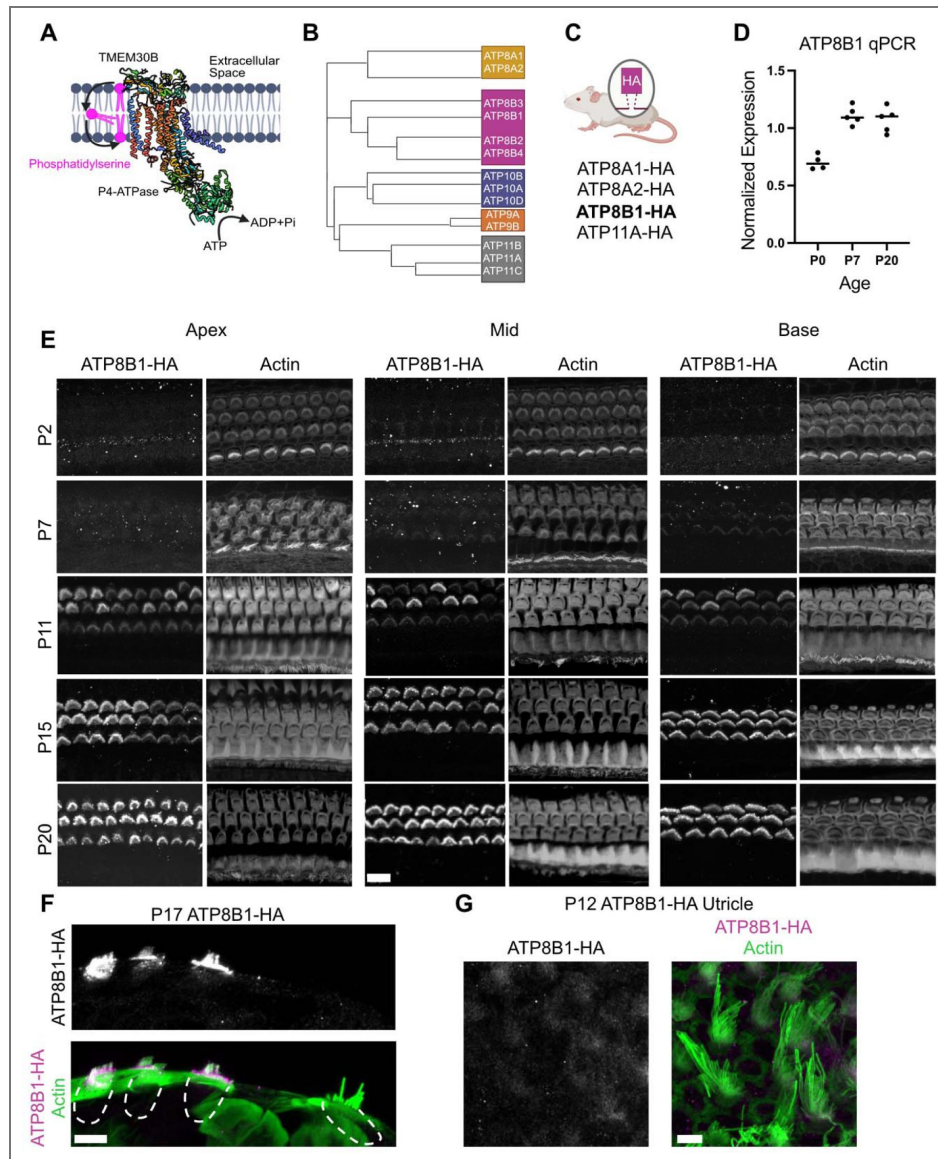


Figure 1. ATP8B1 is a P4-ATPase flippase selectively expressed in outer hair cells.

(A) Schematic illustrating ATP8B1 and its obligate chaperone TMEM30B embedded in the plasma membrane (adopted from PDB, 8OX4) (Dieudonné et al. 2023). **(B)** Cladogram of the 14 P4-ATPase family members expressed in mice. **(C)** Of the 14 P4-ATPases, four were detected in hair cells, and HA knock-in mouse lines were generated for each. ATP8B1-HA (bold) was the only flippase found to be expressed in outer hair cells. **(D)** qPCR analysis of *Atp8b1* expression at P0, P7, and P20. Four to five cochleae were analyzed per age group. **(E)** ATP8B1-HA Immunofluorescence across development and along the cochlear tonotopic axis in the organ of Corti. Organ of Corti were stained for anti-HA and phalloidin for F-actin. **(F)** Orthogonal view of ATP8B1-HA immunofluorescence in a P17 organ of Corti. A Compressstome section was used to facilitate imaging of the hair bundle plane. Scale bars: 5 μ m **(G)** ATP8B1-HA immunofluorescence in P12 utricle showing minimal expression. Scale bars 10 μ m.

ATP8B1-HA knock-in allele for localization studies. Among these candidates, ATP8B1 was the only flippase specifically enriched in the hair bundle of OHCs, consistent with previous studies reporting ATP8B1-dependent hair-cell degeneration and hearing loss (Stapelbroek et al. 2009). Remarkably, no ATP8B1-HA immunoreactivity was detected in IHCs or their hair bundles.

We performed a developmental time course of expression. Organs of Corti from ATP8B1-HA mice were imaged at key stages spanning hair-cell maturation: P2 (around the onset of MET activity), P7 (MET established but prior to hearing onset), P12 (near hearing onset), P17 and P20 (mature auditory epithelium) (Fig. 1E). Across this timeline, ATP8B1 became progressively enriched in OHC stereocilia, coinciding with maturation of MET function and hearing onset. Protein expression patterns matched transcript levels, as qPCR analysis for *Atp8b1* demonstrated a developmental trajectory consistent with our immunofluorescence data (Fig. 1D).

Finally, we assessed whether ATP8B1 is restricted to the stereocilia or distributed throughout the hair cell. ATP8B1-HA localized almost exclusively to OHC bundles and the apical membrane, with minimal staining in the remaining plasma membrane (Fig. 1F). In contrast, staining of the utricle revealed only weak ATP8B1-HA signal in vestibular hair cells, markedly lower than that observed in OHCs (Fig. 1G). Together, these data establish ATP8B1 as a tightly localized flippase that becomes strongly bundle-enriched during OHC maturation, with substantially lower expression in vestibular hair cells, and showed no detectable signal in IHCs.

We next investigated the precise subcellular localization of ATP8B1 in OHC stereocilia. OHCs oriented in profile were identified and imaged. Quantitative fluorescence intensity profiling of ATP8B1-HA and actin along both short (row 2) and tall (row 1) stereocilia revealed that ATP8B1 is present in all stereocilia rows. Notably, ATP8B1 is not uniformly distributed along the length of the stereocilia; instead, it is enriched at the stereociliary base, with signal intensity progressively decreasing toward the tips (Fig. 2A, B, C, D).

Loss of ATP8B1 leads to early onset hearing loss and rapid OHC degeneration

To define the role of ATP8B1 in auditory function, we generated a *Atp8b1* knockout (KO) mouse (19 bp deletion allele disrupting the translational start). Examination of the cochlea revealed a rapid and progressive loss of OHCs, beginning in the basal turn by postnatal day 17 and extending apically over the next several days (Fig. 3A, B). By P20, only a small number of basal OHCs remained (Fig. 3B, C). In contrast, IHCs, in which we did not detect ATP8B1, were preserved (Fig. 3D), indicating a selective requirement for ATP8B1 in OHC survival. Consistent with this cellular phenotype, *Atp8b1* KO mice exhibited early-onset hearing loss. Auditory brainstem response (ABR) thresholds and distortion product otoacoustic emissions (DPOAEs) were significantly elevated across all tested frequencies at P20 compared with littermate controls, consistent with loss of OHC function (Fig. 3E). This phenotype mirrors that previously reported for an independent *Atp8b1* point-mutation allele (Stapelbroek et al. 2009). Together, these findings demonstrate that ATP8B1 is essential for OHC survival and auditory function.

Following the observed hair cell loss of *Atp8b1* KO mice, we performed scanning electron microscopy (SEM) to assess hair-bundle morphology and cell integrity (Fig. 3F). *Atp8b1* KO OHCs showed marked morphological deterioration, characterized by reduced transducing stereocilia number and occasional fusion along the edges of stereocilia rows, a known pathological feature associated with progressive hearing loss (Fig. 3G). In contrast, IHCs did not exhibit detectable bundle abnormalities or cell loss, consistent with our immunohistochemistry results. Notably, stereocilia degeneration and fusion in the KO mice occurred prior to overt hair cell loss, indicating that disruption of bundle architecture is an early consequence of ATP8B1 deficiency.

TMEM30B expression and localization mirrors that of ATP8B1

The targeting and function of P-type ATPase flippases requires association with β -subunits TMEM30A or TMEM30B. To determine which partners operate with ATP8B1 in OHCs, we created HA-tagged KI mice for both proteins. TMEM30B, but not TMEM30A (data not shown), was

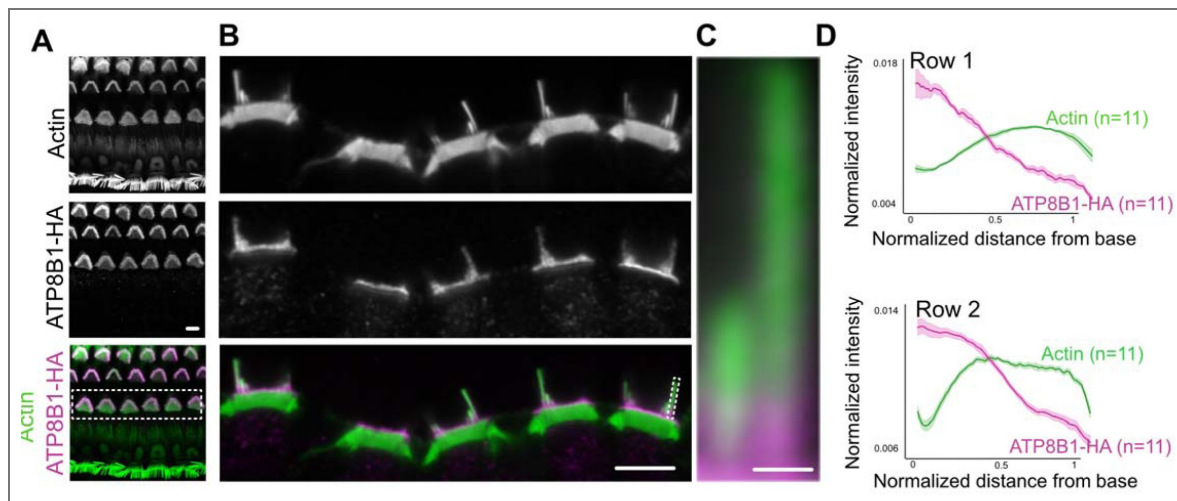


Figure 2. ATP8B1 is enriched toward the base of stereocilia.

(A) ATP8B1-HA immunofluorescence showing OHC specific expression. (B,C) ATP8B1-HA IF is enriched towards the stereocilia base and the apical hair cell membrane. (D) Average normalized fluorescence intensity profiles of actin and ATP8B1 along short (row 2) and long (row 1) stereocilia. Position is normalized from 0 (base) to 1 (tip), and intensities are normalized to total signal per stereocilium. Shaded regions indicate SEM. (Scale bar: 5 μ m and 0.5 μ m in stereocilia inset)

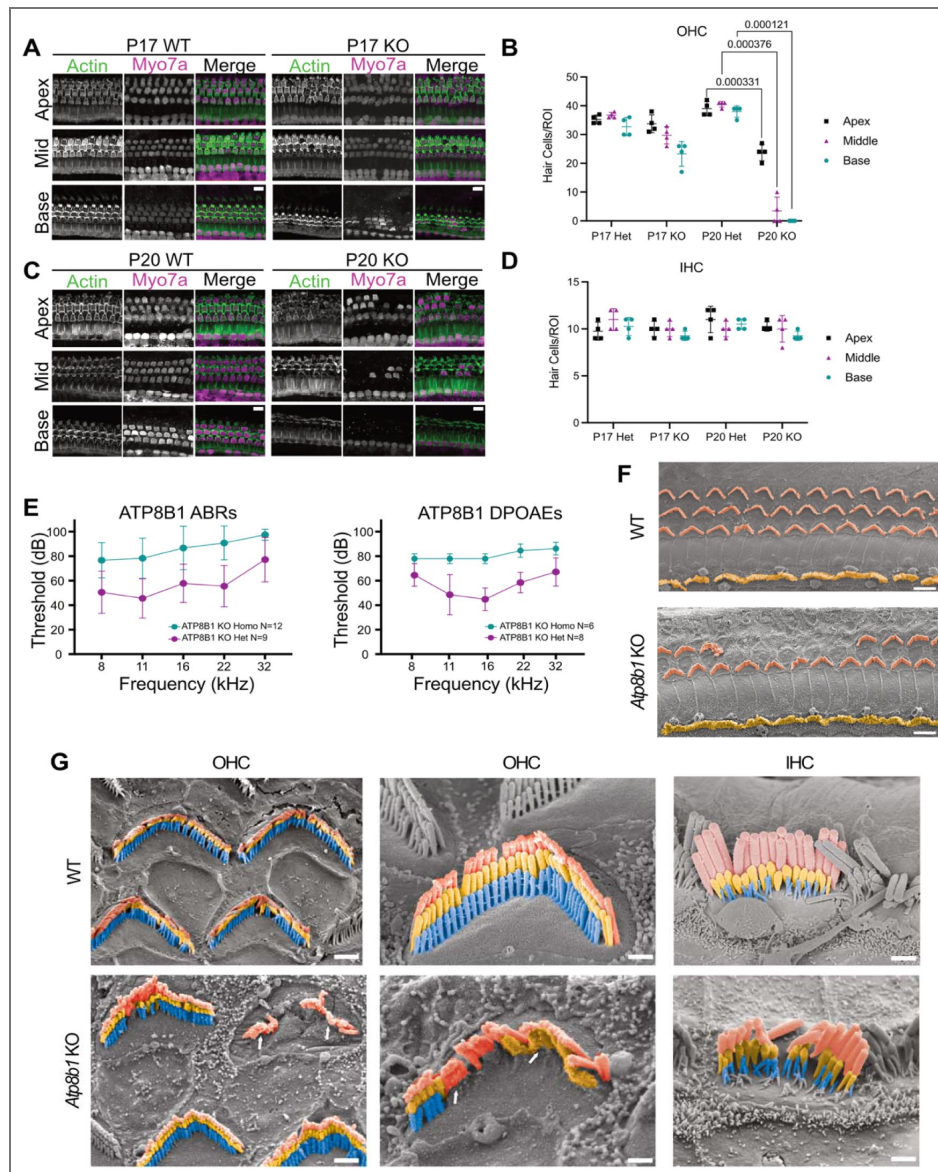


Figure 3. Loss of ATP8B1 causes progressive OHC bundle degeneration, hair cell loss, and elevated auditory thresholds

(A,B) Representative immunofluorescence images of WT and *Atp8b1* KO cochleae at P17 and P20 stained for MYO7A (hair cells) and phalloidin (F-actin). OHC loss is first observed in the basal turn of *Atp8b1* KO cochleae. Scale bar, 10 μ m. (C) Quantification of OHC numbers in WT and *Atp8b1* KO cochleae at P17 and P20 across apical, middle, and basal turns ($n = 3$ –5 cochleae per group). At P20, OHC loss was significant in all tonotopic regions in KO mice compared with WT (Welch's t-test, $p < 0.001$), whereas no significant differences were observed at P17. (D) Quantification of IHC survival in WT and *Atp8b1* KO cochleae at P17 and P20 across apical, middle, and basal turns ($n = 3$ –5 cochleae per group). No significant differences were detected between genotypes at either age (Welch's t-test). (E) Auditory brainstem response (ABR) thresholds at P30 are significantly elevated in *Atp8b1* KO mice compared with littermate controls (KO, $N = 12$; WT, $N = 9$). Distortion product otoacoustic emissions (DPOAE) thresholds at P20 are also significantly elevated, (KO, $N = 6$; WT, $N = 8$). ABR thresholds were increased at all tested frequencies (8, 11, 16, 22, and 32 kHz; Mann-Whitney U test, $p < 0.001$). DPOAE thresholds were increased at all tested frequencies (8, 11, 16, 22, and 32 kHz; Mann-Whitney U test, ($p < .01$, 8kHz), $p < 0.001$, 11–32kHz) (F) Scanning electron microscopy (SEM) images of WT and *Atp8b1* KO organs of Corti at P20. OHCs are pseudo-colored orange and IHCs yellow. Scale bars: 5 μ m. (G) High-magnification SEM images at P20 reveal stereocilia fusion and OHC loss in *Atp8b1* KO mice, features absent in WT. Arrows point out areas of stereocilia fusion and loss. IHCs remain morphologically intact. Scale bars: 1 μ m (left), 0.5 μ m (middle and right).

expressed in hair cells. TMEM30B-HA mice exhibited normal hearing thresholds compared with C57BL/6J controls at P30, indicating that insertion of the HA tag did not disrupt auditory function (Sup. Fig. 1 [↗](#)). Organs of Corti from TMEM30B-HA mice were imaged at P2, P7, P12, P17 and P20 (Fig. 4A [↗](#)). Across this series, TMEM30B-HA showed a progressive enrichment in stereocilia, paralleling the maturation of the MET apparatus and the onset of hearing, similar to ATP8B1. We next studied the subcellular distribution of TMEM30B. While ATP8B1-HA was almost exclusively confined to OHC bundles (Fig. 1 [↗](#) and 2 [↗](#)), TMEM30B-HA was enriched in the stereocilia compartment, but also weakly in the cell body (Fig. 4B [↗](#)). Like ATP8B1-HA, TMEM30B-HA showed modest labeling of utricular hair bundles at P17 (Fig. 4C [↗](#)) and was excluded from IHC bundles. Together, these findings identify ATP8B1 and TMEM30B as a tightly co-localized flippase complex that becomes progressively OHC bundle-specific during auditory maturation.

TMEM30B is required for hearing and OHC maintenance

Next, we generated *Tmem30b* KO mice harboring a 54 bp deletion disrupting the translational start. Similar to *Atp8b1* KO mice, *Tmem30b* KO mice exhibited rapid OHC degeneration, beginning in the basal turn at P17 and advancing apically over the following days. By P20, only a small number of basal OHCs remained (Fig. 5A, B [↗](#)). In contrast, IHCs were preserved, closely mirroring the *Atp8b1* KO phenotype and indicating that loss of TMEM30B selectively compromises OHC survival (Fig. 5C, D [↗](#)). Consistent with this cellular phenotype, *Tmem30b* KO mice exhibited early-onset hearing loss. Auditory brainstem response (ABR) thresholds and distortion product otoacoustic emissions (DPOAEs) were significantly elevated across all tested frequencies at P20 compared with littermate controls, consistent with loss of OHC function (Fig. 5E [↗](#)). Together, these findings confirm TMEM30B as a deafness gene, in agreement with an auditory phenotype previously reported by the IMPC (International Mouse Phenotyping Consortium) (Bowl et al. 2017 [↗](#)).

We next performed SEM analysis to assess the morphological defects in *Tmem30b* KO OHC hair bundles (Fig. 5F [↗](#)). Prior to overt cell loss, *Tmem30b* KO OHCs exhibited fractured and disorganized hair bundles. We did not observe the same hair bundle fusion that occurred in the *Atp8b1* KO OHCs, possibly pointing to a difference in cell deterioration between the two mouse lines (Fig. 5G [↗](#)). IHCs displayed no detectable morphological abnormalities. Overall, the degenerative phenotype in *Tmem30b* KO mice largely parallels that of *Atp8b1* KO mice, supporting a shared requirement for the ATP8B1-TMEM30B flippase complex in maintaining OHC integrity.

To determine how the loss of TMEM30B affects the localization of ATP8B1-HA, we examined ATP8B1-HA localization on the *Tmem30b* KO mouse background. In control mice, ATP8B1-HA is enriched in stereocilia, and not found below the hair bundle and the apical membrane. In contrast, in the absence of TMEM30B, ATP8B1-HA showed markedly increased somatic staining with reduced confinement to the hair bundle, indicating protein mislocalization (Fig. 5H [↗](#)). These findings demonstrate that TMEM30B is necessary for correct targeting of ATP8B1, providing a mechanistic explanation for the closely overlapping phenotypes observed in the two KO models.

ATP8B1 and TMEM30B deficiency causes aberrant PS exposure in OHC bundles

ATP8B1 deficiency is predicted to impair PS internalization from the outer plasma membrane leaflet (Klomp et al. 2004 [↗](#)). Persistent PS exposure could disrupt MET function and/or initiate downstream pathways that contribute to hair-cell degeneration as observed in both KO mouse models. We therefore examined whether *Atp8b1* and *Tmem30b* KO OHCs exhibit abnormal PS exposure. We performed Annexin V (AnV) labeling on apical turns of P15 organs of Corti (Zhang et al. 1997 [↗](#)), a developmental timepoint that precedes overt hair cell loss in these mutants (Koopman et al. 1994 [↗](#)) (Fig. 6A, C [↗](#)). AnV labeling was restricted to stereocilia bundle and apical membrane and not detected along the lateral membrane or within the cell body. Both *Atp8b1* KO and *Tmem30b* KO mice exhibited significantly elevated AnV fluorescence compared with wild-type controls (Fig. 6B, D [↗](#)). We occasionally observed weak AnV labeling also in IHCs, at the apical membrane (often appearing to be enriched at cell junctions) and the hair bundle (Fig. 6A, C [↗](#)).

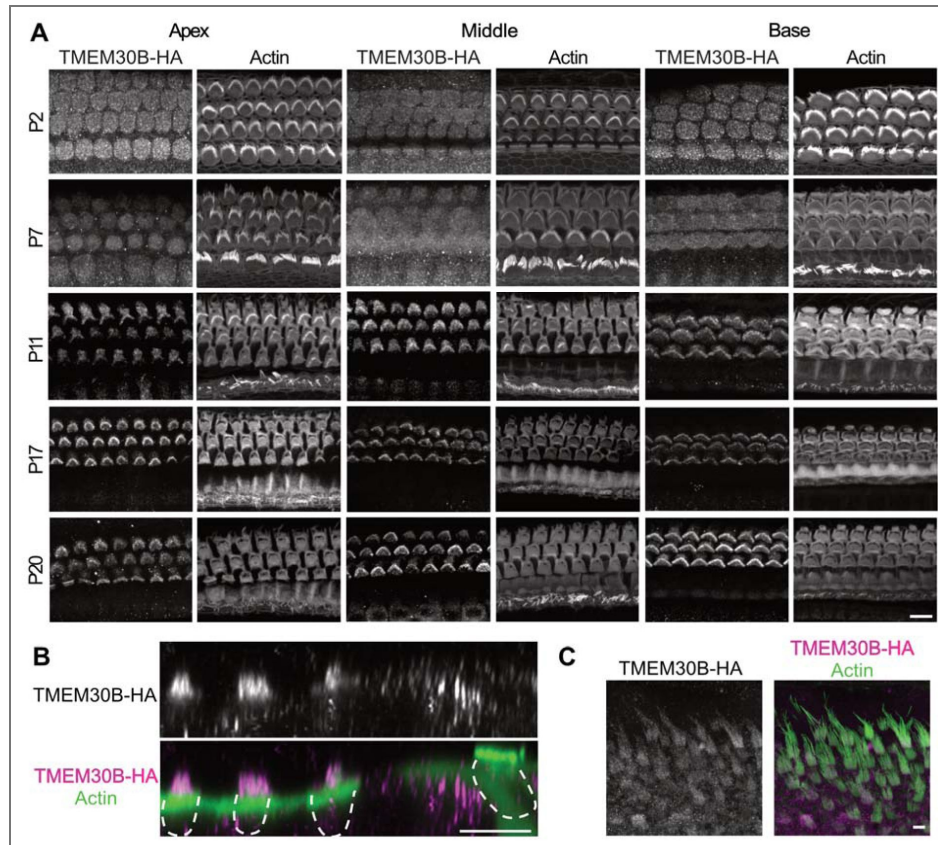


Figure 4. The P4-ATPase chaperone TMEM30B is enriched in OHC bundles.

(A) Immunofluorescence images of TMEM30B-HA across development and along the cochlear tonotopic axis in the organ of Corti. Organ of Corti were stained for anti-HA and phalloidin for F-actin. Scale bars 10 μ m. **(B)** Orthogonal view of TMEM30B-HA immunofluorescence of a P17 organ of Corti. Scale bars 10 μ m. **(C)** Immunofluorescence images of a P17 TMEM30B-HA utricle showing moderate expression in utricular hair cells. Scale bars 10 μ m.

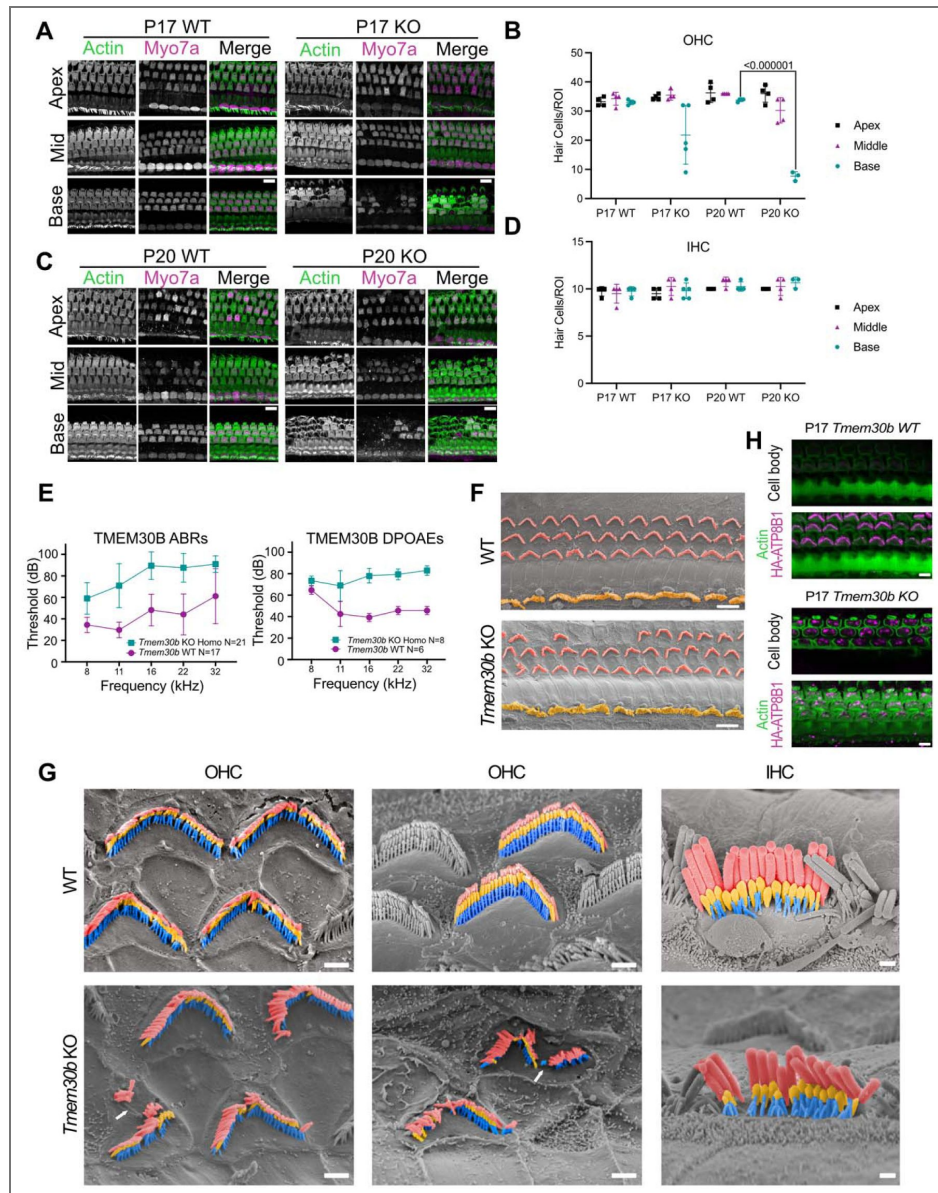


Figure 5. Loss of TMEM30B causes progressive OHC degeneration, hair cell loss, and elevated auditory thresholds.

(A, B) Representative Immunofluorescence images of WT and *Tmem30b* KO cochleae at P17 and P20, stained for MYO7A (hair cells) and phalloidin (F-actin). OHC loss is first detected in the basal turn of *Tmem30b* KO cochleae at P17, and pronounced OHC loss in the basal turn of KO cochleae at P20. Scale bar, 10 μ m. (C) Quantification of OHC numbers in WT and *Tmem30b* KO cochleae at P17 and P20 across apical, middle, and basal turns ($n = 3-5$ cochleae per group). At P20, OHC loss was significant in the basal region of KO mice compared with WT (Welch's t -test, $p < 0.001$), whereas no significant differences were observed at P17. (D) Quantification of IHC numbers in WT and *Tmem30b* KO cochleae at P17 and P20 across apical, middle, and basal turns ($n = 3-5$ cochleae per group). No significant differences were detected between genotypes at either age (Welch's t -test). (E) Auditory brainstem response (ABR) thresholds at P30 are significantly elevated in *Tmem30b* KO mice compared with littermate controls (KO, $N = 21$; WT, $N = 17$). Distortion product otoacoustic emissions (DPOAE) thresholds at P30 are also significantly elevated, (KO, $N = 8$; WT, $N = 6$). ABR and DPOAE thresholds were increased at all tested frequencies (8, 11, 16, 22, and 32 kHz; Mann-Whitney U test, $p < 0.001$). (F) Scanning electron microscopy (SEM) images of WT and *Tmem30b* KO organs of Corti at P20. OHCs are pseudo-colored orange and IHCs yellow. Scale bars: 5 μ m (G) High-magnification SEM images at P20 reveal stereocilia misorientation and OHC loss in *Tmem30b* KO mice, features absent in WT. Arrows point out areas of stereocilia misorientation. IHCs remain morphologically intact. Scale bars: 1 μ m (left), 0.5 μ m (middle and right). (H) Immunofluorescence images of organs of Corti from *Tmem30b* KO / ATP8B1-HA mice at P17 showing aberrant localization of ATP8B1-HA to the hair cell soma in the absence of TMEM30B (magenta). Scale bars: 5 μ m

Together, these findings indicate that loss of ATP8B1-TMEM30B flippase activity leads to aberrant PS exposure predominantly in OHC stereocilia, preceding and likely contributing to hair cell degeneration in both KO models.

Specific enrichment of ATP8B1-TMEM30B in OHC stereocilia requires MET activity

Previous studies have shown that the localization of P4-ATPase β -subunits, such as TMEM30A, can be regulated by neuronal activity (Li et al. 2021 [↗](#)). This raised the possibility that MET activity similarly influences the localization of the ATP8B1-TMEM30B flippase complex in hair cells. To test this idea, we examined ATP8B1 and TMEM30B distribution in *Cib2* KO hair cells, which lack MET currents (Giese et al. 2017 [↗](#); Riazuddin et al. 2012 [↗](#)). At P15, prior to overt hair cell degeneration in *Cib2* KO mice, ATP8B1-HA and TMEM30B-HA remained detectable in hair bundles but showed a pronounced increase in cell-body staining, indicating mislocalization in the absence of MET activity (Fig. 7A, B, C, D [↗](#)). These results demonstrate that MET activity is required for specific targeting of the ATP8B1-TMEM30B flippase complex in stereocilia.

Discussion

In this study, we identify P4-ATPase ATP8B1 and its accessory subunit TMEM30B as essential regulators of OHC survival and auditory function. Our findings establish a direct mechanistic link between OHC stereocilia membrane lipid asymmetry and hearing by demonstrating that disruption of a stereocilia-localized flippase complex leads to aberrant PS exposure, early hair-bundle pathology, progressive OHC degeneration, and rapid hearing loss. Together, these data reveal a previously underappreciated requirement for active lipid homeostasis in OHCs and provide cellular and mechanistic validation of TMEM30B as a novel deafness gene.

A flippase complex is selectively enriched in OHC stereocilia

A central finding of this work is the highly selective localization of ATP8B1 and TMEM30B to OHC stereocilia. Among the P4-ATPases expressed in hair cells, ATP8B1 uniquely localizes to the hair bundle, was not detectably enriched in IHCs, and becomes progressively enriched in hair bundles during postnatal maturation, coinciding with the onset and maturation of hair cell MET. This specificity likely serves to accommodate the uniquely high mechanical and metabolic demands placed on OHC stereocilia. ATP8B1 is strongly enriched within stereocilia membranes, with minimal localization in the plasma membrane below the epithelial barrier level. Loss of TMEM30B results in mislocalization of ATP8B1, demonstrating that this β -subunit is required not only for flippase activity but also for proper targeting and retention of ATP8B1 in the stereocilia membrane.

Lipid asymmetry as a potential structural and functional determinant of MET

Recent cryo-EM studies, together with the discovery that TMC1 and TMC2 possess intrinsic lipid-scrambling activity, have prompted a re-evaluation of MET in which membrane lipids and their asymmetric distribution are re-considered as active determinants of channel function rather than passive structural components (Ballesteros and Swartz 2022 [↗](#); Jeong et al. 2022 [↗](#); Clark et al. 2024 [↗](#); Peineau et al. 2025 [↗](#)). Loss of the asymmetric distribution of membrane with their bulky and charged headgroups such as PS is known to have profound biophysical consequences for the cellular membrane (Kakuda et al. 2022 [↗](#); X. Li et al. 2024 [↗](#); Wang et al. 2025 [↗](#)), and the stereocilia membrane will not be an exception. Redistribution of PS to the outer leaflet alters bilayer thickness (Wang et al. 2025 [↗](#)), curvature stress, and membrane viscosity (Caputo et al. 2025 [↗](#)), parameters that are particularly critical in stereocilia (George et al. 2020 [↗](#); George et al. 2023 [↗](#); George and Ricci 2025 [↗](#)), where MET channel gating is exquisitely sensitive to membrane mechanics.

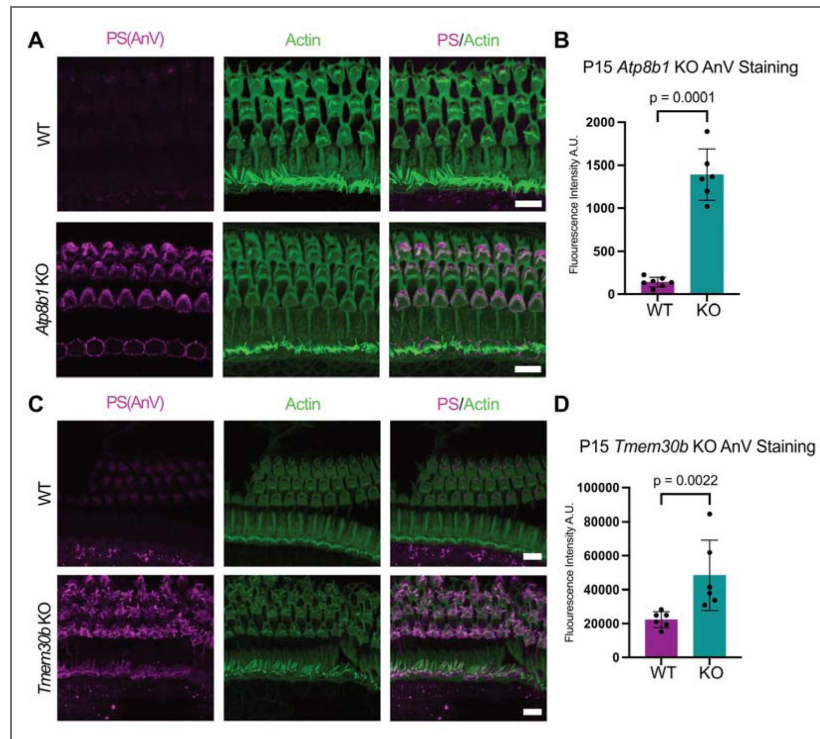
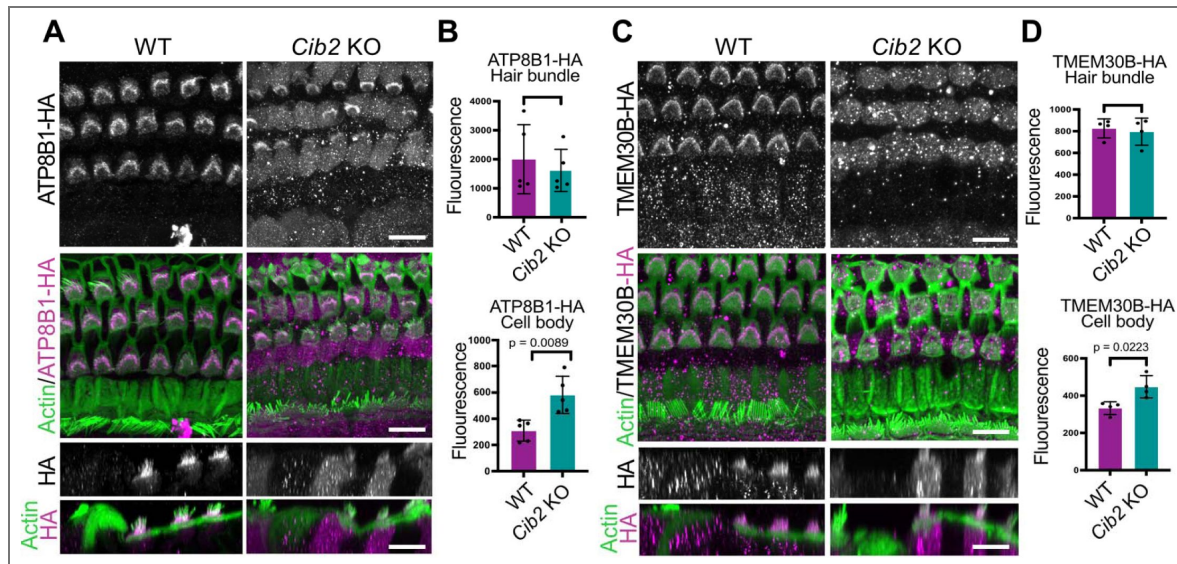


Figure 6. Loss of plasma membrane asymmetry precedes hair cell loss in *Atp8b1* and *Tmem30b* knockout mice.

(A) Representative confocal images of WT and *Atp8b1* KO cochleae at P15 stained with Annexin V to label externalized phosphatidylserine (PS) and phalloidin to visualize F-actin. Increased Annexin V labeling is observed in *Atp8b1* KO hair cells prior to overt cell loss. Scale bar, 10 μ m. (B) Quantification of Annexin V fluorescence intensity in WT and *Atp8b1* KO cochleae ($n = 5-7$ cochleae per group). *Atp8b1* KO hair cells show significantly increased PS externalization compared with WT (Welch's t -test, $p < 0.0001$). (C) Representative confocal images of WT and *Tmem30b* KO cochleae at P15 stained with Annexin V and phalloidin. Increased Annexin V labeling is similarly observed in *Tmem30b* KO hair cells. In *Atp8b1* and *Tmem30b* KO organs, weak AnV labeling was occasionally observed also in IHCs, at the apical cell junctions and the hair bundle. Scale bar, 10 μ m. (D) Quantification of Annexin V fluorescence intensity in WT and *Tmem30b* KO cochleae ($n = 5-7$ cochleae per group), demonstrating significantly elevated PS externalization in KO hair cells (Welch's t -test, $p < 0.01$).

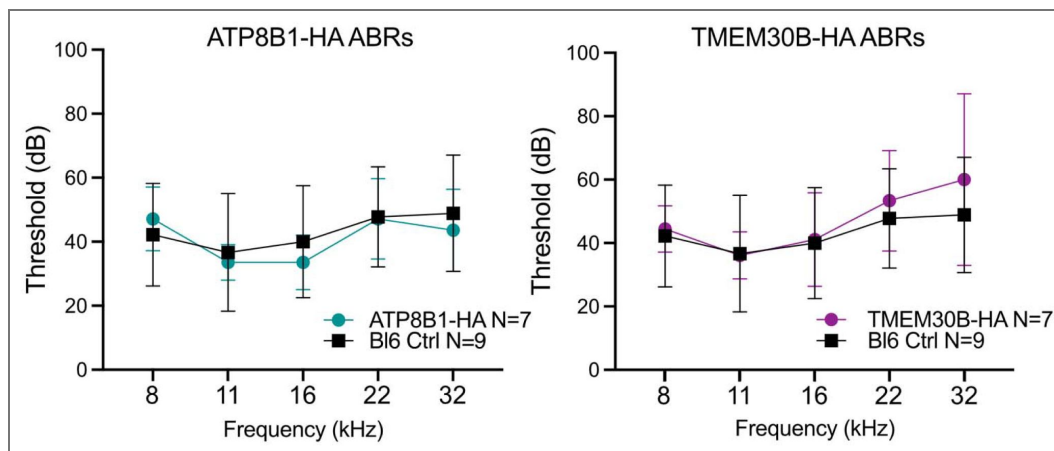
Figure 7. ATP8B1 and TMEM30B require mechanotransduction activity for proper subcellular localization.

(A, C) Representative ATP8B1-HA and TMEM30B-HA immunofluorescence in P15 cochleae (whole mount and profile view), on WT or *Cib2* KO background, counterstained with phalloidin to visualize F-actin. The z-projections shown in the top panels of A and C give the appearance of fewer hair bundles per field of view. However, this is not due to a reduction in hair bundle number; rather, the increased fluorescence signal in the cell bodies obscures the hair bundle staining. Therefore, quantification was performed using the profile views generated by the reslice function in ImageJ shown in the bottom panels of A and C. In the absence of MET activity (*Cib2* KO), ATP8B1-HA and TMEM30B-HA are aberrantly enriched in the hair cell soma of *Cib2* KO mice. (B) Quantification of ATP8B1-HA immunofluorescence intensity in WT and *Cib2* KO cochleae in the hair bundle and cell body ($n = 4-6$ cochleae per group). ATP8B1-HA intensity was significantly increased in the cell body of *Cib2* KO hair cells ($p < 0.01$, Welch's t -test), with no significant difference detected in the hair bundle. (D) Quantification of TMEM30B-HA fluorescence intensity in WT and *Cib2* KO cochleae in the hair bundle and cell body ($n = 4-6$ cochleae per group) (whole mount and profile view). TMEM30B-HA intensity was significantly increased in the cell body of *Cib2* KO hair cells ($p < 0.05$, Welch's t -test), with no significant difference observed in the hair bundle. Scale bar $10 \mu\text{m}$



Supplemental Figure 1. ATP8B1-HA and TMEM30B-HA mice have normal hearing.

Auditory brainstem response (ABR) thresholds at P30 in ATP8B1-HA and TMEM30B-HA (ATP8B1-HA, $N = 7$; TMEM30B-HA, $N = 7$; B6 WT ctrl, $N = 9$). ABR thresholds were not significantly elevated at all tested frequencies (8, 11, 16, 22, and 32 kHz; Mann-Whitney U test, NS)



Building on this framework, a recent model proposed by George and Ricci suggests that TMC1/2 scramblase activity, by lowering membrane viscosity, reduces the energetic cost of channel gating, which may improve both the speed and sensitivity of the mechanotransduction response (George and Ricci 2025 [DOI](#)). If lipid scrambling is an integral feature of MET, however, a compensatory mechanism must exist to prevent unchecked collapse of membrane asymmetry. It is tempting to speculate that the ATP8B1-TMEM30B flippase complex fulfills this role by continuously restoring lipid asymmetry and maintaining OHC membranes within a “Goldilocks zone” of viscosity optimal for OHC MET.

This model is supported by the spatial distribution of ATP8B1, which is most abundant at the base of stereocilia and within the apical OHC membrane, with decreasing levels toward the stereocilia tips where MET occurs. Such a gradient suggests that TMC1-mediated lipid scrambling predominates at the site of MET, while ATP8B1-TMEM30B activity at the bundle base and apical membrane prevents widespread PS exposure, which could be interpreted as an “eat-me” signal and trigger pathological and phagocytic responses by surrounding supporting cells. Consistent with this model, elevated PS exposure in both *Atp8b1* and *Tmem30b* KO hair cells precedes overt structural degeneration, indicating that continuous flippase activity is required to preserve stereociliary membrane composition and stability.

Selective requirement for stereocilia flippase activity in OHCs versus IHCs

A striking aspect of our finding is that in the cochlea, ATP8B1 is selectively expressed in OHC stereocilia and was not detected in IHCs under the conditions examined. One possible explanation is that IHCs stereocilia express another flippase. This seems unlikely as we systematically assessed all P4-ATPases expressed in hair cells and did not find evidence for an alternative stereocilia-enriched P4-ATPase in IHC bundles. Instead, we think it likely that IHCs, at least in the stereocilia, lack flippase activity altogether. It is possible that IHCs maintain lipid asymmetry using a different strategy, for example, by relying on a distinct set of lipid transporters localized outside the bundle, possibly sufficient given the lower MET resting open probability in IHCs (Johnson et al. 2011 [DOI](#); Peng et al. 2013 [DOI](#); Corns et al. 2014 [DOI](#)), resulting in lower basal scrambling pressure. It is also possible that beyond P4-ATPase flippases, complementary regulation could also arise from other enzymes controlling lipid abundance (synthesis and degradation) and compartmentalization (Blom et al. 2011 [DOI](#)).

Early bundle pathology precedes hair-cell loss

Annexin V mediated detection of PS and structural analyses reveal that PS externalization and hair-bundle degeneration precedes hair-cell death in both KO models. Notably, IHCs remain morphologically and functionally intact, consistent with their lack of ATP8B1 and TMEM30B expression. This selective vulnerability underscores the unique lipid homeostasis requirements of OHC stereocilia, likely reflecting an adaptation to their higher MET activity and mechanical load. A recent study reported Annexin V labeling of hair bundles beginning around P7 in wildtype mouse OHCs, suggesting PS exposure on the outer leaflet during normal development (George and Ricci 2025 [DOI](#)). This observation contrasts with our findings, in which robust and consistent Annexin V labeling was observed only in *Atp8b1* and *Tmem30b* KO hair cells. We note, however, that we occasionally detected low-level Annexin V signal in control samples, even in IHCs (as shown in Fig. 6 [DOI](#)). Given the sensitivity of the live Annexin V labeling procedure to rapid deterioration of cochlear hair cells, particularly OHCs after ototoxic insult (Shi et al. 2007 [DOI](#); Goodyear et al. 2008 [DOI](#)), this signal was initially interpreted as background or false-positive staining. Nonetheless, an alternative explanation is that OHC stereocilia possess higher PS exposure under normal physiological conditions, an expected phenomenon if TMC1/2 mediated MET causes phospholipid scrambling. Such low-level exposure of PS may fall near the detection threshold of Annexin V staining, especially if quickly reversed by flippase activity, and thus be difficult to resolve

reproducibly. Further work will be required to determine whether regulated, developmentally controlled phospholipid exposure occurs in normal hair bundles and how it is balanced by flippase activity.

Why do ATP8B1/TMEM30B-deficient OHCs die?

One notable feature of *Atp8b1* and *Tmem30b* KO phenotypes is their fast-progressing, base-to-apex pattern of OHC loss, reminiscent of several models in which MET is disrupted (e.g., TMC1/2 or CIB2 loss and related MET-deficient conditions) (Kawashima et al. 2011 [DOI](#); Riazuddin et al. 2012 [DOI](#); Zhao et al. 2014 [DOI](#); Pan et al. 2018 [DOI](#); Beurg et al. 2025 [DOI](#)). Loss of lipid asymmetry could directly compromise MET by altering bundle membrane mechanics and/or the availability of lipids on the inner leaflet that are required for normal MET. In this model, OHC degeneration would arise downstream of impaired MET signaling. At the same time, the OHC-specific degeneration observed in *Atp8b1* and *Tmem30b* KO mice also parallels, both in kinetics and cellular selectivity, the phenotype reported in mice with impaired stereocilia calcium extrusion via PMCA2 (Street et al. 1998 [DOI](#); Kozel et al. 1998 [DOI](#); Beurg et al. 2025 [DOI](#)). Loss of lipid asymmetry and/or altered phosphoinositide organization could reduce PMCA2 efficiency, compromising calcium clearance from stereocilia and promoting cytotoxic calcium accumulation. Consistent with this general possibility, PMCA activity is known to depend on membrane lipid composition. Specifically, PS on the inner leaflet was shown to be required for optimal PMCA activity (Zhang et al. 2009 [DOI](#)).

Activity-dependent targeting of the flippase complex

This study showed that proper and specific targeting of ATP8B1–TMEM30B to stereocilia requires MET activity. In the absence of MET currents, both proteins are present but mislocalized, accumulating in the cell body rather than being predominantly retained in the bundle. This activity dependence suggests a feedback mechanism in which MET-associated signals, potentially calcium influx, promote stabilization or trafficking of the flippase complex to sites of active MET. It should also be noted that PS externalization does not occur in TMC1/2 double KOs (George and Ricci 2025 [DOI](#)). Such coupling would provide an elegant solution for synchronizing lipid remodeling with channel activity: MET gating triggers scrambling through TMC proteins while simultaneously promoting flippase-mediated restoration of asymmetry, allowing hair cells to exploit lipid dynamics for function without incurring pathological consequences.

Limitations of this study

A limitation of the present study is that loss of lipid asymmetry in OHC stereocilia was assessed primarily through PS externalization, as detected by Annexin V binding. Annexin V is a widely used indicator of disrupted membrane asymmetry, and the absence of PS exposure in WT mice, together with its robust appearance in ATP8B1- and TMEM30B-deficient OHCs, supports a role for ATP8B1 in maintaining membrane lipid organization. However, this approach specifically reports PS exposure and does not directly inform on the asymmetric distribution of other lipid species. ATP8B1 has been reported to transport PS in several experimental contexts, and our data are therefore fully consistent with the possibility that PS is a direct substrate of ATP8B1 in OHC stereocilia membranes. At the same time, independent evidence from cell-based, biochemical, and structural studies indicates that ATP8B1 can also transport phosphatidylcholine (PC) and phosphoinositides, with recent work demonstrating ATP8B1-mediated flipping of PI(4,5)P₂ (Takatsu et al. 2014 [DOI](#); Gómez-Mellado et al. 2022 [DOI](#); Dieudonné et al. 2022 [DOI](#); Bhandari et al. 2025 [DOI](#)). Consequently, the PS externalization observed in *Atp8b1* and *Tmem30b* KO mice may reflect loss of asymmetry of PS itself, of other ATP8B1 substrates, or of multiple lipid species simultaneously. Because robust in situ reporters for leaflet-specific PC or phosphoinositide distribution are currently limited, our study cannot directly distinguish among these possibilities. Thus, while PS exposure provides a clear and biologically meaningful readout of altered lipid asymmetry, it should not be interpreted as indicating that ATP8B1 function in stereocilia is

exclusively or primarily PS-centric. Instead, our findings support a broader role for ATP8B1 in maintaining the asymmetric organization of stereocilia membranes, with PS externalization serving as a sensitive marker of this process.

Future studies employing lipid-specific biosensors, reconstitution-based approaches, or advanced lipidomic strategies may be important for defining the full spectrum of ATP8B1 substrates in stereocilia and for determining how perturbations in distinct lipid species contribute to OHC structure and function.

TMEM30B as a deafness gene and therapeutic implications

While ATP8B1 has been previously implicated in hearing loss, evidence was limited and mechanistic insight lacking (Stapelbroek et al. 2009 [\[1\]](#)). Our data provide strong validation of ATP8B1's role in hearing and identify TMEM30B as an essential partner whose loss phenocopies ATP8B1 deficiency at molecular, cellular, and functional levels. These findings align with a previous large-scale mouse mutagenesis screen reporting hearing loss in *Tmem30b* KO mice (Bowl et al. 2017 [\[2\]](#)). Importantly, ATP8B1 and TMEM30B present attractive therapeutic features. Their expression begins postnatally and peaks after hearing onset in mice, and hair-cell loss in KO mice occurs after ~P16, providing a potential window for intervention. Furthermore, TMEM30B is a single-exon gene of modest size (~3.5 kb including UTRs), making it well suited for AAV mediated gene delivery and faithful recapitulation of endogenous expression.

Conclusions and future directions

In summary, this study establishes ATP8B1 and TMEM30B as important regulators of membrane lipid asymmetry in OHC stereocilia and hair-cell survival, and points to a potentially essential role for lipid flipping in hair cell MET. Future studies will be required to quantify, using cellular electrophysiology, how lipid asymmetry influences MET channel gating and sensitivity, and to determine which additional lipid species are regulated by ATP8B1 in stereocilia.

Methods

Animal Care and Handling

All murine experiments conducted for this study adhered to the guidelines established by the University of Virginia Institutional Animal Care and Use Committee (IACUC) and the NIH. Animal protocols were reviewed and approved by the University of Virginia IACUC. Mice were housed in an AAALAC-accredited vivarium located in the basement of our research building. Animals were maintained on a 12-hour light/dark cycle, with cage changes performed every five days. Mice had ad libitum access to standard rodent chow and water provided through lixit systems. Control mice were C57BL/6J mice obtained from Jackson Laboratory. Experimental mice ranged from postnatal day 0 (P0) to P5 and were euthanized via rapid decapitation. Mice aged P6 and older were euthanized using CO₂ asphyxiation, followed by cervical dislocation as a secondary method to ensure humane euthanasia.

Generation of Experimental Mice

Genetically modified mouse models used in this study were generated using CRISPR/Cas9-mediated genome editing to create knockout or knock-in alleles of ATP8B1 and TMEM30B. Guide RNA target sequences were). CRISPR/Cas9 target sequences were designed using the online tool CRISPOR (<http://crispor.tefor.net/crispor.py> [\[3\]](#)) to generate KI and KO alleles for *Atp8b1* and *Tmem30b* (Haeussler et al. 2016 [\[4\]](#)). The selected guide RNA target sequences were **AAGCACAGAA AGAGACT** for *Atp8b1* and **CGCCGTGGCGCTCCAGGTCA** for *Tmem30b*.

For *Atp8b1*, the genomic sequence surrounding the target site and illustrating the KO modification is shown below: TCAGCCCAACACAGGAAAGTCACGACTACTCTGTTTCCTCTTTCTCTCCAGGTAGTTC CAATTTGCC AGCAGA**ATGAGCACAGAAAGAGACTCG**GAACAACATTTGATGAGGAGTCTCAGCCCAAC GATGAAGTG GTCCCTACAGTGATGATGA

The HA epitope-coding sequence (TACCCATACGATGTTCCAGATTACGCT) was inserted immediately downstream of the start codon (ATG, bold). The KO allele was generated by deletion of 19 nucleotides (underlined).

For *Tmem30b*, the following CRISPR target sequence was identified: **CGCCGTGGCGCTCCAGGTCA**. The genomic sequence surrounding the target site and illustrating the KO modification is shown below: GCGAGCGGAGGCGGGAGGCTCGCCGCTGATCCTGGGTTGAGAGTCGGCGGCCGAGGCCCGGCTGA GATCCCCGCCATGACCTGGAGCGCCACGGCGCGGGGCGCGACCAGCCGACAACACCGCCTTCACTC AGCAGCGCCTCCCCGCTGGCAGCC

As for *ATP8B1*, the HA epitope-coding sequence was inserted immediately downstream of the start codon (ATG, bold). The KO allele was generated by deletion of 54 nucleotides (underlined).

Genotyping

Genotyping of *Atp8b1* alleles yielded PCR products of 239 bp (wt), 266 bp (HA KI), and 220 bp (KO). Primers used were:

Forward: AGGGGTCTTCTGACAGGATG
Reverse: TCTGTTCTGGTTCAACCGTGG

Genotyping of *Tmem30b* alleles yielded PCR products of 232 bp (wt), 259 bp (HA KI), and 178 bp (KO). Primers used were:

Forward: CGCTGATCCTGGGTTGAGAG
Reverse: TGCCGTTGGAGGAGTAGAAG

Mouse colony maintenance

Atp8b1 KO mice were maintained as heterozygous breeders. All other lines (*Atp8b1* HA KI, *Tmem30b* KO, and *Tmem30b* HA KI) were maintained as homozygous breeding colonies.

Immunofluorescence

Mice were sacrificed via decapitation (for P5 and younger) or CO₂ asphyxiation followed by cervical dislocation (for P6 and older). Cochleae were carefully dissected and placed into a dish containing phosphate-buffered saline (PBS; GIBCO, Thermo Fisher Scientific). Under a dissecting microscope, the ossicles were removed, and the cochleae were transferred to a well containing 4% paraformaldehyde (PFA; Electron Microscopy Services). The cochleae were flushed through the oval and round windows using a transfer pipette to ensure proper fixation and incubated in the fixative for 20 minutes at room temperature. After fixation, the cochleae were washed three times for five minutes each in PBS. For P7 and younger mice, cochleae were immediately dissected. For P8 and older mice, cochleae were decalcified in 0.5 M ethylenediaminetetraacetic acid (EDTA; Bioland Scientific LLC), pH 8.0, for up to two weeks, depending on bone density. Organs of Corti were either dissected as whole mounts under a stereomicroscope using forceps and micro-dissection scissors or sectioned into 50 µm slices using a Compressstome (Precisionary Instruments, Ashland, MA). Dissected or sliced organs of Corti were transferred to blocking buffer and incubated for two hours at room temperature. The blocking buffer consisted of PBS supplemented with 1% bovine serum albumin (BSA), 3% normal donkey serum, and 0.2% saponin. Following blocking, tissues were incubated overnight at 4°C with primary antibodies diluted in the same blocking buffer. The following primary antibody was used: rabbit anti-HA (C29F4, Cell Signaling Technology, Danvers, MA). The next day, tissues were washed three times for five minutes each in PBS, then incubated with fluorescently labeled secondary antibodies and phalloidin in blocking buffer for two hours at room temperature. After secondary antibody incubation, tissues were washed three additional times in PBS, mounted onto glass slides using ProLong Gold Antifade Mounting Media (Thermo Fisher Scientific), and allowed to cure overnight. Confocal imaging was performed using a Leica Stellaris 5 confocal microscope equipped with a 63x oil-immersion objective.

ATP8B1-HA Fluorescence Line Scan Analysis

Cochleae from ATP8B1-HA knock-in mice were immunostained with anti-HA antibody and labeled with phalloidin to visualize F-actin as described in the immunofluorescence protocol above. Confocal images were acquired using a Leica Stellaris 5 confocal microscope equipped with a 63× oil-immersion objective. High-resolution images of outer hair cell stereocilia were collected using identical acquisition parameters across samples. To assess sub-stereociliary localization of ATP8B1-HA, fluorescence intensity line scans were performed along individual stereocilia using ImageJ (NIH). Line scans were drawn manually along the length of both long (row 1) and short (row 2) stereocilia from the base at the cuticular plate to the stereocilia tip. Fluorescence intensity profiles for ATP8B1-HA and phalloidin were extracted and background-subtracted. For each stereocilium, positional distance was normalized from 0 (base) to 1 (tip), and fluorescence intensity was normalized to the total signal per stereocilium to allow comparison across cells. Normalized intensity profiles were averaged across stereocilia, and shaded regions represent the standard error of the mean (SEM).

Fluorescence Intensity Quantifications of ATP8B1-HA and TMEM30B-HA

Cochleae were immunostained with anti-HA antibody and labeled with phalloidin as described in the immunofluorescence protocol above. Confocal images were acquired using a Leica Stellaris 5 confocal microscope equipped with a 63× oil-immersion objective. For each experiment, all images were acquired using identical acquisition parameters, including zoom and laser power, across genotypes within that experiment. Fluorescence intensity of the HA signal was quantified in ImageJ (NIH). Whole-mount images were resliced to generate orthogonal views, and regions of interest (ROIs) were manually drawn around the hair bundle and hair cell soma. Background fluorescence was measured in adjacent regions lacking specific signal and subtracted from all measurements. For all analyses, measurements were restricted to the middle cochlear turn, as *Cib2* knockout cochleae fail to mature tonotopically. For each cochlea, fluorescence intensity was measured in five outer hair cells and averaged to generate a single biological replicate (n = 4–6 cochleae per genotype). Data analysis and statistical testing were performed using GraphPad Prism.

Quantitative PCR

qPCR primers were designed to detect ATP8B1 expression levels. Total RNA was extracted using TRIzol reagent (15596026, ThermoFisher Scientific). Reverse transcription was performed using the SuperScript™IV Reverse Transcription system (18090010, ThermoFisher Scientific). qPCR reactions were conducted using iTaq Universal SYBR Green Supermix (1725121, Bio-Rad, CA, USA) on a CFX Opus 384 Real-Time PCR System (12011319, Bio-Rad) for fluorescence measurement. Transcript level of *Atp8b1* is normalized to the transcript level of *Gapdh*. Expression analysis was performed in GraphPad Prism.

Hair Cell Counts

Cochleae were immunostained for MYO7A and labeled with phalloidin as described in the immunofluorescence protocol above. Confocal images were acquired using a Leica Stellaris 5 confocal microscope equipped with a 63× oil-immersion objective. For each experiment, all images were acquired using identical acquisition parameters, including zoom and laser power, across genotypes within that experiment.

Hair cells were quantified using the Cell Counter plugin in ImageJ (NIH) based on the presence of MYO7A-positive cells. Counts were performed separately for apical, middle, and basal cochlear turns, defined by their relative position along the cochlear spiral. Data analysis and statistical testing were performed using GraphPad Prism.

Annexin V Staining and Quantification

P15 mice were euthanized and cochleae were rapidly dissected and gently opened to facilitate Annexin V penetration. Cochleae were flushed with Alexa Fluor 488–conjugated Annexin V (Thermo Fisher Scientific) diluted 1:25 (10 μ L Annexin V in 250 μ L L-15 medium, GIBCO, Thermo Fisher Scientific; total volume $\sim$ 260 μ L) and then incubated for 30 min at room temperature. Following incubation, cochleae were washed twice in L-15 medium, fixed in 4% paraformaldehyde, and washed three times in PBS (5 min each). Samples were then dissected and mounted using ProLong Gold antifade mounting medium. Confocal images were acquired using a Leica Stellaris 5 confocal microscope equipped with a 63 $\times$ oil-immersion objective for the *Atp8b1* knockout experiments and a Leica Stellaris 8 confocal microscope for the *Tmem30b* knockout experiments due to instrument availability. For each experiment, all images were acquired using identical acquisition parameters, including zoom and laser power, across genotypes within that experiment. Fluorescence intensity of the Annexin V signal was quantified in ImageJ. ROIs were manually drawn around individual outer hair cells, and mean fluorescence intensity was measured using the ImageJ (NIH) measurement function. Background fluorescence was measured in adjacent regions lacking specific signal and subtracted from all measurements. Analyses were restricted to the apical cochlear turn, as decalcification was not performed prior to Annexin V incubation. For each cochlea, fluorescence intensity was measured in five outer hair cells and averaged to generate a single biological replicate ($n = 5$ –7 cochleae per genotype). Data analysis and statistical testing were performed using GraphPad Prism.

Scanning Electron Microscopy

Mice were sacrificed via decapitation (P5 and younger) or CO₂ asphyxiation followed by cervical dislocation (P6 and older). Cochleae were carefully dissected and placed in a dish containing phosphate-buffered saline (PBS; GIBCO, Thermo Fisher Scientific). Under a dissecting microscope, ossicles were removed, and the cochleae were transferred to conical tubes containing 2.5% glutaraldehyde and 2% paraformaldehyde (PFA; Electron Microscopy Services). Samples were incubated in this fixative solution overnight at 4°C. For adult mice, cochleae were subsequently incubated in 0.5 M ethylenediaminetetraacetic acid (EDTA; Bioland Scientific LLC), pH 8.0, for up to two weeks to facilitate decalcification, depending on bone density. Following decalcification, the cochleae were further dissected to expose the organ of Corti, ensuring the complete removal of the tectorial membrane. Samples were prepared for scanning electron microscopy using the OTOTO procedure (Davies and Forge 1987 [DOI](#)). This process included sequential osmium tetroxide and thiocarbohydrazide treatments, followed by dehydration through a graded ethanol series and critical point drying using hexamethyldisilazane (HMDS; Electron Microscopy Services). Once dried, samples were sputter-coated with a thin layer of platinum to enhance conductivity. Imaging was performed on a JEOL JSM-IT710HR InTouchScope™ Scanning Electron Microscope housed in our microscopy core facility. SEM images were pseudocolored using Affinity (Canva Pty Ltd.)

Hearing Tests in Mice

Prior to Auditory Brainstem Response (ABR) tests mice were anesthetized with an intraperitoneal injection of 20 mg/kg ketamine hydrochloride (Covetrus Inc.) and 10 mg/kg xylazine hydrochloride (Covetrus Inc.). ABR tests were performed in a sound isolating cubicle (Med-Associates, product number: ENV-022MD-WF). To maintain body temperature mice are kept on a Deltaphase isothermal heating pad (Braintree Scientific). ABR recording equipment was purchased from Tucker Davis Technologies (Alachua, FL). Recordings were captured by subdermal needle electrodes (FE-7; Grass Technologies). The noninverting electrode was placed at the vertex of the midline, the inverting electrode over the mastoid of the right ear, and the ground electrode on the upper thigh. Stimulus tones (pure tones) were presented at a rate of 21.1/s through a high-frequency transducer (Tucker Davis Technologies). Responses were filtered at 300–3000 Hz and threshold levels were determined from 1024 stimulus presentations at 8, 11.3, 16, 22.4, and 32 kHz. Stimulus intensity was decreased in 5–10 dB steps until a response waveform could no longer be identified. Stimulus intensity was then increased in 5 dB steps until a waveform could again be

identified. If a waveform could not be identified at the maximum output of the transducer, a value of 5 dB was added to the maximum output as the threshold. DPOAEs of the same group of WT and *Atp8b1* and *Tmem30b* KO mice were recorded. While under anesthesia for ABR testing, DPOAE was recorded using SmartOAE ver. 5.20 (Intelligent Hearing Systems). A range of pure tones from 8 to 32 kHz (16 sweeps) was used to obtain the DPOAE for the right ear. DPOAE recordings were made for f2 frequencies from 8.8 to 35.3 kHz using a paradigm set as follows: L1 = 65 dB, L2 = 55 dB SPL, and f2/f1 = 1.22. Mice were separated by sex when tested for initial ABR and DPOAE experiments. We did not find any significant difference in ABR and DPOAE thresholds based on sex (not shown). ABR analysis for ATP8B1-HA, TMEM30B-HA, *Atp8b1* Ko, and *Tmem30b* Ko mice contain both males and females. DPOAE analysis for *Atp8b1* Ko, and *Tmem30b* Ko mice contain both males and females.

Statistics and Reproducibility

All statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Auditory brainstem response (ABR) thresholds were compared between genotypes using Mann–Whitney U tests. For all statistical analyses, significance was defined as $p < 0.05$. In figures, statistically significant p -values are indicated, whereas non-significant comparisons are not shown. Unless otherwise indicated, error bars represent standard deviation. Power analyses were conducted using preliminary means and standard deviations to determine appropriate sample sizes. Based on an effect size of Cohen's $d = 1.4$ calculated from preliminary ABR data, a one-tailed test with $\alpha = 0.05$ and a desired power of 0.8 (80%) indicated that a sample size of 10 mice per group (20 total) was sufficient to detect statistically significant differences between genotypes (Faul et al. 2007 [DOI](#), 2009 [DOI](#)).

Data Availability

Raw data used to create this manuscript are available on <https://doi.org/10.17605/OSF.IO/GQBMF> [DOI](#). Mouse lines and other reagents are available from the corresponding author upon request.

Data availability

The data supporting the findings of this study are being prepared for public release and will be made available on the Open Science Framework (OSF) upon acceptance of the manuscript.

Acknowledgements

We thank Wenhao Xu and Daniel Grigsby at Genetically Engineered Murine Model Core (University of Virginia, *RRID:SCR_025473* [DOI](#)) for generating mouse models used in this study. We thank Sijie Hao from Advanced Microscopy Facility (University of Virginia, *RRID:SCR_018736* [DOI](#)) for the support of SEM imaging Funding sources: J.B.S is supported by NIH grant R01DC018842 and R01DC021176-03. H.N.D is supported by NIH grant F30DC023139-01

Additional information

Author Contributions

Conceptualization, H.N.D., S.L., J.B. Shin; methodology, H.N.D., S.L., J.B. Shin; experiments, H.N.D., S.L., J.S.I., A.L.R., T.F., B.S., E.K., N.A., J.B. Shin; writing, review & editing, H.N.D. and J.B. Shin; supervision, J.B. Shin.

Funding

Funder	Grant reference number	Author
HHS NIH National Institute on Deafness and Other Communication Disorders (NIDCD)	R01DC018842	Jung-Bum Shin

HHS NIH National Institute on Deafness and Other Communication Disorders (NIDCD)	R01DC021176-03	Jung-Bum Shin
HHS NIH National Institute on Deafness and Other Communication Disorders (NIDCD)	F30DC023139-01	Henry Newkirk De Hoyos

Author ORCID iDs

Henry N De Hoyos:  <https://orcid.org/0000-0002-2083-1261>

Jung-Bum Shin:  <https://orcid.org/0000-0003-3047-0874>

References

- Ahmed Zubair M**, Goodyear Richard, Riazuddin Sheikh Saima, et al. (2006) The Tip-Link Antigen, a Protein Associated with the Transduction Complex of Sensory Hair Cells, Is Protocadherin-15. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* **26**:7022-34 <https://doi.org/10.1523/JNEUROSCI.1163-06.2006> | [PubMed](#)
- Assad John A**, Shepherd Gordon MG, Corey David P (1991) Tip-Link Integrity and Mechanical Transduction in Vertebrate Hair Cells. *Neuron* **7**:985-94 [https://doi.org/10.1016/0896-6273\(91\)90343-X](https://doi.org/10.1016/0896-6273(91)90343-X) | [PubMed](#)
- Ballesteros Angela**, Fenollar-Ferrer Cristina, Swartz Kenton Jon (2018) Structural Relationship between the Putative Hair Cell Mechanotransduction Channel TMC1 and TMEM16 Proteins. *eLife* **7** <https://doi.org/10.7554/eLife.38433> | [PubMed](#)
- Ballesteros Angela**, Swartz Kenton J (2022) Regulation of Membrane Homeostasis by TMC1 Mechano-electrical Transduction Channels Is Essential for Hearing. *Science Advances* **8**:eabm5550 <https://doi.org/10.1126/sciadv.abm5550> | [PubMed](#)
- Beurg Maryline**, Konrad Dakota Elle, Fettiplace Robert (2025) Hair Cell Apoptosis and Deafness in Tmc1 Mutations. *Proceedings of the National Academy of Sciences* **122**:e2425215122 <https://doi.org/10.1073/pnas.2425215122> | [PubMed](#)
- Bhandari Nilam**, Prince Ashutosh, Khan Mariam R, et al. (2025) ATP8B1 Regulates PIP2 Localization and Cleavage of Pyroptotic Executioner Gasdermin D. *Proceedings of the National Academy of Sciences* **122**:e2502798122 <https://doi.org/10.1073/pnas.2502798122> | [PubMed](#)
- Blom Tomas**, Somerharju Pentti, Ikonen Elina (2011) Synthesis and Biosynthetic Trafficking of Membrane Lipids. *Cold Spring Harbor Perspectives in Biology* **3**:a004713 <https://doi.org/10.1101/cshperspect.a004713> | [PubMed](#)
- Bowl Michael R**, Simon Michelle M, Ingham Neil J, et al. (2017) A Large Scale Hearing Loss Screen Reveals an Extensive Unexplored Genetic Landscape for Auditory Dysfunction. *Nature Communications* **8**:886 <https://doi.org/10.1038/s41467-017-00595-4> | [PubMed](#)
- Bretscher Mark S** (1972) Asymmetrical Lipid Bilayer Structure for Biological Membranes. *Nature New Biology* **236**:11-12 <https://doi.org/10.1038/newbio236011a0> | [PubMed](#)
- Bryde Susanne**, Hennrich Hanka, Verhulst Patricia M, Devaux Philippe F, Lenoir Guillaume, Holthuis Joost CM (2010) CDC50 Proteins Are Critical Components of the Human Class-1 P4-ATPase Transport Machinery. *The Journal of Biological Chemistry* **285**:40562-72 <https://doi.org/10.1074/jbc.M110.139543> | [PubMed](#)
- Caputo Margherita**, Gubar Olga, Tóth Petra, Vitale Nicolas, Gasman Stéphane, Ory Stéphane (2025) Lipid Asymmetry and Membrane Trafficking: Transbilayer Distribution of Structural Phospholipids as Regulators of Exocytosis and Endocytosis. *The Journal of Biological Chemistry* **301**:110441 <https://doi.org/10.1016/j.jbc.2025.110441> | [PubMed](#)
- Clark Sarah**, Jeong Hanbin, Posert Rich, Goehring April, Gouaux Eric (2024) The Structure of the Caenorhabditis Elegans TMC-2 Complex Suggests Roles of Lipid-Mediated Subunit Contacts in Mechanosensory Transduction. *Proceedings of the National Academy of Sciences* **121**:e2314096121 <https://doi.org/10.1073/pnas.2314096121> | [PubMed](#)

- Coleman Jonathan A**, Zhu Xianjun, Djajadi Hidayat R, et al. (2014) Phospholipid Flippase ATP8A2 Is Required for Normal Visual and Auditory Function and Photoreceptor and Spiral Ganglion Cell Survival. *Journal of Cell Science* **127**:1138 <https://doi.org/10.1242/jcs.145052> | [PubMed](#)
- Cooper Geoffrey M** (2000) Cell Membranes. In: *The Cell: A Molecular Approach* (2nd Edition) Sinauer Associates. <https://www.ncbi.nlm.nih.gov/books/NBK9928/>
- Corns LF**, Johnson SL, Kros CJ, Marcotti W (2014) Calcium Entry into Stereocilia Drives Adaptation of the Mechanoelectrical Transducer Current of Mammalian Cochlear Hair Cells. *Proceedings of the National Academy of Sciences* **111**:14918-23 <https://doi.org/10.1073/pnas.1409920111> | [PubMed](#)
- Davies Stephen**, Forge Andrew (1987) Preparation of the Mammalian Organ of Corti for Scanning Electron Microscopy. *Journal of Microscopy* **147**:89-101 <https://doi.org/10.1111/j.1365-2818.1987.tb02821.x> | [PubMed](#)
- Dieudonné Thibaud**, Herrera Sara Abad, Laursen Michelle Juknaviciute, et al. (2022) Autoinhibition and Regulation by Phosphoinositides of ATP8B1, a Human Lipid Flippase Associated with Intrahepatic Cholestatic Disorders. *eLife* **11**:e75272 <https://doi.org/10.7554/eLife.75272> | [PubMed](#)
- Dieudonné Thibaud**, Kümmerer Felix, Laursen Michelle Juknaviciute, et al. (2023) Activation and Substrate Specificity of the Human P4-ATPase ATP8B1. *Nature Communications* **14**:7492 <https://doi.org/10.1038/s41467-023-42828-9> | [PubMed](#)
- Doktorova Milka**, Symons Jessica, Levental Ilya (2020) Structural and Functional Consequences of Reversible Lipid Asymmetry in Living Membranes. *Nature Chemical Biology* **16**:1321-30 <https://doi.org/10.1038/s41589-020-00688-0> | [PubMed](#)
- Faul Franz**, Erdfelder Edgar, Buchner Axel, Lang Albert-Georg (2009) Statistical Power Analyses Using G*Power 3.1: Tests for Correlation and Regression Analyses. *Behavior Research Methods* **41**:1149-60 <https://doi.org/10.3758/BRM.41.4.1149> | [PubMed](#)
- Faul Franz**, Erdfelder Edgar, Lang Albert-Georg, Buchner Axel (2007) G*Power 3: A Flexible Statistical Power Analysis Program for the Social, Behavioral, and Biomedical Sciences. *Behavior Research Methods* **39**:175-91 <https://doi.org/10.3758/bf03193146> | [PubMed](#)
- Fettiplace Robert**, Kim Kyunghee X (2014) The Physiology of Mechanoelectrical Transduction Channels in Hearing. *Physiological Reviews* **94**:951-86 <https://doi.org/10.1152/physrev.00038.2013> | [PubMed](#)
- George Shefin S**, Ricci Anthony J (2025) “Auditory Hair Cell Mechanotransduction Channels Dynamically Shape the Mechanical Properties of Their Membrane Environment.”. *Advanced Science* **4**:e08268 <https://doi.org/10.1002/advs.202508268> | [PubMed](#)
- George Shefin S**, Steele Charles R, Ricci Anthony J (2020) Rat Auditory Inner Hair Cell Mechanotransduction and Stereociliary Membrane Diffusivity Are Similarly Modulated by Calcium. *iScience* **23**:101773 <https://doi.org/10.1016/j.isci.2020.101773> | [PubMed](#)
- George Shefin Sam**, Bahloul-Jaziri Amel El, Ricci Anthony J (2023) Tmc Regulates Membrane Viscosity in Mammalian Cochlear Hair Cells. *Biophysical Journal* **122**:91a <https://doi.org/10.1016/j.bpj.2022.11.692>
- Giese Arnaud PJ**, Tang Yi Quan, Sinha Ghanshyam P, et al. (2017) CIB2 Interacts with TMC1 and TMC2 and Is Essential for Mechanotransduction in Auditory Hair Cells. *Nature Communications* **43** <https://doi.org/10.1038/s41467-017-00061-1> | [PubMed](#)
- Gillespie Peter G**, Müller Ulrich (2009) Mechanotransduction by Hair Cells: Models, Molecules, and Mechanisms. *Cell* **139**:33-44 <https://doi.org/10.1016/j.cell.2009.09.010> | [PubMed](#)
- Gómez-Mellado Valentina E**, Chang Jung-Chin, Ho-Mok Kam S, et al. (2022) ATP8B1 Deficiency Results in Elevated Mitochondrial Phosphatidylethanolamine Levels and Increased Mitochondrial Oxidative Phosphorylation in Human Hepatoma Cells. *International Journal of Molecular Sciences* **23**:12344 <https://doi.org/10.3390/ijms232012344> | [PubMed](#)

- Goodyear Richard J**, Gale Jonathan E, Ranatunga Kishani M, Kros Corn   J, Richardson Guy P (2008) Aminoglycoside-Induced Phosphatidylserine Externalization in Sensory Hair Cells Is Regionally Restricted, Rapid, and Reversible. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* **28**:9939-52 <https://doi.org/10.1523/JNEUROSCI.1124-08.2008> | PubMed
- Haeussler Maximilian**, Sch  nig Kai, Eckert H  l  ne, et al. (2016) Evaluation of Off-Target and on-Target Scoring Algorithms and Integration into the Guide RNA Selection Tool CRISPOR. *Genome Biology* **17**:148 <https://doi.org/10.1186/s13059-016-1012-2> | PubMed
- Hossein Amirali**, Deserno Markus (2020) Spontaneous Curvature, Differential Stress, and Bending Modulus of Asymmetric Lipid Membranes. *Biophysical Journal* **118**:624-42 <https://doi.org/10.1016/j.bpj.2019.11.3398> | PubMed
- Hudspeth A James** (1989) How the Ear's Works Work. *Nature* **341**:397-404 <https://doi.org/10.1038/341397a0> | PubMed
- Jeong Hanbin**, Clark Sarah, Goehring April, et al. (2022) Structures of the TMC-1 Complex Illuminate Mechanosensory Transduction. *Nature* **610**:796-803 <https://doi.org/10.1038/s41586-022-05314-8> | PubMed
- Johnson Stuart L**, Beurg Maryline, Marcotti Walter, Fettiplace Robert (2011) Prestin-Driven Cochlear Amplification Is Not Limited by the Outer Hair Cell Membrane Time Constant. *Neuron* **70**:1143-54 <https://doi.org/10.1016/j.neuron.2011.04.024> | PubMed
- Kakuda Shinako**, Suresh Pavana, Li Guangtao, London Erwin (2022) Loss of Plasma Membrane Lipid Asymmetry Can Induce Ordered Domain (Raft) Formation. *Journal of Lipid Research* **63** <https://doi.org/10.1016/j.jlr.2021.100155> | PubMed
- Kawashima Yoshiyuki**, G  l  oc Gwena  lle SG, Kurima Kiyoto, et al. (2011) Mechanotransduction in Mouse Inner Ear Hair Cells Requires Transmembrane Channel-like Genes. *The Journal of Clinical Investigation* **121**:4796-809 <https://doi.org/10.1172/JCI60405> | PubMed
- Kazmierczak Piotr**, Sakaguchi Hirofumi, Tokita Joshua, et al. (2007) Cadherin 23 and Protocadherin 15 Interact to Form Tip-Link Filaments in Sensory Hair Cells. *Nature* **449**:87-91 <https://doi.org/10.1038/nature06091> | PubMed
- Klomp Leo WJ**, Vargas Julie C, van Mil Saskia WC, et al. (2004) Characterization of Mutations in ATP8B1 Associated with Hereditary Cholestasis. *Hepatology* **40**:27-38 <https://doi.org/10.1002/hep.20285> | PubMed
- Kolla Likhitha**, Kelly Michael C, Mann Zoe F, et al. (2020) Characterization of the Development of the Mouse Cochlear Epithelium at the Single Cell Level. *Nature Communications* **11**:1 <https://doi.org/10.1038/s41467-020-16113-y> | PubMed
- Koopman G**, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, van Oers MH (1994) Annexin V for Flow Cytometric Detection of Phosphatidylserine Expression on B Cells Undergoing Apoptosis. *Blood* **84**:1415-20 <https://doi.org/10.1182/blood.v84.5.1415.bloodjournal8451415>
- Kozel PJ**, Friedman RA, Erway LC, et al. (1998) Balance and Hearing Deficits in Mice with a Null Mutation in the Gene Encoding Plasma Membrane Ca²⁺-ATPase Isoform 2. *The Journal of Biological Chemistry* **273**:18693-96 <https://doi.org/10.1074/jbc.273.30.18693> | PubMed
- Le Son C**, Yang Huanghe (2021) Structure-Function of TMEM16 Ion Channels and Lipid Scramblases. *Advances in Experimental Medicine and Biology* **1349**:87-109 https://doi.org/10.1007/978-981-16-4254-8_6 | PubMed
- Lenoir Guillaume**, Williamson Patrick, Puts Cath  leyne F, Holthuis Joost CM (2009) Cdc50p Plays a Vital Role in the ATPase Reaction Cycle of the Putative Aminophospholipid Transporter Drs2p [∗]. *Journal of Biological Chemistry* **284**:17956-67 <https://doi.org/10.1074/jbc.M109.013722> | PubMed
- Li Tao**, Yu Diankun, Oak Hayeon C, et al. (2021) Phospholipid-flippase Chaperone CDC50A Is Required for Synapse Maintenance by Regulating Phosphatidylserine Exposure. *The EMBO Journal* **40**:e107915 <https://doi.org/10.15252/emboj.2021107915> | PubMed

- Li Xinyu, Li Shuzhen, Zhang Weipu, Wang Qi, Zou Wei (2024) Impacts of P4-ATPase Deletion on Membrane Asymmetry and Disease Development. *Cell Biochemistry and Function* **42**:e70004 <https://doi.org/10.1002/cbf.70004> | PubMed
- Liu Huizhan, Chen Lei, Giffen Kimberlee P, et al. (2018) Cell-Specific Transcriptome Analysis Shows That Adult Pillar and Deiters' Cells Express Genes Encoding Machinery for Specializations of Cochlear Hair Cells. *Frontiers in Molecular Neuroscience* **11**:356 <https://doi.org/10.3389/fnmol.2018.00356> | PubMed
- Lorent JH, Levental KR, Ganesan L, et al. (2020) Plasma Membranes Are Asymmetric in Lipid Unsaturation, Packing and Protein Shape. *Nature Chemical Biology* **16**:644-52 <https://doi.org/10.1038/s41589-020-0529-6> | PubMed
- Mark Vincent A, Van der Ronald PJ, Oude Elferink Coen C, Paulusma Vincent A, Van der Mark Ronald PJ, Oude Elferink Coen C, Paulusma (2013) P4 ATPases: Flippases in Health and Disease. *International Journal of Molecular Sciences* **14**:7897-922 <https://doi.org/10.3390/ijms14047897> | PubMed
- Medrano-Soto Arturo, Moreno-Hagelsieb Gabriel, McLaughlin Daniel, Ye Zachary S, Hendargo Kevin J, Saier Milton H (2018) Bioinformatic Characterization of the Anoctamin Superfamily of Ca²⁺-Activated Ion Channels and Lipid Scramblases. *PLOS One* **13**:e0192851 <https://doi.org/10.1371/journal.pone.0192851> | PubMed
- Nagata Shigekazu, Hanayama Rikinari, Kawane Kohki (2010) Autoimmunity and the Clearance of Dead Cells. *Cell* **140**:619-30 <https://doi.org/10.1016/j.cell.2010.02.014> | PubMed
- Nagata Shigekazu, Sakuragi Takaharu, Segawa Katsumori (2020) "Flippase and Scramblase for Phosphatidylserine Exposure.". *Current Opinion in Immunology, Innate immunity* **62**:31-38 <https://doi.org/10.1016/j.coi.2019.11.009> | PubMed
- Orvis Joshua, Gottfried Brian, Kancherla Jayaram, et al. (2021) gEAR: Gene Expression Analysis Resource Portal for Community-Driven, Multi-Omic Data Exploration. *Nature Methods* **18**:8 <https://doi.org/10.1038/s41592-021-01200-9> | PubMed
- Osada Naoki, Hashimoto Katsuyuki, Hirai Momoki, Kusuda Jun (2007) Aberrant Termination of Reproduction-Related TMEM30C Transcripts in the Hominoids. *Gene* **392**:151-56 <https://doi.org/10.1016/j.gene.2006.11.021> | PubMed
- Pan Bifeng, Akyuz Nurunisa, Liu Xiao Ping, et al. (2018) TMC1 Forms the Pore of Mechanosensory Transduction Channels in Vertebrate Inner Ear Hair Cells. *Neuron* **99**:736-753.e6 <https://doi.org/10.1016/j.neuron.2018.07.033> | PubMed
- Pan Bifeng, Géléoc Gwenaëlle S, Asai Yukako, et al. (2013) TMC1 and TMC2 Are Components of the Mechanotransduction Channel in Hair Cells of the Mammalian Inner Ear. *Neuron* **79**:504-15 <https://doi.org/10.1016/j.neuron.2013.06.019> | PubMed
- Pater Justin A, Penney Cindy, O'Rielly Darren D, et al. (2022) Autosomal Dominant Non-Syndromic Hearing Loss Maps to DFNA33 (13q34) and Co-Segregates with Splice and Frameshift Variants in ATP11A, a Phospholipid Flippase Gene. *Human Genetics* **141**:431-44 <https://doi.org/10.1007/s00439-022-02444-x> | PubMed
- Peineau Thibault, Marcovich Irina, von Muhlenbrock Rodriguez Cristobal, et al. (2025) Mammalian TMC1 or 2 Are Necessary for Scramblase Activity in Auditory Hair Cells. *Hearing Research* **460** <https://doi.org/10.1016/j.heares.2025.109229> | PubMed
- Peng Anthony W, Effertz Thomas, Ricci Anthony J (2013) Adaptation of Mammalian Auditory Hair Cell Mechanotransduction Is Independent of Calcium Entry. *Neuron* **80**:960-72 <https://doi.org/10.1016/j.neuron.2013.08.025> | PubMed
- Pickles JO, Comis SD, Osborne MP (1984) Cross-Links between Stereocilia in the Guinea Pig Organ of Corti, and Their Possible Relation to Sensory Transduction. *Hearing Research* **15**:103-12 [https://doi.org/10.1016/0378-5955\(84\)90041-8](https://doi.org/10.1016/0378-5955(84)90041-8) | PubMed
- Reddy A Srinivas, Warshaviak Dora Toledo, Chachisvilis Mirianas (2012) Effect of Membrane Tension on the Physical Properties of DOPC Lipid Bilayer Membrane. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1818**:2271-81 <https://doi.org/10.1016/j.bbamem.2012.05.006> | PubMed

- Riazuddin Saima, Belyantseva Inna A, Giese Arnaud, et al. (2012) Mutations in CIB2, a Calcium and Integrin Binding Protein, Cause Usher Syndrome Type 1J and Nonsyndromic Deafness DFNB48. *Nature Genetics* **44**:1265-71 <https://doi.org/10.1038/ng.2426> | PubMed
- Scheffer Déborah I, Shen Jun, Corey David P, Chen Zheng-Yi (2015) Gene Expression by Mouse Inner Ear Hair Cells during Development. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* **35**:6366-80 <https://doi.org/10.1523/JNEUROSCI.5126-14.2015> | PubMed
- Segawa Katsumori, Kurata Sachiko, Nagata Shigekazu (2016) Human Type IV P-Type ATPases That Work as Plasma Membrane Phospholipid Flippases and Their Regulation by Caspase and Calcium. *The Journal of Biological Chemistry* **291**:762-72 <https://doi.org/10.1074/jbc.M115.690727> | PubMed
- Segawa Katsumori, Kurata Sachiko, Nagata Shigekazu (2018) The CDC50A Extracellular Domain Is Required for Forming a Functional Complex with and Chaperoning Phospholipid Flippases to the Plasma Membrane. *The Journal of Biological Chemistry* **293**:2172-82 <https://doi.org/10.1074/jbc.RA117.000289> | PubMed
- Shi Xiaorui, Gillespie Peter G, Nuttall Alfred L (2007) Apical Phosphatidylserine Externalization in Auditory Hair Cells. *Molecular Membrane Biology* **24**:16-27 <https://doi.org/10.1080/09687860600926883> | PubMed
- Shin Hye-Won, Takatsu Hiroyuki (2020) Phosphatidylserine Exposure in Living Cells. *Critical Reviews in Biochemistry and Molecular Biology* **55**:166-78 <https://doi.org/10.1080/10409238.2020.1758624> | PubMed
- Shin Hye-Won, Takatsu Hiroyuki (2025) Substrates, Regulation, Cellular Functions, and Disease Associations of P4-ATPases. *Communications Biology* **8**:135 <https://doi.org/10.1038/s42003-025-07549-3> | PubMed
- Siemens Jan, Lillo Concepcion, Dumont Rachel A, et al. (2004) Cadherin 23 Is a Component of the Tip Link in Hair-Cell Stereocilia. *Nature* **428**:950-55 <https://doi.org/10.1038/nature02483> | PubMed
- Stapelbroek Janneke M, Peters Theo A, Van Beurden Denis HA, et al. (2009) ATP8B1 Is Essential for Maintaining Normal Hearing. *Proceedings of the National Academy of Sciences of the United States of America* **106**:9709-14 <https://doi.org/10.1073/PNAS.0807919106> | PubMed
- Street VA, McKee-Johnson JW, Fonseca RC, Tempel BL, Noben-Trauth K (1998) Mutations in a Plasma Membrane Ca²⁺-ATPase Gene Cause Deafness in Deafwaddler Mice. *Nature Genetics* **19**:390-94 <https://doi.org/10.1038/1284> | PubMed
- Suzuki Jun, Umeda Masato, Sims Peter J, Nagata Shigekazu (2010) Calcium-Dependent Phospholipid Scrambling by TMEM16F. *Nature* **468**:834-38 <https://doi.org/10.1038/nature09583> | PubMed
- Takatsu Hiroyuki, Tanaka Gaku, Segawa Katsumori, et al. (2014) Phospholipid Flippase Activities and Substrate Specificities of Human Type IV P-Type ATPases Localized to the Plasma Membrane. *The Journal of Biological Chemistry* **289**:33543-56 <https://doi.org/10.1074/jbc.M114.593012> | PubMed
- Velden Lieke M. van der, Wichers Catharina GK, van Breevoort Adriana ED, et al. (2010) Heteromeric Interactions Required for Abundance and Subcellular Localization of Human CDC50 Proteins and Class 1 P4-ATPases. *The Journal of Biological Chemistry* **285**:40088-96 <https://doi.org/10.1074/jbc.M110.139006> | PubMed
- Wang Hong-Yin, Rumin Alissa, Doktorova Milka, et al. (2025) Loss of Lipid Asymmetry Facilitates Plasma Membrane Blebbing by Decreasing Membrane Lipid Packing. *Proceedings of the National Academy of Sciences* **122**:e2417145122 <https://doi.org/10.1073/pnas.2417145122> | PubMed
- Whitlock Jarred M, Chernomordik Leonid V (2021) Flagging Fusion: Phosphatidylserine Signaling in Cell-Cell Fusion. *The Journal of Biological Chemistry* **296**:100411 <https://doi.org/10.1016/j.jbc.2021.100411> | PubMed
- Xing Yazhi, Peng Kun, Yi Qian, et al. (2023) TMEM30A Is Essential for Hair Cell Polarity Maintenance in Postnatal Mouse Cochlea. *Cellular & Molecular Biology Letters* **28**:23 <https://doi.org/10.1186/s11658-023-00437-w> | PubMed

Xiong Wei, Grillet Nicolas, Elledge Heather M, et al. (2012) TMHS Is an Integral Component of the Mechanotransduction Machinery of Cochlear Hair Cells. *Cell* **151**:1283-95

<https://doi.org/10.1016/j.cell.2012.10.041> | PubMed

Zhang G, Gurtu V, Kain SR, Yan G (1997) Early Detection of Apoptosis Using a Fluorescent Conjugate of Annexin V. *BioTechniques* **23**:525-31 <https://doi.org/10.2144/97233pf01> | PubMed

Zhang Jie, Xiao Ping, Zhang Xujia (2009) Phosphatidylserine Externalization in Caveolae Inhibits Ca²⁺ Efflux through Plasma Membrane Ca²⁺-ATPase in ECV304. *Cell Calcium* **45**:177-84

<https://doi.org/10.1016/j.ceca.2008.09.002> | PubMed

Zhao Bo, Wu Zizhen, Grillet Nicolas, et al. (2014) TMIE Is an Essential Component of the Mechanotransduction Machinery of Cochlear Hair Cells. *Neuron* **84**:954-76

<https://doi.org/10.1016/j.neuron.2014.10.041> | PubMed

Peer reviews

Reviewer #1 (Public review):

Sensory hair cells of the inner ear convert mechanical sound vibrations into electrical signals through mechano-electrical transduction (MET). While the protein components of the MET machinery have been studied extensively, much less is known about how the surrounding membrane lipid environment contributes to hair cell function. The recent discovery that TMC1 and TMC2 also function as lipid scramblases has brought renewed attention to the importance of membrane lipid asymmetry and the mechanisms that maintain it in sensory hair cells.

In this study, the authors identify the P4-ATPase ATP8B1 and its partner TMEM30B as key regulators of membrane lipid asymmetry in outer hair cells. Using complementary genetic models, HA-tagged knock-in mice, localization analyses, and functional experiments, they show that ATP8B1-TMEM30B is enriched in stereocilia and the apical membrane of outer hair cells and is required to maintain phosphatidylserine asymmetry, support hair cell survival, and preserve normal hearing. The parallels between the ATP8B1/TMEM30B loss-of-function phenotypes and TMC1 deafness-associated mutants with constitutive scrambling support a model in which ATP8B1-TMEM30B flippase activity maintains membrane lipid asymmetry and homeostasis, whereas constitutive TMC1-mediated phospholipid scrambling disrupts this balance and contributes to membrane instability.

The authors have addressed the points raised during the initial review thoroughly. The revised manuscript includes clearer methodological details, additional physiological characterization, improved presentation and quantification of several datasets, and a more balanced interpretation of the localization and mechanistic findings. These changes improve both the clarity and rigor of the study while leaving its main conclusions unchanged.

As with any study that opens a new area of investigation, important mechanistic questions remain. In particular, it will be interesting to determine how disruption of membrane lipid asymmetry ultimately impairs MET function and triggers hair cell degeneration, how flippase and scramblase activities are coordinated in vivo, and how these pathways are integrated with the broader molecular machinery underlying mechanotransduction. These questions highlight the exciting directions that this study opens for the field.

Overall, this work provides evidence that ATP8B1-TMEM30B is a critical regulator of stereocilia membrane lipid asymmetry and represents an important contribution to our understanding of membrane homeostasis in auditory hair cells. I have no further major concerns and support publication.

<https://doi.org/10.7554/eLife.110466.2.sa2>

Reviewer #2 (Public review):

Summary:

Prior work identified TMEM30B (knockout mice) as well as ATP8B1 (human genetics and mouse model), ATP8A2 (knockout mice), and ATP11A (human genetics) as relevant for hearing. The authors also reasoned that given the recent discovery of TMC1 and TMC2's dual function as mechanotransduction channels of the inner ear and as lipid scramblases, a counterpart flippase should be in the sensory hair-cell stereocilia bundle where mechanotransduction happens. They use CRISPR/CAS to modify the endogenous mouse genes and add an HA tag at the N-terminus of the ATP8B1, ATP8A1, ATP8A2, and ATP11A proteins. Their experiments with these mice unambiguously localized ATP8B1 at the base of outer hair cell stereocilia bundles. Knockout of ATP8B1 results in loss of outer hair cells, deficient auditory function (ABR), and degeneration of outer hair cell stereocilia bundles. Similarly, hair cells from genetically modified mice with endogenous HA-tagged TMEM30B proteins show localization of this protein to outer hair cell stereocilia bundles. TMEM30B knock out mice phenocopy the ATP8B1 knock out model. Interestingly, the authors show that annexin V staining precedes hair cell loss in ATP8B1 and TMEM30B knockout mice and that proper localization of these proteins is lost in mice that lack CIB2, a protein essential for hair cell mechanotransduction.

Strengths:

- (1) Use of knock-in HA-tagged proteins to unambiguously localize ATP8B1 and TMEM30B
- (2) Systematic characterization of auditory function (ABR), hair cell loss, and hair-cell stereocilia bundle morphology.
- (3) Advances our understanding of the role played by lipid homeostasis in auditory function.
- (4) Reports on mouse models that will be helpful to further understand the mechanistic role played by ATP8B1 and TMEM30B in normal hearing and hereditary deafness.

Weaknesses:

- (1) Are the HA tags causing any functional issues? Function and localization of tagged proteins can sometimes be compromised. This is checked for TMEM30B and ATP8B1, but not for ATP8A1, ATP8A2, and ATP11A.
- (2) Following on the point above, is it possible that ATP8B1-HA is well localized, but localization for the other three flippases (ATP8A1-HA, ATP8A2-HA, and ATP11A-HA) is compromised by the tag? Is this potential miss-localization causing any functional phenotypes? I find surprising that there are flippases only in outer hair cells and only formed by ATP8B1. A possible explanation is that the tag is interfering with trafficking. If so, there should be a phenotype (ABRs), although this might be masked by redundancy among these flippases or caused by systemic issues (admittedly difficult to sort out).

<https://doi.org/10.7554/eLife.110466.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Summary of Revisions Performed:

We have clarified the qPCR methodology in the methods section and stated the housekeeping gene GAPDH to address potential misunderstandings.

We have assessed hearing in the generated HA-tagged mouse lines and included an adequately powered ABR measurements analysis in the revised manuscript as a supplemental figure.

We have included powered DPOAE experiments in both ATP8B1 and TMEM30B KO mice to strengthen the findings of the ABRs.

We have clarified the presentation of the z-stack in Figure 1F.

We have elaborated on the analysis for Figure 7B to strengthen comprehension by readers.

We have revised the statement to read: “No IHC stereocilia-enriched P4-ATPases were detected under the conditions examined.”

While we appreciate the suggestion to examine TMEM30B localization on the ATP8B1 KO background, this is not feasible within a reasonable timeframe; we have clarified this limitation in the manuscript.

We have incorporated relevant prior work (e.g., George and Ricci, 2026) demonstrating minimal Annexin V labeling prior to P6 and lack of PS externalization in TMC1/2 double knockout models.

We have clarified that hearing thresholds for TMEM30B-HA and ATP8B1-HA lines were addressed in this study, while additional HA-tagged flippase lines (ATP8A1, ATP8A2, ATP11A) are part of ongoing work to be reported separately.

We have softened statements regarding HA-tag insertion and clarified that, to our knowledge, localization and function are not disrupted, while acknowledging this as a potential limitation.

We have revised the Methods section to clarify differences in fluorescence measurements across experiments.

Public Reviews:

Reviewer #1 (Public review):

Figure 1D.

The authors should clarify how the qPCR data were normalized and specify the reference (housekeeping) genes used. This information is necessary to evaluate the robustness and comparability of the gene expression data.

We thank the reviewer for this comment. qPCR data were normalized to GAPDH as the reference (housekeeping) gene. We have clarified this in the Methods section to ensure transparency and reproducibility.

(2) Figure 1F.

The lack of F-actin staining at the hair cell base raises the possibility that the permeabilization conditions may have limited antibody access to certain membrane regions. This is especially important given that the authors used a gentle permeabilization agent such as saponin to preserve membrane integrity. Because the authors conclude that ATP8B1 and TMEM30B are localized “almost exclusively to OHC bundles and the apical membrane, with minimal staining in the remaining plasma membrane,” (line 128). Including co-labeling with a plasma membrane marker or more comprehensive F-actin visualization of lateral and basal regions would help ensure that the restricted localization is biological rather than technical. In the absence of such

controls, the localization claim may be somewhat overstated and should be tempered accordingly.

We thank the reviewer for this important point. The image shown represents a single z-slice from a larger stack, and the hair cell body lies outside the plane of this section. To clarify this, we revised the accompanying text.

(3) Figure 7B.

Although quantification of ATP8B1-HA intensity at the bundle appears similar between WT and Cib2 KO samples, the representative image suggests that some bundles lack detectable labeling. To better capture phenotype variability, it would be helpful to include an additional quantification showing the fraction or number of bundles with detectable ATP8B1-HA signal in Cib2 KO mice.

We thank the reviewer for this suggestion. We have clarified the quantification of the fraction of hair cell bundles with detectable ATP8B1-HA and TMEM30B-HA signal per field of view. Although the representative images may give the impression that some hair bundles lack staining, this is due to changes in ATP8B1-HA and TMEM30B-HA distribution within the cell body. In all cases, detectable ATP8B1-HA and TMEM30B-HA signal remained present in the hair bundles.

(4) Lines 346-349

The manuscript suggests that IHCs lack stereocilia-enriched P4-ATPases. However, this conclusion is not directly supported by the presented data. The authors should either provide supporting localization or expression data for other P4-ATPases or soften the statement to indicate that no stereocilia-enriched P4-ATPases were detected under the conditions examined.

We agree with the reviewer and have revised this statement to read: “No IHC stereocilia-enriched P4-ATPases were detected under the conditions examined.”

Recommendations:

(5) The authors convincingly demonstrate that TMEM30B loss results in ATP8B1 mislocalization. While not essential to the central conclusions, examining TMEM30B localization in ATP8B1 KO hair cells would clarify whether this interdependence is reciprocal, as described for other P4-ATPase-CDC50 complexes.

While we agree that this experiment would provide valuable information, performing it would require generation of a compound mouse line carrying both the TMEM30B-HA allele and the ATP8B1 knockout allele. This work is beyond the scope of the current revision and cannot be completed within a reasonable timeframe.

(6) Lines 359-374. The discussion of Annexin V labeling is careful and balanced. This paragraph would benefit from referencing other studies that showed minimal Annexin V labeling in healthy P6 organ of Corti, reinforcing that robust PS externalization in the present study is pathological rather than developmental.

We thank the reviewer for this suggestion and have incorporated relevant prior work, including George and Ricci (2026), which demonstrates minimal Annexin V labeling prior to P6 and further supports our interpretation.

(7) Lines 392-399.

The proposed feedback model linking MET activity and ATP8B1-TMEM30B localization is compelling. The discussion could be strengthened by noting that in TMC1/2 double

knockout hair cells, PS externalization is not observed, consistent with the idea that flippase activity becomes critical specifically when scrambling occurs. The mislocalization observed in Cib2 KO hair cells further supports the coupling between TMC-mediated scrambling and flippase-mediated membrane restoration.

We agree and have revised the text to include that TMC1/2 double knockout hair cells do not exhibit phosphatidylserine externalization, supporting the idea that flippase activity becomes critical in the context of scrambling.

Reviewer #2 (Public review):

Weaknesses:

(1) Are the HA tags causing any functional issues? Function and localization of tagged proteins can sometimes be compromised. It would be good to know, for each knock-in model (TMEM30B, ATP8B1, ATP8A1, ATP8A2, and ATP11A), whether the HA-tagged protein is causing any issues with the mice and particularly with hearing (ABRs). Are these mice normal? Can they hear? These data are missing.

We thank the reviewer for raising this important point. In this study, we focus on TMEM30B-HA and ATP8B1-HA mouse lines, while additional HA-tagged flippase lines (ATP8A1, ATP8A2, ATP11A) are part of ongoing work to be reported separately.

Both TMEM30B-HA and ATP8B1-HA mice are viable and exhibit normal breeding and ageing. We have included adequately powered ABR measurements of both TMEM30B-HA and ATP8B1-HA which indicate wild-type-like hearing thresholds.

(2) Following on the point above, is it possible that ATP8B1-HA is well localized, but localization for the other three flippases (ATP8A1-HA, ATP8A2-HA, and ATP11A-HA) is compromised by the tag? Is this potential mislocalization causing any functional phenotypes? (ABRs of point 1). I find it surprising that there are flippases only in outer hair cells and only formed by ATP8B1. A possible explanation is that the tag is interfering with trafficking. If so, there should be a phenotype (ABRs), although this might be masked by redundancy among these flippases or caused by systemic issues (admittedly difficult to sort out). Given that this manuscript will likely become foundational, and that there is evidence that at least two of the other flippases are involved in hearing loss, it would be good to provide more information about the mice and HA-tagged proteins in the other knock-ins (ATP8A1-HA, ATP8A2-HA, and ATP11A-HA). Depending on the data available for the knock-ins, the authors may want to discuss these scenarios and soften the statement indicating that inner-hair cells may lack flippase activity altogether.

We appreciate this concern. To our knowledge, the HA tag does not appear to disrupt localization or function of the tagged proteins. However, we agree that this cannot be fully excluded. We have therefore softened our conclusions about IHC flippases and clarified that additional flippases (ATP8A1, ATP8A2, ATP11A) are under investigation and will be described in a separate study.

(3) Expression of ATP8B1 at P0 (Figure 1D), when there should not be protein in outer hair cells yet seems high. Does this mean that other cells in the cochlea also express ATP8B1? Is this a concern?

We thank the reviewer for this observation. We interpret the elevated ATP8B1 transcript levels at P0 as reflecting transcription that precedes detectable protein accumulation in OHC stereocilia. While expression in other cochlear cell types cannot be excluded, we did not detect ATP8B1-HA immunolabeling outside hair cells in the knock-in model.

(4) Fluorescence scales in Figure 6 B and D and Figure 7 B and D are very different. So are the values for WT. One would expect that the WT would be similar in all cases (at least within the same compartments), given that the methods section indicates that "All images were collected using identical acquisition parameters, including zoom and laser power, across genotypes". If WT shows such variability, how can we compare?

We appreciate the need for clarification. Identical acquisition parameters were maintained within each experiment used for direct comparison (e.g., within a given panel). However, different panels (e.g., Figures 6B vs. 6D) were acquired on different days using different imaging settings.

We have revised the Methods section to explicitly state this and clarify that comparisons are intended only within panels, not across experiments.

Recommendations for the authors:

Reviewer #1 (Recommendations for the authors):

(1) Line 42: When discussing TMC similarity to TMEM16 scramblases, it may be helpful to mention that some TMEM16 family members (TMEM16A and B) function as ion channels, highlighting the dual ion/lipid functionality within the superfamily. The similarity to TMEM63/OSCA ion channels and lipid scramblases could also be noted. The fact that TMC, TMEM16, and TMEM63/OSCA belong to the same superfamily would provide a broader context. I also suggest referencing the work that initially suggested this relationship: (<https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0192851>)

We have included this citation and expanded on this discussion in the revised version.

(2) Line 45: Consider including the recent Cryo-EM structure of CeTMC2 (PNAS, 2023), which provides structural insight into TMC-lipid interactions.

We have included this citation in the revised version.

(3) Line 62: For precision, consider removing "calcium-activated," as caspase-activated scramblases also disrupt membrane asymmetry.

For precision, we have removed "calcium-activated" in the revised version.

(4) Line 71: Clarify whether this refers to "fusion of membranes" or "cell fusion."

We have clarified this statement to mean cell-cell fusion.

(5) Line 85: Consider citing studies showing constitutive PS externalization in TMC1 mutant mouse models linked to deafness.

We have added a citation to show that constitutive PS externalization is linked to deafness (Ballesteros and Swartz, 2022, and Beurg et al. 2025).

(6) Line 93: TMEM30C is not discussed. A brief comment on its expression or relevance in hair cells would provide completeness.

We have added a brief statement regarding TMEM30C and cited prior work describing its expression pattern (Osada et al. 2007).

(7) Figure 1A: Use distinct colors for the P4-ATPase and CDC50 subunit rather than a rainbow scheme to improve clarity.

We have retained the original color scheme in this panel.

(8) *Figures 3C-D and 5C-D: Increase legend symbol size for clarity. Update Y-axis labels to "Number of OHCs/100 μm " and "Number of IHCs/100 μm ." Correct "um" to " μm ."*

We changed the legend to improve the presentation of these panels to be more legible and changed the measurement to μm .

(9) *Figures 3F, 5F, 5H: Add scale bars.*

We have added scale bars to these figures.

(10) *Figure 7: The confocal images (A, C) show the bundle on top and cell body below, but the quantification (B, D) is in the opposite order. Reorganizing the panels for consistent orientation would improve clarity.*

We have reorganized the panels to improve clarity.

(11) *ABR measurements: Please specify the sex of the mice tested or clarify whether both sexes were included.*

We have included both male and female mice in this study as there were no differences in hearing function. We have added this clarification to the methods section under hearing tests.

Reviewer #2 (Recommendations for the authors):

(1) *In Figure 1A, the panels show CDC50. I would either change to TMEM30B or mention in the caption that TMEM30B is also known as CDC50 as labeled in the figure.*

We have changed CDC50 to TMEM30B.

(2) *Figures 1F and 1G are missing scale bars.*

We have added scale bars to these figures.

(3) *Figures 2 C, D, and 5 C, D - difficult to tell what's what in the legend. Perhaps make symbols larger in front of WT P17, KO P17, etc.?*

We changed the legend to improve the presentation of these panels.

(4) *Text under "TMEM30B is required for hearing and OHC maintenance". There is a difference in phenotype between the TMEM30B (Figure 5C) and ATP8B1 (Figure 3C) knockouts that is not discussed, as apical and middle cells seem to be okay. Should this be discussed?*

We appreciate this observation. We have elected not to expand the discussion of these regional differences because apical and middle hair cells also undergo degeneration at later ages (after P30), suggesting that the observed differences primarily reflect the timing of degeneration rather than distinct underlying mechanisms.

(5) *In the discussion text, under "Why do ATP8B1/TMEM30B-deficient OHCs die?", "Tmc1/2 or Cib2" should probably be "TMC1/2 or CIB2" or "Tmc1/2 or Cib2"*

We have changed this to read TMC1/2 or CIB2.

(6) *The methods section states "..., whereas non-significant comparisons are not shown." However, non-significant p values are shown in Figures 7B and D (bottom panels).*

We have removed the nonsignificant comparisons from Fig 7B and D to be consistent with the rest of the paper.

