

Complexes of vertebrate TMC1/2 and CIB2/3 proteins form hair-cell mechanotransduction cation channels

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Abstract

Calcium and integrin-binding protein 2 (CIB2) and CIB3 bind to transmembrane channel-like 1 (TMC1) and TMC2, the pore-forming subunits of the inner-ear mechano-electrical transduction (MET) apparatus. These interactions have been proposed to be functionally relevant across mechanosensory organs and vertebrate species. Here we show that both CIB2 and CIB3 can form heteromeric complexes with TMC1 and TMC2 and are integral for MET function in mouse cochlea and vestibular end organs as well as in zebrafish inner ear and lateral line. Our AlphaFold 2 models suggest that vertebrate CIB proteins can simultaneously interact with at least two cytoplasmic domains of TMC1 and TMC2 as validated using nuclear magnetic resonance spectroscopy of TMC1 fragments interacting with CIB2 and CIB3. Molecular dynamics simulations of TMC1/2 complexes with CIB2/3 predict that TMCs are structurally stabilized by CIB proteins to form cation channels. Overall, our work demonstrates that intact CIB2/3 and TMC1/2 complexes are integral to hair-cell MET function in vertebrate mechanosensory epithelia.

eLife Assessment

This paper, on the role of calcium and integrin-binding protein 2 and 3 in the hair-cell in the mechano-electrical transduction (MET) apparatus, is a mix of confirmatory studies with new and potentially **important** data. Some parts, such as zebrafish studies, the modelling and simulations, are regarded as necessary and **convincing**. Other parts of the paper do not have the same novelty. Both Liang et al. (2021) and Wang et al. (2023) had previously demonstrated a role for CIB2/CIB3 in auditory and vestibular cells in mice. Moreover, there are also data in Riazuddin et al. (2012) paper that demonstrates the importance of CIB2 in zebrafish and *Drosophila*. Breaking the manuscript up to focus on specific aspects of the problem might alleviate the limitations of this multi-faceted study.

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Introduction

The vertebrate senses of hearing and balance depend on the process of mechano-electrical transduction (MET) in inner-ear hair cells. This process is initiated when mechanical forces from sound waves and head movements deflect specialized mechanosensory projections at the apical surface of hair cells, known as stereocilia (reviewed in Fettiplace, 2017 [Fettiplace, 2017](#); Frolenkov et al., 2004 [Frolenkov et al., 2004](#); Gillespie and Müller, 2009 [Gillespie and Müller, 2009](#)). Deflections of stereocilia result in tensioning of the extracellular tip links that convey mechanical force to the MET channels. Mature tip links are heterotetrameric filaments composed of cadherin 23 (CDH23) and protocadherin 15 (PCDH15) (Ahmed et al., 2006 [Ahmed et al., 2006](#); Kazmierczak et al., 2007 [Kazmierczak et al., 2007](#); Siemens et al., 2004 [Siemens et al., 2004](#); Söllner et al., 2004 [Söllner et al., 2004](#); Sotomayor et al., 2012 [Sotomayor et al., 2012](#)). In mammalian auditory hair cells, MET channels are localized at the PCDH15 end of the tip link (Beurg et al., 2009 [Beurg et al., 2009](#)). Significant progress in our understanding of MET has been recently made by identifying the transmembrane channel-like proteins 1 and 2 (TMC1 and TMC2) as the pore-forming subunits of the MET channel (Kawashima et al., 2011 [Kawashima et al., 2011](#); Pan et al., 2018 [Pan et al., 2018](#)). In hair cells, two other transmembrane proteins, LHFPL5 and TMIE, are essential for sustained hearing. LHFPL5 binds to PCDH15 and may regulate force transmission to TMC1 and TMC2 (Qiu et al., 2023 [Qiu et al., 2023](#); Xiong et al., 2012 [Xiong et al., 2012](#)). TMIE also interacts with TMC1/2 and regulates unitary conductance and ion selectivity of the MET channels (Cunningham et al., 2020 [Cunningham et al., 2020](#); Naz et al., 2002 [Naz et al., 2002](#)). Interestingly, MET currents can still be recorded from hair cells lacking LHFPL5 or TMIE, suggesting that neither are essential for channel function (Fettiplace et al., 2022 [Fettiplace et al., 2022](#)), yet all are needed for sustained hair-cell transduction and normal hearing. In zebrafish, *Tmcs*, *Lhfpl5*, *Tmie*, and *Pcdh15* are also essential for sensory transduction, suggesting that these molecules form the core MET complex in all vertebrate hair cells (Chen et al., 2020 [Chen et al., 2020](#); Erickson et al., 2019 [Erickson et al., 2019](#), 2017 [Erickson et al., 2017](#); Ernest et al., 2000 [Ernest et al., 2000](#); Gleason et al., 2009 [Gleason et al., 2009](#); Gopal et al., 2015 [Gopal et al., 2015](#); Maeda et al., 2017 [Maeda et al., 2017](#), 2014 [Maeda et al., 2014](#); Pacentine and Nicolson, 2019; Phillips et al., 2011 [Phillips et al., 2011](#); Seiler et al., 2004 [Seiler et al., 2004](#); Söllner et al., 2004 [Söllner et al., 2004](#)).

We previously showed that calcium (Ca^{2+}) and integrin-binding protein 2 (CIB2), and the closely related CIB3, interact with TMC1/2 (Giese et al., 2017 [Giese et al., 2017](#)). Furthermore, we also demonstrated that CIB2 is essential for MET function in auditory hair cells (Giese et al., 2017 [Giese et al., 2017](#)). Recessive variants of *CIB2* predominantly cause nonsyndromic DFNB48 hearing loss in humans (Booth et al., 2018 [Booth et al., 2018](#); Michel et al., 2017 [Michel et al., 2017](#); Riazuddin et al., 2012; Seco et al., 2016 [Seco et al., 2016](#); Wang et al., 2017 [Wang et al., 2017](#)). Similarly, recessive alleles of *Cib2* cause hearing loss in mice but no vestibular deficits (Giese et al., 2017 [Giese et al., 2017](#); Michel et al., 2017 [Michel et al., 2017](#); Wang et al., 2017 [Wang et al., 2017](#)). In zebrafish, knockdown of *Cib2* diminishes both the acoustic startle response and mechanosensitive responses of lateral-line hair cells (Riazuddin et al., 2012). CIB2 belongs to a family of four closely related proteins (CIB1-4) that have partial

functional redundancy and similar structural domains, with at least two $\text{Ca}^{2+}/\text{Mg}^{2+}$ -binding EF-hand motifs that are highly conserved for CIB2/3 (Huang et al., 2012). Wang and co-authors investigated the function of *Cib1* in cochlear hair cells through generation of a knockout strain and concluded that although *Cib1* is expressed in the mouse cochlear hair cells, loss of CIB1 protein did not affect auditory function (Wang et al., 2017). A recent study demonstrated that while CIB2 is essential for proper localization of TMC1/2 at the tips of auditory hair cell stereocilia, CIB3 can restore MET currents in cochlear hair cells lacking CIB2 (Liang et al., 2021). The same study reported a crystal structure of a TMC1 intracellular linker in complex with CIB3, which suggests that CIB2/3 proteins are likely to be auxiliary subunits of hair cell MET channels (Liang et al., 2021).

Previous work on CIB proteins primarily focused on auditory hair cells, leaving open the question of whether they play a role in vestibular function. This question was addressed in part by another recent study that reported vestibular deficits in mice lacking both CIB2 and CIB3 (Wang et al., 2023). However, additional evidence showing that CIB2/3 can function and interact with TMC1/2 proteins across sensory organs, hair-cell types, and species is still needed. Here we provide further evidence that CIB2 and CIB3 are indeed evolutionarily conserved and functionally redundant components of the vertebrate transduction apparatus necessary for MET channel activity in mouse vestibular hair cells, as well as in zebrafish mechanosensory epithelia. Moreover, based on our structural analyses and simulations, we propose that CIB2 and CIB3 are required for stabilization and channel function of the MET proteins TMC1 and TMC2.

Results

CIB2 and CIB3 are expressed in mouse vestibular hair cells

We and others previously demonstrated that CIB2 is essential for MET in auditory hair cells (Giese et al., 2017; Michel et al., 2017; Wang et al., 2017). In contrast to auditory hair cells, we found that the vestibular hair cells in *Cib2*^{KO/KO} mice apparently have MET. We assessed MET via uptake of FM 1-43 (Figure 1A), a styryl dye that mostly permeates into hair cells through functional MET channels (Meyers et al., 2003), indicating that there may be another CIB protein playing a functionally redundant role. Bulk RNA sequencing revealed that all four members of the *Cib* family are expressed in mouse inner ear tissues (Figure 1B). However, when assessed by single cell RNA sequencing analysis, only *Cib2* and *Cib3* are expressed in immature hair cells in the mouse utricle (Figure 1C), a vestibular sensory organ required to detect linear acceleration (McInturff et al., 2018). Further, we find that the expression of *Cib2* and *Cib3* is maintained in mature type 1 and type 2 utricular hair cells (Figure 1C). We also examined the expression of *Tmc1* and *Tmc2* in utricular hair cells and found that in immature hair cells *Tmc1* is highly expressed, while *Tmc2* expression is low (Figure 1C). *Tmc2* expression increases during development but remains below *Tmc1* levels in both type 1 and type 2 hair cells upon maturation (Figure 1C). CIB2 and CIB3 have been reported to interact with TMC1/2, and CIB2 is essential for auditory hair cell MET (Giese et al., 2017; Liang et al., 2021). The co-expression of *Cib2* and *Cib3* in developing and mature vestibular hair bundles, and the ability of CIB2/3 to interact with TMC1 and TMC2, all make them attractive candidates to regulate MET function in vestibular hair cells.

CIB3 is not required for hearing function in mice

To evaluate whether CIB3 regulates MET function both in auditory and vestibular hair cells, we generated *Cib3* knockout (*Cib3*^{KO}) mice using CRISPR/Cas9 technology (Figure 1D). An indel in exon 4 of *Cib3* was introduced leading to a nonsense mutation (p.Asp54*), thus causing a loss of allele function (Figure 1E-F). We first characterized hearing function in *Cib3*^{KO/KO} and control littermate mice at P16 by measuring auditory-evoked brainstem responses (ABRs). Normal ABR

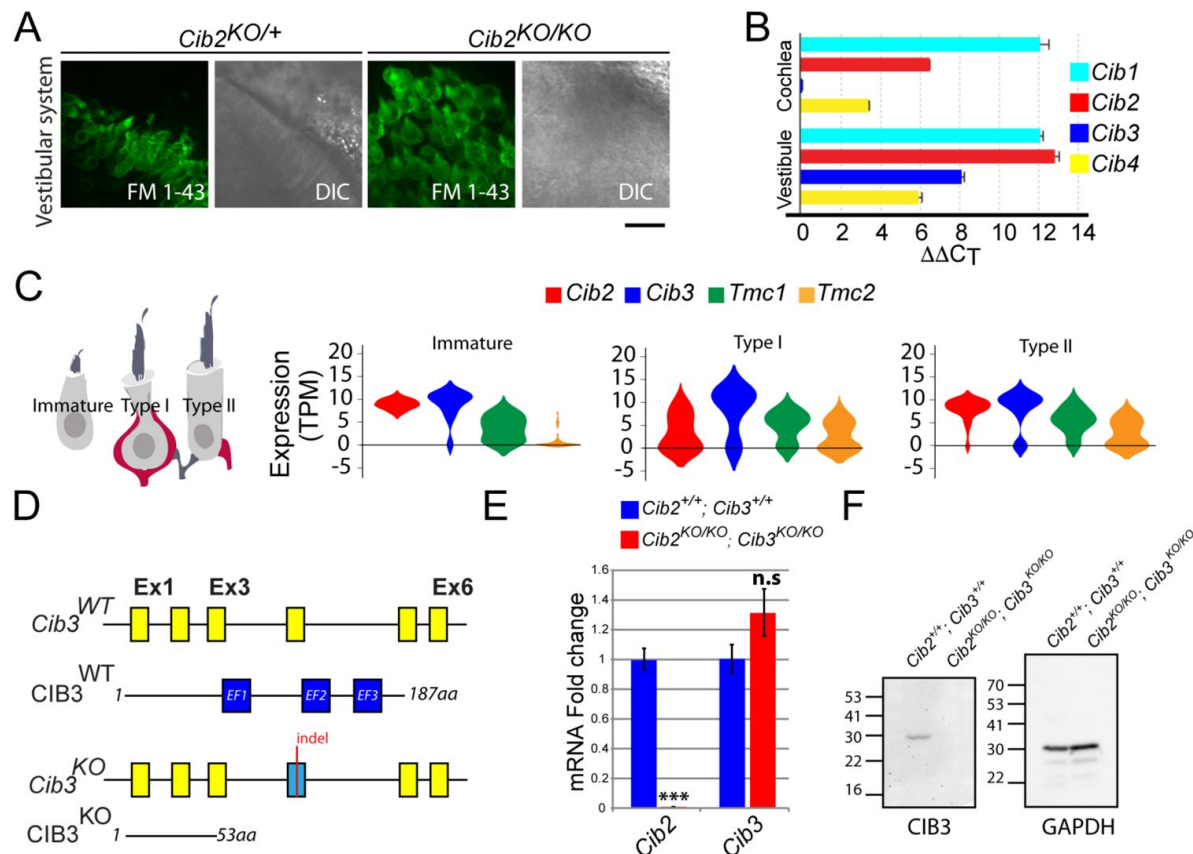


Figure 1.

Expression of *Cib* genes in the mouse inner ear and generation of *Cib3* knockout mice.

(A) Maximum intensity projections of Z-stacks of confocal fluorescent images and corresponding DIC images of *Cib2*^{KO/+} and *Cib2*^{KO/KO} cultured vestibular end organ explants imaged after exposure to 3 μ M of FM 1-43 for 10 s. The samples were dissected at P5 and kept 2 days *in vitro* (P5 + 2div). Scale bar: 20 μ m. (B) Real-time quantitative RT-PCR analysis of mRNA levels in the cochlear and vestibular sensory epithelia at P12 revealed different expression of *Cib1*, *Cib2*, *Cib3*, and *Cib4* in the auditory and vestibular end organs. (C) Violin plots generated by gEAR portal showing the expression levels of *Cib2*, *Cib3*, *Tmc1*, and *Tmc2* by scRNA-seq in immature, type 1, and type 2 utricular vestibular hair cells. TPM: transcript per million. (D) Gene structure of the wild-type and *Cib3*^{KO} alleles. Exons are shown in yellow. Indel in exon 4 leads to a nonsense mutation and a premature stop at the protein level (p. Asp54*). (E) Real-time quantitative RT-PCR analysis using cDNA from brain tissue of *Cib2*;*Cib3* mutant mice was used to analyze the expression of *Cib2* and *Cib3*. Contrary to *Cib2* mRNA, *Cib3* mRNA appears to be stable in the *Cib2*^{KO/KO};*Cib3*^{KO/KO} mice and does not go through nonsense mediated decay process. The relative expression of *Cib2* and *Cib3* members were normalized against *hprt*. Asterisks indicate statistical significance: ***, $p < 0.001$ (Student's *t*-test). n.s.: not significant. Error bars represent SEM. (F) Western Blot analysis on *Cib2*;*Cib3* mouse heart tissue was performed using CIB3 antibody. No CIB3 protein was detected in the *Cib2*^{KO/KO};*Cib3*^{KO/KO} mice, validating our mouse model. GAPDH detection was used as a loading control.

waveforms and thresholds were observed in *Cib3*^{KO/KO} indicating normal hearing. Next, we investigated if there was a functional redundancy between CIB2 and CIB3 in auditory hair cells. For this analysis, the *Cib3*^{KO} allele was introduced into the *Cib2*^{KO} background. Similar to what has been previously reported in *Cib2*^{KO/KO} mice (Giese et al., 2017 [↗](#); Michel et al., 2017 [↗](#); Wang et al., 2017 [↗](#)), the *Cib2:Cib3* double knockout (*Cib2*^{KO/KO};*Cib3*^{KO/KO}) mice were also profoundly deaf. The *Cib2*^{KO/KO};*Cib3*^{KO/KO} mice did not respond to click or strong tone burst stimuli at 100 dB sound pressure level (SPL) (Figure 2A-B [↗](#)). As reported previously for *Cib2*^{KO/KO} mice, we observed that auditory hair cells in *Cib2*^{KO/KO};*Cib3*^{KO/KO} mice did not accumulate FM 1-43 dye (Figure 2C [↗](#)). Also, similar to *Cib2*^{KO/KO} mice (Giese et al., 2017 [↗](#); Michel et al., 2017 [↗](#); Wang et al., 2017 [↗](#)), we observed progressive degeneration of auditory hair cells in *Cib2*^{KO/KO};*Cib3*^{KO/KO} mice (Figure 2D [↗](#)). Taken together with previous studies, our results confirm that CIB2 is indispensable for hearing and MET function in the auditory hair cells.

CIB2 and CIB3 are required for vestibular function in mice

After we assessed auditory function, we turned our analyses toward vestibular function. Similar to *Cib2*^{KO/KO} mice (Giese et al., 2017 [↗](#)), *Cib3*^{KO/KO} mice did not display any obvious indications of vestibular dysfunction, such as circling, hyperactivity or head bobbing, performed well when exploratory behavior was tested, and had normal vestibulo-ocular reflexes (Figure 3 [↗](#)). In contrast, visual observation of the *Cib2*^{KO/KO};*Cib3*^{KO/KO} mice indicated altered exploratory behavior in an open-field test, suggesting an impairment of the vestibular system (Figure 3A-B [↗](#)). To assess the implications of the *Cib2*^{KO/KO};*Cib3*^{KO/KO} mice on vestibular function, we next performed a series of tests, including: the vestibulo-ocular reflex (VOR), performance in a rotating rod, and head movement analysis (Figure 3C-H [↗](#)). The VOR is mediated by a direct three neuron pathway linking the vestibular nerve to the extraocular muscles and produces compensatory eye movements in the direction opposite of the head movement to provide stable gaze (reviewed in Cullen, 2019 [↗](#)). The dynamic response properties (i.e., gain and phase) of the VOR can be precisely quantified in response to rotations applied at different frequencies in darkness (VORd). In response to VORd testing, *Cib2*^{+/+};*Cib3*^{KO/KO} mice generated robust compensatory eye movements (Figure 3C [↗](#), black trace). In contrast, *Cib2*^{KO/KO};*Cib3*^{KO/KO} mice generated negligible eye movements (Figure 3C [↗](#), red trace). Consistent with this observation, quantification of the VORd across frequencies (Figure 3D [↗](#)) revealed gains near zero in the *Cib2*^{KO/KO};*Cib3*^{KO/KO} mice that were significantly reduced ($p < 0.05$) compared to those measured in *Cib2*^{+/+};*Cib3*^{KO/KO} mice. Interestingly, the VORd gains of *Cib2*^{KO/+};*Cib3*^{KO/KO} mice also showed attenuation with a significant decrease at 2 Hz ($p < 0.01$) compared to *Cib2*^{+/+};*Cib3*^{KO/KO} mice. On the other hand, the response phase of VORd eye movements were comparable for *Cib2*^{KO/+};*Cib3*^{KO/KO} and *Cib2*^{+/+};*Cib3*^{KO/KO} mice at all frequencies ($p > 0.05$) (Figure 3D [↗](#)).

As a control for possible changes in visual system and/or ocular motor pathway, we also quantified the optokinetic reflex (OKR), which generates eye movements in responses to a moving visual scene and in light. The OKR normally works together with the VOR to stabilize the visual field on the retina. Both *Cib2*^{+/+};*Cib3*^{KO/KO} and *Cib2*^{KO/+};*Cib3*^{KO/KO} showed robust OKN responses which similarly decreased as a function of frequency (Figure 3E [↗](#); $p > 0.05$). Likewise, OKR gain values in *Cib2*^{KO/KO};*Cib3*^{KO/KO} were comparable with the single exception of a significant gain decrease at 0.2 Hz ($p < 0.05$). Analysis of OKN similarly revealed comparable response phase for all 3 groups of mice at all frequencies ($p > 0.05$) with the exception that the *Cib2*^{KO/KO};*Cib3*^{KO/KO} mice demonstrated less lag at 0.8 and 2 Hz (Figure 3E-F [↗](#)). Overall, the OKR responses of mutant and control mice were generally comparable.

In addition to generating compensatory VOR eye movements to stabilize gaze, the vestibular system also plays a key role in the maintenance of posture and control of balance. Thus, we also tested all 3 groups of mice on several vestibular tasks that assessed balance and postural defects. First, quantification of open-field testing experiments confirmed that mice exhibited circling – a behavior that is commonly associated with loss of vestibular function in mice (Figure 3B [↗](#);

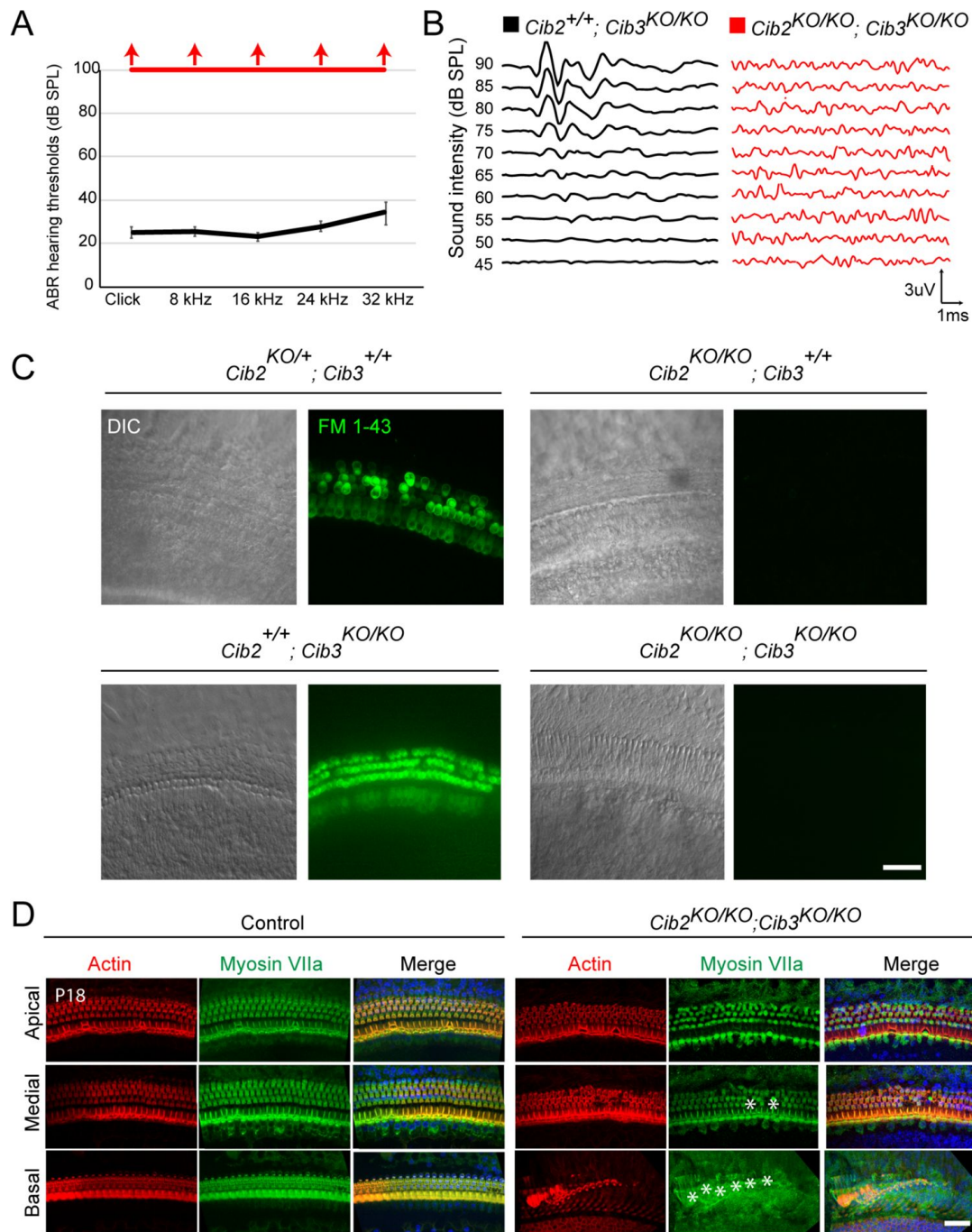


Figure 2.

CIB2/3 double mutant mice have profound hearing loss.

(A) ABR thresholds to broadband clicks and tone-pips with frequencies of 8 kHz, 16 kHz, 24 kHz and 32 kHz in *Cib2*^{+/+}; *Cib3*^{KO/KO} (black; *n* = 5) and *Cib2*^{KO/KO}; *Cib3*^{KO/KO} (red; *n* = 5) mice at P16. The same animals were tested with clicks and tone pips. (B) ABR pure tone traces of P32 *Cib2*^{+/+}; *Cib3*^{KO/KO} (black) and *Cib2*^{KO/KO}; *Cib3*^{KO/KO} (red) mice measured at 32 kHz. Hearing thresholds are in Decibels (dB SPL). (C) Maximum intensity projections of Z-stacks of confocal fluorescent images (right) and corresponding DIC images (left) of *Cib2*^{+/+}; *Cib3*^{KO/KO}, *Cib2*^{KO/KO}; *Cib3*^{+/+}, *Cib2*^{+/+}; *Cib3*^{+/+} and *Cib2*^{KO/KO}; *Cib3*^{KO/KO} cultured organ of Corti explants imaged after exposure to 3 μM of FM 1-43 for 10 s. The samples were dissected at P5 and kept 2 days in vitro (P5 + 2div). Scale bar: 20 μm. (D) Maximum intensity projections of confocal Z-stacks of apical, medial and basal turns of organ of Corti of control and *Cib2*^{KO/KO}; *Cib3*^{KO/KO} mice immunostained with an anti-myosin VIIa antibody (green) and counterstained with phalloidin (red) at P18. Asterisks indicate hair cell loss. Scale bar: 10 μm.

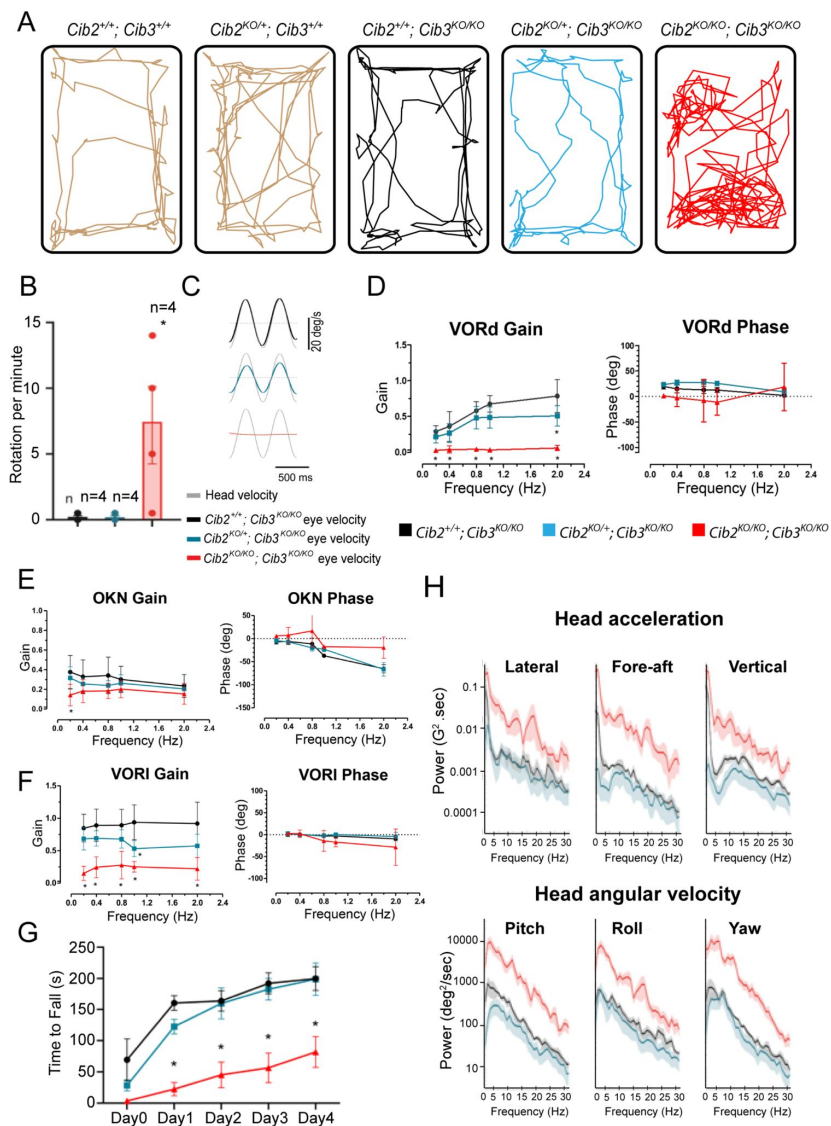


Figure 3.

CIB2/3 double mutant mice have vestibular dysfunction.

(A) Traces showing the open-field exploratory behavior of P60 *Cib2*^{+/+};*Cib3*^{+/+}, *Cib2*^{KO/+};*Cib3*^{+/+}, *Cib2*^{+/+};*Cib3*^{KO/KO}, *Cib2*^{KO/+};*Cib3*^{KO/KO} and *Cib2*^{KO/KO};*Cib3*^{KO/KO} mutant mice. (B) Quantification of the number of rotations in 120 seconds (mean $\pm$ SEM), showing that, unlike *Cib2*^{+/+};*Cib3*^{KO/KO} and *Cib2*^{KO/+};*Cib3*^{KO/KO} mice, *Cib2*^{KO/KO};*Cib3*^{KO/KO} mutant mice display a circling behavior and a vestibular defect (*t*-test) ($n = 4$ for each genotype). (C) Examples of head-velocity (grey) and resultant eye-velocity traces evoked during VORd testing. Note, the eye movement is compensatory, and the trace has been inverted to facilitate comparison with head velocity. (D) VORd Gain and phase (mean $\pm$ SD) plotted as a function of frequency for *Cib2*^{+/+};*Cib3*^{KO/KO} ($n = 4$), *Cib2*^{KO/+};*Cib3*^{KO/KO} ($n = 5$), *Cib2*^{KO/KO};*Cib3*^{KO/KO} ($n = 4$). (E) OKN gain and phase (mean $\pm$ SD) plotted as a function of frequency for *Cib2*^{+/+};*Cib3*^{KO/KO} ($n = 4$), *Cib2*^{KO/+};*Cib3*^{KO/KO} ($n = 5$), *Cib2*^{KO/KO};*Cib3*^{KO/KO} ($n = 4$). (F) VORI gain and phase (mean $\pm$ SD) plotted as a function of frequency for *Cib2*^{+/+};*Cib3*^{KO/KO} ($n = 4$), *Cib2*^{KO/+};*Cib3*^{KO/KO} ($n = 5$), *Cib2*^{KO/KO};*Cib3*^{KO/KO} ($n = 4$). Comparisons made with two-way ANOVA followed by Bonferroni post-hoc test for (A-D); $\ast p < 0.05$. (G) Quantification of the time mice remained on the rotating rod with increasing acceleration (mean $\pm$ SEM). Comparisons made with two-way ANOVA followed by Bonferroni post-hoc test for (F-K); $\ast P < 0.002$. (*Cib2*^{+/+};*Cib3*^{KO/KO} ($n = 6$), *Cib2*^{KO/+};*Cib3*^{KO/KO} ($n = 6$), *Cib2*^{KO/KO};*Cib3*^{KO/KO} ($n = 6$)). (H) Comparison of power spectra density of head movements in translational axes (top) and rotational axes (bottom) between wild type (*Cib2*^{+/+};*Cib3*^{KO/KO}, black), heterozygous mutants (*Cib2*^{KO/+};*Cib3*^{KO/KO}, blue), and homozygous mutants (*Cib2*^{KO/KO};*Cib3*^{KO/KO}, red). The double knockout *Cib2*^{KO/KO};*Cib3*^{KO/KO} exhibit significantly higher power than *Cib2*^{+/+};*Cib3*^{KO/KO} and *Cib2*^{KO/+};*Cib3*^{KO/KO} across all frequencies (0–30 Hz), in all six translational and rotational axes.

$p < 0.002$). We also completed rotarod testing in which performance was quantified by measuring the amount of time the mouse remained on the rotating rod as its acceleration increased. While $Cib2^{+/+};Cib3^{KO/KO}$ and $Cib2^{KO/+};Cib3^{KO/KO}$ mice exhibited similar performance, $Cib2^{KO/KO};Cib3^{KO/KO}$ mice exhibited significantly poorer performance across all time points from day 1 through day 4 (**Figure 3G** [↗](#), $p < 0.002$). Finally, it was also possible to distinguish $Cib2^{KO/KO};Cib3^{KO/KO}$ mice from the other two groups in their home cages since they commonly displayed head bobbing, a trait also generally associated with vestibular dysfunction in mice. To quantify head movement in our mice, we attached a 6D MEMS module consisting of three gyroscopes and three linear accelerometers to the mouse's head implant (see Methods). Analysis of power spectra of head motion revealed significantly higher power for $Cib2^{KO/KO};Cib3^{KO/KO}$ mice in all six dimensions of motion, as compared to $Cib2^{KO/+};Cib3^{KO/KO}$ and $Cib2^{+/+};Cib3^{KO/KO}$ mice (**Figure 3H** [↗](#), compare red vs. blue and black). Further, there were no significant differences in the head movements power spectra of $Cib2^{KO/+};Cib3^{KO/KO}$ and $Cib2^{+/+};Cib3^{KO/KO}$ mice (**Figure 3H** [↗](#), blue vs. black). Taken together our findings indicate that $Cib2^{KO/KO};Cib3^{KO/KO}$ mice show marked deficits in vestibulomotor function when compared to both $Cib2^{KO/+};Cib3^{KO/KO}$ and $Cib2^{+/+};Cib3^{KO/KO}$ mice.

CIB2 and CIB3 are functional subunits of the MET apparatus in mouse vestibular hair cells

To understand the root cause of vestibulomotor function deficits described above for $Cib2^{KO/KO};Cib3^{KO/KO}$ mice, we first analyzed vestibular hair bundle morphology using confocal microscopy. High-resolution confocal imaging did not reveal any obvious vestibular hair cell loss in $Cib2^{KO/KO};Cib3^{KO/KO}$ mice (**Figure 4A** [↗](#)). Next, we visualized MET activity in vestibular hair cells of saccular and utricular end organs of $Cib2^{KO/KO};Cib3^{KO/KO}$ mice using the styryl dye FM 1-43.

These vestibular end organs are divided into two main physically and functionally distinct domains, a central striolar domain and a peripheral extrastriolar domain. At P5, FM 1-43 labeling of $Cib2^{KO/KO};Cib3^{KO/KO}$ tissue failed to label vestibular hair cells in appreciable numbers (**Figure 4B** [↗](#)). Similarly, fixable FM 1-43FX intraperitoneal injections at P60 also showed no dye uptake in $Cib2^{KO/KO};Cib3^{KO/KO}$ mice (**Figure 4C** [↗](#)). $Cib2^{KO/KO}$ and $Cib3^{KO/KO}$ single mutant mice had dye uptake both in striolar and extrastriolar hair cells, although a subtle reduction in FM 1-43FX was observed in extrastriolar hair cells of both single mutants at P60 (**Figure 4C** [↗](#)). Taken together, these results support compulsory but functionally redundant roles for CIB2 and CIB3 in the vestibular hair cell MET complex.

Cib2 and Cib3 function is evolutionary conserved in zebrafish

Numerous studies have shown that the same proteins are required for hair cell MET in zebrafish and mammals (Chen et al., 2020 [↗](#); Erickson et al., 2017 [↗](#); Ernest et al., 2000 [↗](#); Gleason et al., 2009 [↗](#); Gopal et al., 2015 [↗](#); Maeda et al., 2017 [↗](#), 2014 [↗](#); Phillips et al., 2011 [↗](#); Seiler et al., 2004 [↗](#); Söllner et al., 2004 [↗](#)). Unlike mammals, the zebrafish inner ear does not have a cochlea. Instead, zebrafish rely on their saccule for hearing and utricle for balance. Zebrafish also utilize hair cells in a specialized sensory system, the lateral line, to detect local water movement. The hair cells within both the zebrafish inner ear and lateral line share many morphological similarities to mammalian vestibular hair cells (Nicolson, 2017 [↗](#)). Zebrafish scRNAseq data show that both *cib2* and *cib3* are expressed in inner ear while primarily *cib2* is expressed in lateral-line hair cells (Baek et al., 2022 [↗](#); Shi et al., 2023 [↗](#)). Given the zebrafish reliance on the saccule for hearing and the similarities between zebrafish hair cells to mammalian vestibular hair cells, we tested whether Cib2 and/or Cib3 were required for hair cell MET in the zebrafish inner ear or lateral line.

To examine the impact of Cib2 and Cib3 loss in zebrafish, we created mutants using CRISPR-Cas9 based methods to target each homologue (Varshney et al., 2016 [↗](#)). Both mutants lead to a stop codon in the last EF-hand domain (**Figure 5** [↗](#)-figure supplement 1). We first examined auditory function by testing the acoustic startle reflex in *cib2* and *cib3* mutants. For our experiments we

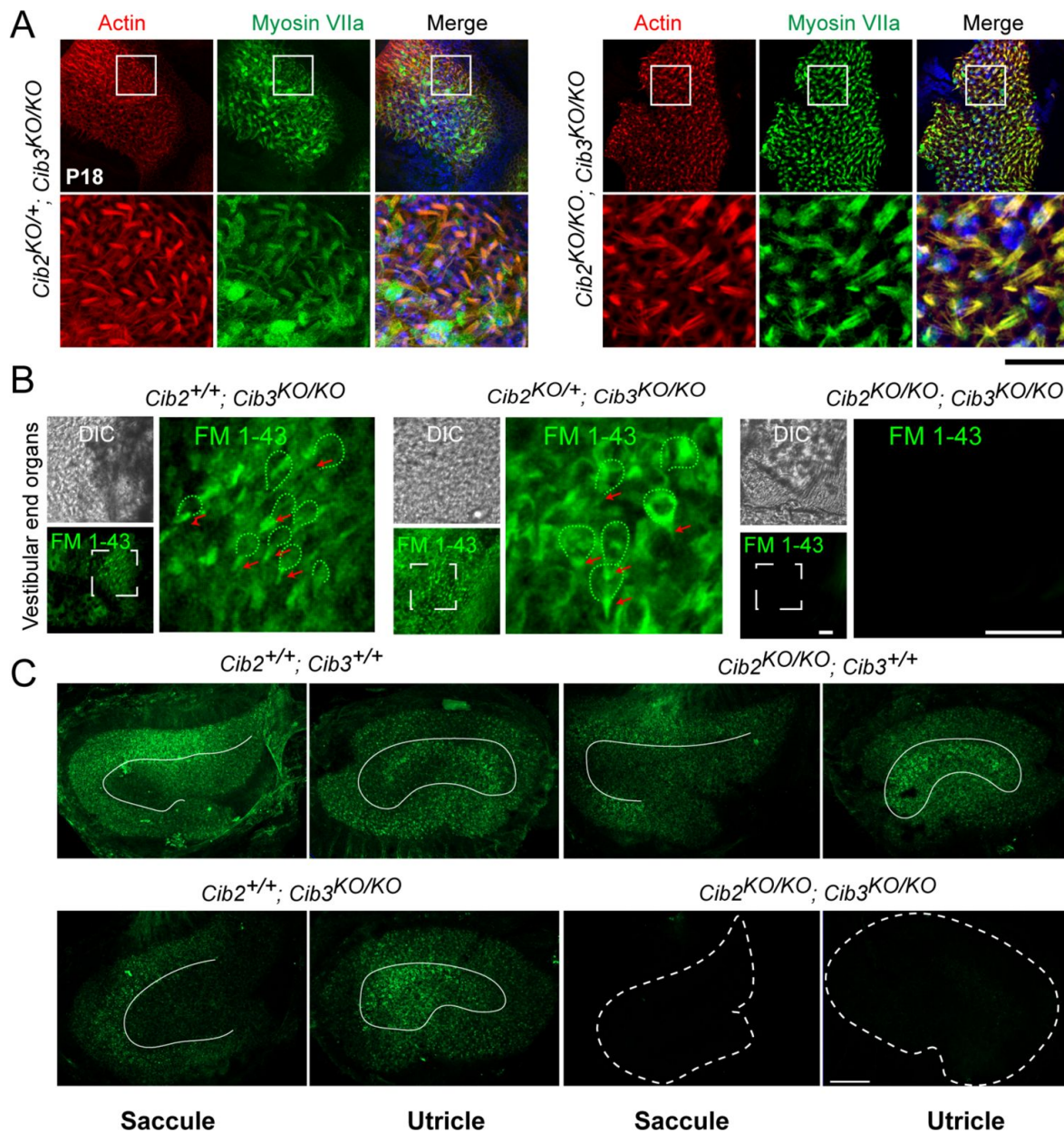


Figure 4.

Vestibular hair cells do not have functional MET channels at rest in CIB2/3 double mutant mice.

(A) Maximum intensity projections of confocal Z-stacks of the vestibular end organs of P18 *Cib2*^{KO/+}; *Cib3*^{KO/KO} and *Cib2*^{KO/KO}; *Cib3*^{KO/KO} mutant mice immunostained with myosin VIIa antibody (green) and counterstained with phalloidin (red) and DAPI (blue). Scale bar = 50 μm. (B) Adult vestibular end organs of *Cib2*^{+/+}; *Cib3*^{KO/KO}, *Cib2*^{KO/+}; *Cib3*^{KO/KO} and *Cib2*^{KO/KO}; *Cib3*^{KO/KO} mutant mice imaged after exposure to 3 μM of FM 1-43 for 10 s. Vestibular hair cell bodies (green dotted lines), and stereocilia bundles (red arrows) are shown. Scale bar: 100 μm. (C) FM 1-43FX (fixable form) labeling of saccules and utricles by IP injection at P60. Obvious reduction in signal in *Cib2*^{KO/KO}; *Cib3*^{KO/KO}, and subtle extrastriolar reduction in *Cib2*^{+/+}; *Cib3*^{KO/KO} mice was observed. Extrastriolar regions are delimited with white lines. Scale bar = 100 μm.

used a Zantiks behavioral system to quantify the probability of generating a startle in response to a vibrational tap stimulus. This response is thought to rely primarily on hair cells in the zebrafish saccule and lateral-line system. We found that, compared to controls, the probability of a startle response was significantly reduced in *cib2* mutants (**Figure 5A**, control: 0.71 ± 0.04 ; *cib2*: 0.28 ± 0.06 , $p = 0.005$). In contrast, *cib3* mutants startle with a similar frequency compared to controls (**Figure 5A**, $p = 0.36$). Interestingly, contrary to mammals, where CIB2 is crucial for hearing, acoustic startle responses were not completely eliminated in *cib2* mutants. One possibility for this partial loss of acoustic startle is that Cib3 may be able to compensate for Cib2 in the zebrafish. Therefore, we examined acoustic startle responses in *cib2;cib3* mutants. In *cib2;cib3* mutant animals we observed a complete absence of startle responses (**Figure 5A**). Furthermore, we observed that *cib2;cib3* mutants but not *cib2* or *cib3* mutants exhibited spontaneous circling behavior, an additional indicator of inner ear dysfunction in zebrafish (Nicolson et al., 1998). Together our behavioral results indicate that Cib2 and Cib3 are both required to ensure normal acoustic startle responses in zebrafish. Furthermore, just loss of Cib2, but not Cib3 can impair auditory function in zebrafish.

Cib2 and Cib3 are required for MET in the zebrafish lateral line

Our results demonstrate that both Cib2 and Cib3 are required for proper auditory function in zebrafish. This raises the question of whether, similar to mice, these proteins are critical for MET function in zebrafish hair cells. Therefore, we next turned our analysis to hair cells in neuromast organs of the lateral line where methods to assess MET are well-documented and straightforward (Lukasz and Kindt, 2018; Seiler et al., 2004).

First, to assess whether there were gross alterations to MET in lateral-line hair cells we examined uptake of the FM 1-43. We found that, compared to controls, the percent of hair cells per neuromast labeling with FM 1-43 was significantly reduced in *cib2* mutants (**Figure 5B,D**, control: 97.6 ± 1.2 ; *cib2*: 37.5 ± 6.9 , $p < 0.0001$). In contrast, a similar percent of hair cells per neuromast labeled with FM 1-43 in *cib3* mutants, compared to controls (**Figure 5B,D**, *cib3*: 95.7 ± 1.9 , $p = 1.0$). We also quantified the average intensity of the FM 1-43 in hair cells (**Figure 5C**). We found that, compared to controls, the average FM 1-43 label was reduced in *cib2* but not *cib3* mutants (**Figure 5C**). In *cib2* mutants we did observe residual hair cells that labeled with FM 1-43. We hypothesized that in lateral-line hair cells, similar to its role in zebrafish auditory function, Cib3 may be able to substitute for Cib2 in MET. To assess whether Cib3 can substitute for Cib2, we examined FM 1-43 labeling in *cib2;cib3* mutants. In *cib2;cib3* mutant animals we observed a complete absence of FM 1-43 labeling in lateral-line hair cells (**Figure 5B,D**). Thus, taken together the results of our FM 1-43 labeling analysis are consistent with a requirement for both Cib2 and Cib3 to ensure normal MET in all lateral-line hair cells.

To obtain a more quantitative assessment of MET we used Ca^{2+} imaging to perform evoked measurements of hair cell function. For this assessment, we used a transgenic line that expresses a membrane-localized GCaMP6s (memGCaMP6s) specifically in hair cells (**Figure 5H-I**). This transgenic line has previously been used to measure Ca^{2+} influx into apical hair bundles while stimulating lateral-line hair cells with a fluid-jet (**Figure 5H-I**) (Lukasz and Kindt, 2018). Using this approach, we were able to stimulate and monitor stimulus-evoked mechanosensitive Ca^{2+} responses in hair bundles within neuromast organs of each genotype (Representative responses: **Figure 5I-L**). We quantified the number of hair bundles per neuromast with mechanosensitive Ca^{2+} responses, and found that compared to controls, significantly fewer cells were mechanosensitive in *cib2* and *cib2;cib3* mutants (**Figure 5**-figure supplement 2A, control: 92.2 ± 2.5 ; *cib2*: 49.9 ± 5.8 , *cib2;cib3*: 19.0 ± 6.6 , $p > 0.0001$). In contrast, in *cib3* mutants, the percent of mechanosensitive cells per neuromast was similar compared to controls (**Figure 5**-figure supplement 2A, *cib3*: 94.5 ± 1.8 , $p = 1.0$). We also quantified the magnitude of the mechanosensitive Ca^{2+} signal in the hair bundles, focusing on only the responsive hair cells for each genotype. This quantification revealed that, while *cib3* mechanosensitive responses were not different compared

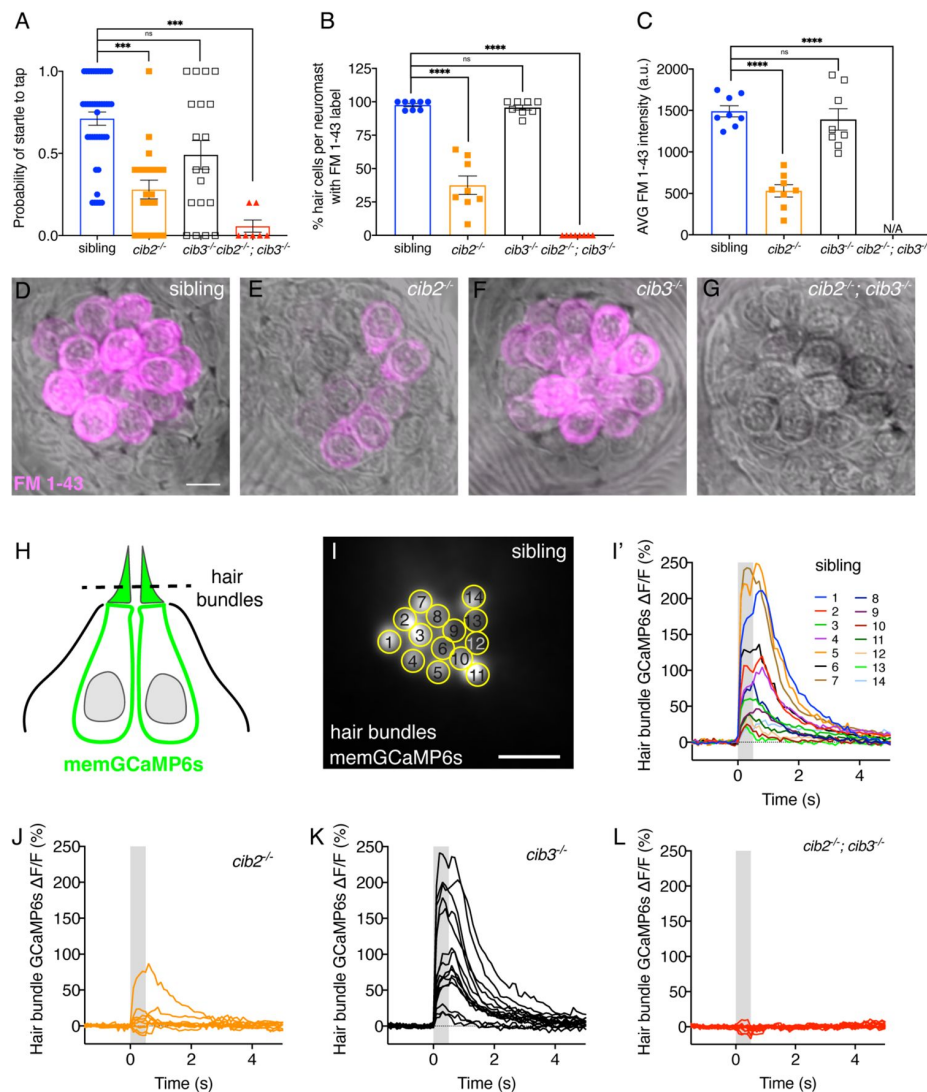


Figure 5.

Both *Cib2* and *Cib3* are required in zebrafish for acoustic startle and MET function.

(**A**) Compared to controls (siblings) *cib3* mutants have a normal acoustic startle response. *Cib2;cib3* double mutants completely lack an acoustic startle response, while *cib2* mutants have a reduced probability to startle compared to controls ($n = 43$ sibling, 20 *cib2*, 19 *cib3* and 7 *cib2;cib3* animals). (**B**) Compared to controls *cib3* mutants have a normal % of hair cells that label with FM 1-43 per neuromast. In *cib2;cib3* mutants no hair cells label with FM 1-43, and in *cib2* mutants a significantly reduced % of hair cells label with FM 1-43 per neuromast. (**C**) The average intensity of FM 1-43 labeling in *cib3* mutant hair cells is similar to controls. In contrast, in *cib2* mutants, hair cells that label with FM 1-43, have a significantly reduced intensity compared to controls ($n = 8$ neuromasts per genotype in B and C). (**D-G**) Representative neuromasts labeled with FM 1-43 for each genotype. FM 1-43 label is overlaid onto laser scanning DIC images. (**H**) Schematic showing the localization of membrane-localized GCaMP6s (memGCaMP6s); the indicator used to measure Ca^{2+} influx into lateral-line hair bundles. The plane used for Ca^{2+} imaging and to determine if a hair cell is mechanosensitive is indicated by the dashed line. (**I-I'**) Representative example of a hair bundle imaging plane (I), along with the resulting Ca^{2+} responses (I') from hair bundles of a control neuromast. The ROIs used to measure Ca^{2+} signals in each hair bundle are indicated in (I). (**J-L**) Representative examples of Ca^{2+} responses from neuromasts in *cib2* (J), *cib3* (K) and *cib2;cib3* mutants (L). FM 1-43 imaging and behavior were performed at 5 dpf. Ca^{2+} imaging was performed at 5 or 6 dpf. A Kruskal-Wallis test was used in A; A one-way ANOVA was used in B and C. The scale bar in D and I = 5 μm . The online version of this article includes source data and the following figure supplement(s) for **Figure 5**. The online version of this article includes source data and the following figure supplement(s) for **Figure 5**. **Figure supplement 1.** Summary of zebrafish *cib2* and *cib3* alleles used in this study. **Figure supplement 2.** Summary of mechanosensitive Ca^{2+} responses in zebrafish.

to controls (**Figure 5** [figure supplement 2B-C](#), GCaMP6s % $\Delta F/F$ control: 70.6 ± 5.9 ; *cib3*: 71.6 ± 5.2 , $p > 1.0$), the magnitude of responses in *cib2* and *cib2;cib3* hair bundles was significantly reduced (**Figure 5** [figure supplement 2B-C](#), GCaMP6s % $\Delta F/F$ control: 70.6 ± 5.9 ; *cib2*: 33.0 ± 5.8 , *cib2;cib3*: 14.7 ± 1.9 , $p < 0.0001$). Overall, our *in vivo* FM 1-43 and Ca^{2+} -imaging analyses revealed that loss of Cib2 alone, but not Cib3 can impair mechanosensory function in the majority of zebrafish lateral-line hair cells. Furthermore, Cib2 and Cib3 are both required to ensure normal MET function in all lateral-line hair cells.

Cib2 is required in specific subsets of hair cells in the zebrafish lateral line and inner ear

Previous work has shown that within the zebrafish inner ear and lateral line there are distinct hair cell subtypes that rely on different complements of the mechanosensitive ion channel Tmc: Tmc1, 2a and 2b. In the primary posterior lateral line (pLL) hair cells are oriented to respond to either anterior or posterior fluid flow. The subset of hair cells that detects posterior flow relies on Tmc2b while the subset that detects anterior flow relies on both Tmc2b and Tmc2a (**Figure 6A** [figure supplement 1](#)) (Chou et al., 2017 [figure supplement 1](#)). Further, work with the crista of the zebrafish inner ear has shown that there are two morphologically distinct types of hair cells: short, teardrop-shaped cells with taller hair bundles and tall, gourd-shaped cells with shorter hair bundles (Smith et al., 2020 [figure supplement 1](#); Zhu et al., 2021 [figure supplement 1](#)). Short cells rely on Tmc2a, while tall cells rely on either Tmc1 or Tmc2b for mechanosensitive function (**Figure 6E** [figure supplement 1](#)) (Smith et al., 2020 [figure supplement 1](#)). Based on this work, we hypothesized that Cib2 or Cib3 could have specific roles in zebrafish hair cell sensory organs linked to hair cell orientation in the lateral line or morphology type in the crista of the inner ear.

Our FM 1-43 labeling in neuromasts revealed no defect in *cib3* mutants, suggesting that Cib2 can compensate for Cib3 in all pLL hair cells. In contrast to *cib3* mutants, only a subset of hair cells per neuromast were labeled with FM 1-43 in *cib2* mutants (**Figure 5E** [figure supplement 1](#)). Previous work has shown that in the pLL of *tmc2b* mutants, a subset of hair cells that respond to anterior flow label with FM 1-43 (Chou et al., 2017 [figure supplement 1](#)). This residual FM 1-43 label is dependent on Tmc2a. Therefore, we examined whether the remaining, FM 1-43 positive hair cells in *cib2* mutants have a specific hair cell orientation. For this analysis, we used a fixable form of FM 1-43, FM 1-43FX to label hair cells. After FM 1-43FX labeling, we fixed the larvae and labeled the apical hair bundles with phalloidin to reveal hair cell orientation. This analysis revealed that in sibling controls, a similar percentage of FM 1-43FX positive hair cells are oriented to respond to anterior and posterior flow (**Figure 6B,B'** [figure supplement 1](#),D). In contrast, when we examined *cib2* mutants, we found that significantly more of the FM 1-43 positive hair cells were oriented to respond to posterior flow (**Figure 6C-D** [figure supplement 1](#)). This indicates that in *cib2* mutants, the residual Cib3 present in pLL hair cells preferentially functions in hair cells that express primarily Tmc2b and are oriented to respond to posterior flow (**Figure 6A** [figure supplement 1](#)). On the other hand, Cib2 can function with hair cells that express Tmc2b (respond to posterior flow) as well as hair cells that express Tmc2b and Tmc2a (respond to anterior flow).

Next, we examined whether different hair-cell subtypes (short versus tall morphology) in the zebrafish inner ear preferentially rely on Cib2 or Cib3 for mechanosensitive function. To examine MET function, we injected FM 4-64 into the zebrafish ear in a transgenic background that labels the hair cell membrane (memGCaMP6s). FM 4-64 is a redshifted version of FM 1-43 and provides a similar readout of MET (Erickson et al., 2017 [figure supplement 1](#); Smith et al., 2020 [figure supplement 1](#)). After injection, we imaged FM 4-64 label in the medial cristae, an inner ear sensory organ that allows for excellent delineation of short and tall cell types (**Figure 6F-F'** [figure supplement 1](#)). We found that similar to our FM 1-43 results in the pLL, no hair cells in the medial cristae were FM 4-64 positive in *cib2;cib3* double mutants (**Figure 6I-I'** [figure supplement 1](#)). In addition, similar to the pLL we observed no defects in FM 4-64 labeling in the crista of *cib3* mutants compared to controls (**Figure 6G-G'** [figure supplement 1](#)). Interestingly we found that short hair cells in the crista of *cib2* mutants failed to label with FM 4-64 (**Figure 6H-H'** [figure supplement 1](#)). Previous work has shown that short hair cells rely on Tmc2a for mechanosensitive function

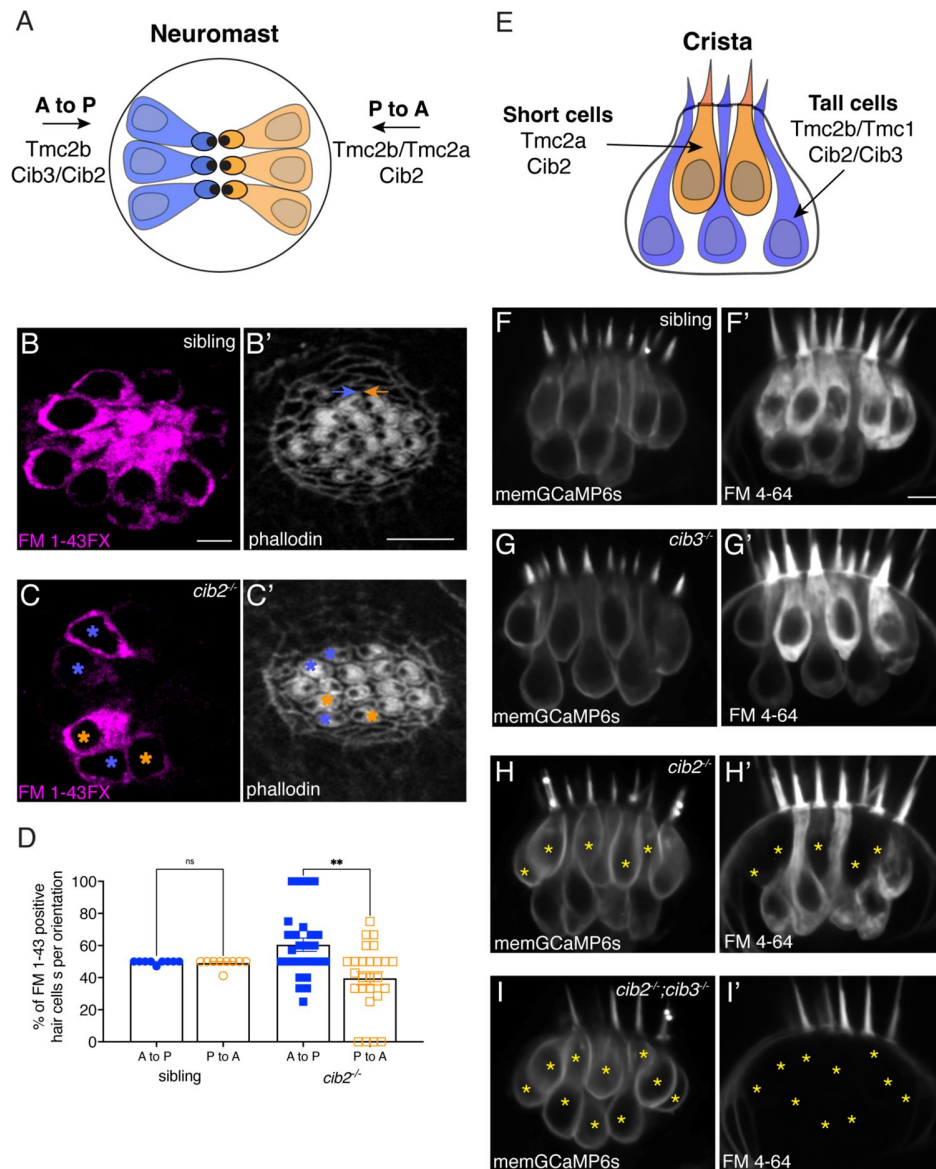


Figure 6.

Cib2 is required in specific subsets of hair cells in the zebrafish pLL and inner ear.

(A) Overview of Tmc requirements in pLL hair cells. Hair cells that sense posterior flow (orange) rely on Tmc2b, while hair cells that sense anterior flow (blue) can rely on Tmc2b and/or Tmc2a for mechanosensitive function. (B-C') FM 1-43FX labeling in pLL neuromasts from sibling control (B) and *cib2* mutants (C). The residual cells in *cib2* mutants rely on Cib3 for mechanosensitive function. Phalloidin label can be used to link FM 1-43FX label to the orientation of pLL hair cells (C,C'; see colored asterisks). In B', orange and blue arrows rest above hair cells that are oriented to respond to anterior and posterior flow, respectively. (D) Quantification reveals that a similar percentage of anterior and posterior responsive pLL hair cells label with FM 1-43FX in sibling controls (posterior flow: 49.7 %, anterior flow: 49.2 %, $n = 9$ neuromasts). In contrast, in *cib2* mutants, a significantly higher percentage of posterior responsive pLL hair cells label with FM 1-43FX in *cib2* mutants (posterior flow: 60.5 %, anterior flow: 39.5 %, $n = 26$ neuromasts). (E) Overview of Tmc and Cib requirements in the hair cells of zebrafish cristae. Short teardrop-shaped cells rely primarily on Tmc2a, while tall gourd-shaped cells rely primarily on Tmc2b and Tmc1. (F-I') Medial cristae for each genotype. MemGCaMP6s labels all hair cells, while FM 4-64 labels hair cells with intact mechanosensitive function. In siblings and *cib3* mutants, both teardrop and gourd cells label with FM 4-64 (F-F', G-G'). In *cib2*;*cib3* double mutants, no hair cells label with FM 4-64 (asterisks) (I-I'). In *cib2* mutants many short cells fail to label with FM 4-64 (asterisks) (H-H'), ($n = 13$ double het sibling, 7 *cib3*, 14 *cib2* and 4 double mutant crista). All images were acquired at 5 dpf. A Kruskal-Wallis test was used in D; $P > 0.01$. The scale bars in B, B', and F' = 5 μ m.

(Smith et al., 2020 [DOI](#)). Overall, our functional assessment of MET based on hair cell subtypes within the zebrafish pLL and inner ear suggests that Cib2 is required for mechanosensitive function in hair cells that express Tmc2a.

CIB proteins simultaneously interact with at least two cytoplasmic domains of TMC1

To gain molecular insight into the role played by CIB proteins in hair-cell MET, we generated AlphaFold 2 (AF2) (Jumper et al., 2021 [DOI](#); Tunyasuvunakool et al., 2021 [DOI](#)) models of the human TMC1 dimer, alone and in complex with human CIB2/3 (**Figure 7A-B** [DOI](#); **Figure 7** [DOI](#)-figure supplement 1; **Figure 7** [DOI](#)-video 1). The AF2 model of TMC1 alone resembles previous TMEM16/OSCA-based models of TMC1 and is also consistent with a recent cryo-EM structure of the worm TMC-1 protein (Ballesteros et al., 2018 [DOI](#); Jeong et al., 2022 [DOI](#); Pan et al., 2018 [DOI](#); Walujkar et al., 2021 [DOI](#)). In these models, TMC1 has ten transmembrane domains ($\alpha 1$ to $\alpha 10$; **Figure 7** [DOI](#)-figure supplements 1-2) and a putative pore is formed by transmembrane helices $\alpha 4$, $\alpha 5$, $\alpha 6$, and $\alpha 7$ at the periphery of each monomer (Pan et al., 2018 [DOI](#); Walujkar et al., 2021 [DOI](#)), away from the dimeric interface involving $\alpha 10$. Our new models feature an additional amphipathic helix, which we denote $\alpha 0$, extending almost parallel to the expected plane of the membrane bilayer without crossing towards the extracellular side (as observed for a mostly hydrophobic $\alpha 0$ in OSCA channels and labeled as H3 in the worm TMC-1 structure) (Jeong et al., 2022 [DOI](#); Jojoa-Cruz et al., 2018 [DOI](#); Liu et al., 2018 [DOI](#); M. Zhang et al., 2018 [DOI](#)). Extracellular and intracellular domains linking transmembrane helices, which were either omitted or poorly predicted in previous work (Ballesteros et al., 2018 [DOI](#); Pan et al., 2018 [DOI](#); Walujkar et al., 2021 [DOI](#)), have well-defined secondary structure and a high confidence score (pLDDT > 70 for most regions; **Figure 7** [DOI](#)-figure supplement 1A). The intracellular domain linking helices $\alpha 2$ and $\alpha 3$, denoted here as IL1, adopts a helix-loop-helix with the two helices running parallel to each other and differing in length (**Figure 7** [DOI](#)-figure supplements 1-5). This is the same fold observed in its crystal structure in complex with CIB3 (Liang et al., 2021 [DOI](#)), which validated the modeling approach.

Interestingly, AF2 models of TMC1 in complex with CIB2 suggest that the N-terminus (NT) of TMC1 also interacts with CIB2 (**Figure 7A-B** [DOI](#)). In our model, CIB2 is clamped between TMC1-NT and TMC1-IL1 while the first helix of TMC1-IL1 runs parallel to the membrane plane interacting with the base of $\alpha 1$ and the intracellular linkers between $\alpha 4$ and $\alpha 5$, $\alpha 6$ and $\alpha 7$, and $\alpha 8$ and $\alpha 9$. Notably, the predicted interaction between TMC1-NT and CIB2 is consistent with previous biochemical data (Giese et al., 2017 [DOI](#)), while the overall model resolves seemingly contradictory results indicating that either the NT or IL1 of TMC1 were responsible for interactions with CIB proteins (Giese et al., 2017 [DOI](#); Liang et al., 2021 [DOI](#)). Additional AF2 models of TMC1 with CIB3 and of TMC2 with CIB2/3 (**Figure 7** [DOI](#)-figure supplement 1) predicted the same architecture for the TMC1/2 + CIB2/3 complexes where CIB proteins are clamped between the IL1 and NT domains of TMCs. Predictions for fish complexes also show the same general architecture (**Figure 7** [DOI](#)-figure supplement 1A).

Nuclear magnetic resonance (NMR) experiments were used to validate the clamp model for *Homo sapiens* (hs) TMC + CIB interactions. We produced uniformly ^{15}N labeled hs CIB2 (22.7 kDa) and CIB3 (22.9 kDa) proteins ([U- ^{15}N]-hs CIB2/3), alone or co-refolded with unlabeled hs TMC1-IL1 (6.3 kDa). In parallel, we produced an unlabeled soluble TMC1-NT fragment (6.9 kDa) and used these CIB and TMC1 samples to test protein-protein interactions by monitoring their effects on 2D ^1H - ^{15}N -correlation spectra of the labeled CIB2 and CIB3 proteins. All protein fragments were purified using size exclusion chromatography (SEC) (**Figure 7** [DOI](#)-figure supplement 6), revealing a single monodisperse peak for [U- ^{15}N]-hs CIB2 (69.2 mL elution volume in S75 16/600 column) and a slightly displaced peak for [U- ^{15}N]-hs CIB2 + hs TMC1-IL1 (68.5 mL). In contrast, SEC experiments revealed two peaks for [U- ^{15}N]-hs CIB3 alone (potential dimer at 62.8 mL and potential monomer at 73.1 mL) and a single peak for [U- ^{15}N]-hs CIB3 + hs TMC1-IL1 (71.3 mL). ^1H - ^{15}N -correlation spectra of [U- ^{15}N]-hs CIB2 alone were consistent with prior data indicating that many, but not all

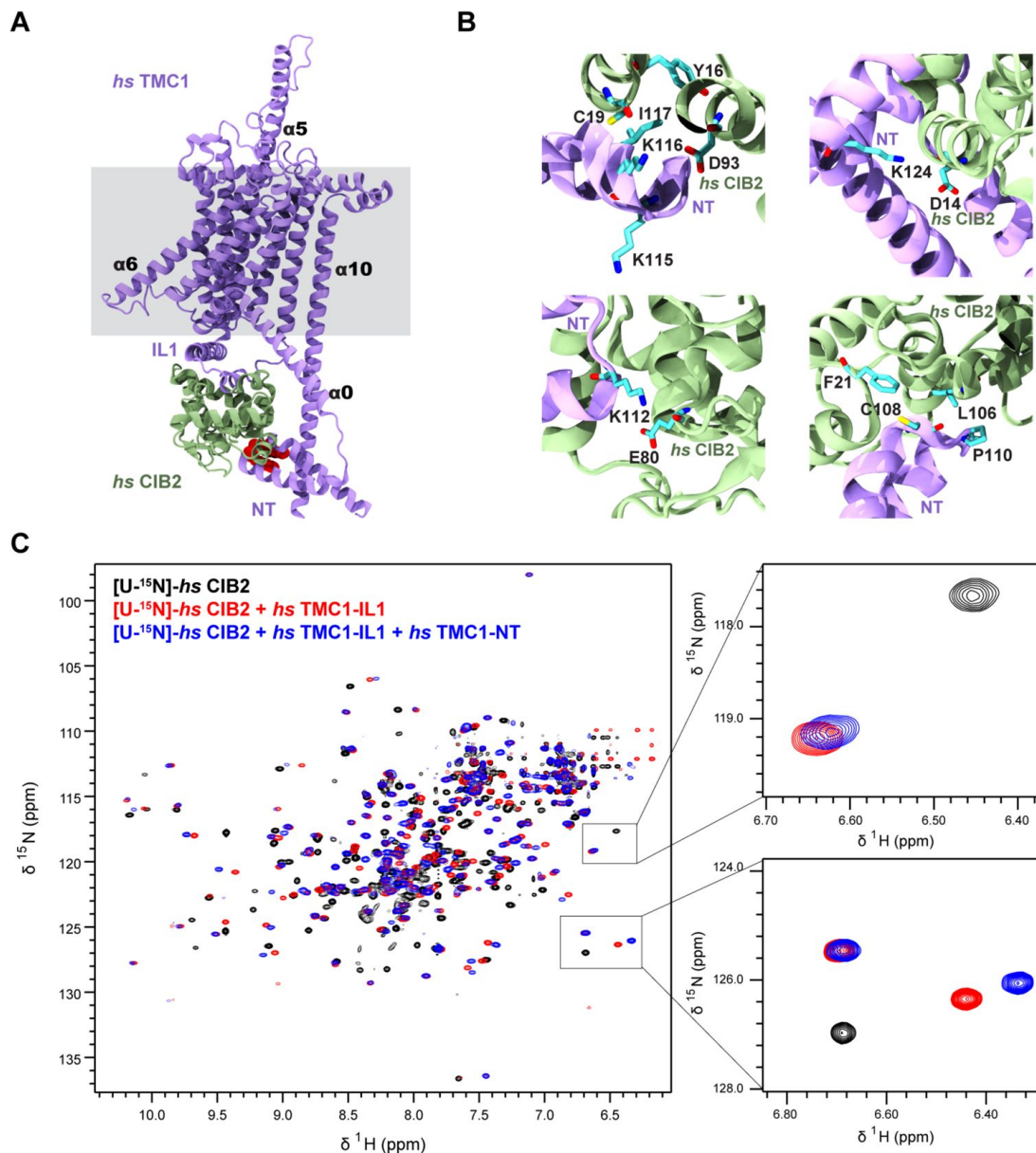


Figure 7.

AF2 predictions and NMR data support a clamp-like model for the human TMC1 and CIB2 complex.

(A) AF2 model of *hs* TMC1 in complex with *hs* CIB2. Side view of monomeric *hs* TMC1 (light purple) in complex with *hs* CIB2 (light sea green). N-terminal residues of TMC1 known to interact with CIB2 are in red. (B) Top left panel shows residues at the *hs* TMC1-NT fragment (115-117) shown to weaken interaction with CIB2 upon mutation (Liang et al., 2021). Additional panels show details of contacts between TMC1 and CIB2 with formation of K124:D14 and K112:E80 salt bridges, as well as hydrophobic interactions. (C) Overlay of ^1H - ^{15}N TROSY-HSQC spectra of *hs* [U- ^{15}N]-CIB2 (black) alone and bound to either only *hs* TMC1-IL (red) or both *hs* TMC1-IL and *hs* TMC1-NT (blue). NMR data were obtained in the presence of 3 mM CaCl_2 . These data are also shown in Figure 7-figure supplement 7. The online version of this article includes source data and the following figure supplement(s) for Figure 7: **Figure supplement 1.** Overview of TMC1/2 and CIB2/3 AF2 models. **Figure supplement 2.** TMC sequence alignment. **Figure supplement 3.** CIB2 sequence alignment. **Figure supplement 4.** CIB3 sequence alignment. **Figure supplement 5.** CIB sequence alignment. **Figure supplement 6.** SEC of *hs* CIB2 and *hs* CIB3 either refolded alone or co-refolded with *hs* TMC1-IL1, and *hs* TMC1-NT. **Figure supplement 7.** ^1H - ^{15}N TROSY-HSQC spectra. **Figure 7-video 1.** Overview of *hs* TMC1 (light and dark purple) in complex with *hs* CIB2 (light sea green)- Ca^{2+} (green spheres). K^+ ions (pink) were shown to be permeating the pore region between $\alpha 4$ and $\alpha 6$. Water molecules, lipids, and protein side chains were omitted for clarity.

potential peaks are resolved (**Figure 7C**, **Figure 7**-figure supplement 7A) (Huang et al., 2012). Spectra for the [U-¹⁵N]-*hs* CIB2 + *hs* TMC1-IL1 complex exhibit widespread perturbations, consistent with extensive remodeling of the CIB2 structure.

Moreover, addition of *hs* TMC1-NT induced additional chemical shift perturbations in many, but not all peaks, demonstrating that the TMC1-IL1 and TMC1-NT ligands bind simultaneously to [U-¹⁵N]-*hs* CIB2 (**Figure 7C**, **Figure 7**-figure supplement 7B). We observed similar results when comparing spectra for [U-¹⁵N]-*hs* CIB3 alone, co-refolded with *hs* TMC1-IL1, and upon addition of *hs* TMC1-NT (**Figure 7**-figure supplement 7C-D). A control experiment with the co-refolded mix of *hs* CIB3 + *hs* TMC1-IL1 and [U-¹⁵N]-*hs* CIB2 (**Figure 7**-figure supplement 7E) revealed no change in chemical shifts indicating that direct heteromeric interactions between CIB2 and CIB3 are unlikely. Thus, taken together, the NMR data strongly support a model in which human CIB2/3 proteins are clamped between TMC1-IL1 and TMC1-NT as suggested by AF2 and consistent with prior biochemical data revealing interactions with either TMC1-NT or TMC1-IL1 (Giese et al., 2017; Liang et al., 2021).

Simulations reveal CIB proteins stabilize TMC1 and TMC2

Our dimeric AF2 models of TMC1/2 alone and in complex with CIB2/3 are consistent with previous experimental data that validated the location of the putative pore in similar TMEM16/OSCA-based homology models of TMC1 (Pan et al., 2018). Our models are also consistent with our NMR data supporting the clamp model for the TMC + CIB complex (**Figure 7C**). To improve the AF2 predictions and to gain mechanistic insights into TMC and CIB function we turned to all-atom equilibrium molecular dynamics (MD) simulations (Karplus and Petsko, 1990). We built ten different models that included dimeric TMC1 or TMC2 alone, in complex with CIB2 or CIB3 with and without bound Ca²⁺. These models were embedded in either pure POPC or stereocilia-like mixed composition bilayers and solvated (150 mM KCl) to generate a total of twenty systems that were equilibrated for 100 ns each (**Figure 8A**, **Figure 8**-table supplement 1).

Equilibrium simulations of TMC1 and TMC2 alone revealed stable conformations for the transmembrane domains of these proteins, but a highly mobile NT domain (**Figure 8B**, **Figure 8**-figure supplement 1). In simulations where CIB2 or CIB3 were present, with or without bound Ca²⁺, we observed more stable conformations of N-termini along with stable salt bridges (**Figure 8C**). Buried surface area (BSA) for the TMC + CIB complexes (**Figure 8**-figure supplement 2A,C,E, **Figure 8**-table supplement 2) revealed a possible trend in which TMC1 and TMC2 have more contacts with CIB3 than with CIB2. While additional sampling and longer simulations might be needed for a robust prediction, these results suggest variable affinities for TMC + CIB protein complexes that might be modulated by Ca²⁺ (**Figure 8**-figure supplement 2F).

Additional analysis of the putative pore in our simulations of TMC1 and TMC2 revealed hydration as observed in previous simulations of TMEM16/OSCA-based TMC1 models, as well as movement of lipids that lined up the predicted permeation pathway for cations suggesting a membrane-based gating mechanism (Pan et al., 2018; Sukharev and Corey, 2004; Walujkar et al., 2021). Longer, Anton 2, 960-ns long equilibrium trajectories of TMC1 with CIB2 and bound Ca²⁺ in a mixed bilayer and TMC1 with CIB2 and (un)bound Ca²⁺ in a POPC bilayer revealed how parts of the amphipathic α0 helices spontaneously partitioned at the membrane interface with their hydrophobic sides pointing toward the lipid bilayer core (**Figure 8D**). The N-terminal cytoplasmic domain of LHFPL5 has been shown to interact with this TMC1 amphipathic helix, which might be essential for channel function in mice (Qiu et al., 2023). The BSA for the dimeric TMC1 + CIB2 complex in these longer simulations (**Figure 8**-figure supplement 2E) decreased as contacts between TMC1-α0 and CIB2 were lost for one of the monomers in the mixed bilayer system. In the monomer in which contacts were maintained, a single cholesterol was situated in the middle of the putative pore. Similarly, TMC1-α0 and CIB2 contacts were lost for both

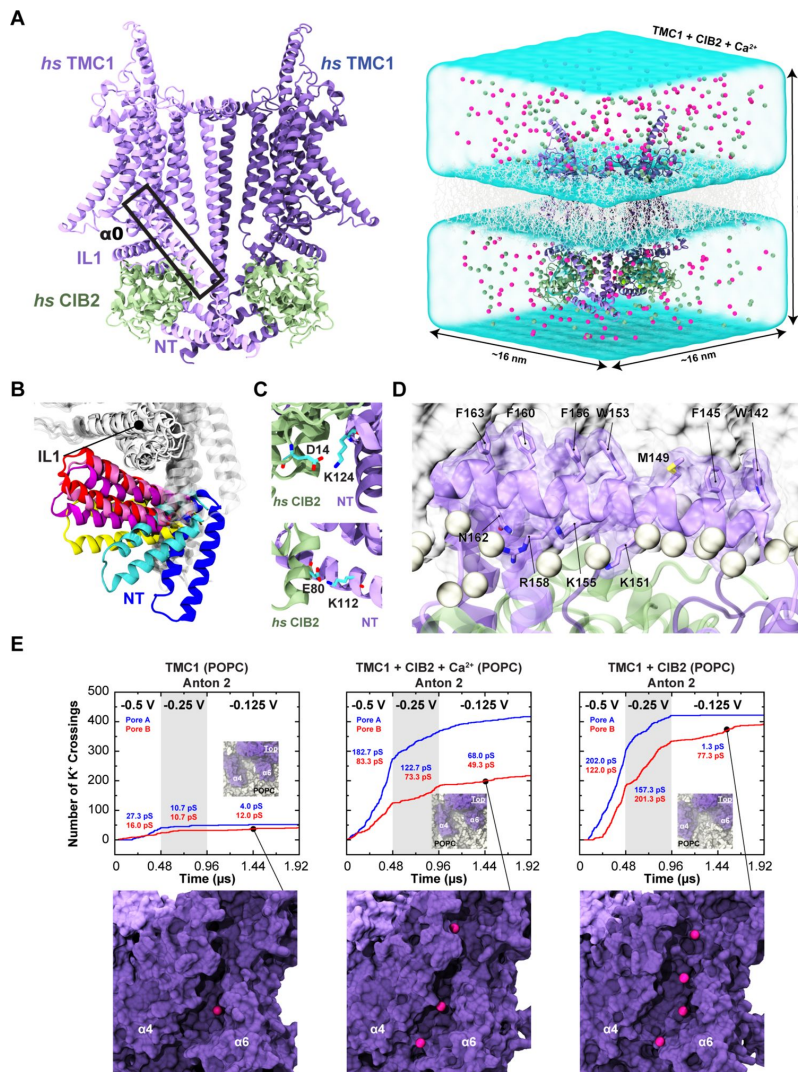


Figure 8.

Simulations of AF2 predictions show that human TMC1 and CIB2 complexes form cation channels.

(A) *Left:* AF2 model of the dimeric *hs* TMC1 + *hs* CIB2 complex. *Right:* A complete simulation system of *hs* TMC1 in complex with *hs* CIB2- Ca^{2+} is shown in surface representation. *hs* TMC1 is shown in light (monomer A) and dark (monomer B) purple, *hs* CIB2 in light sea green, water in transparent light blue, lipid membrane in gray lines, and K^+ and Cl^- ions are shown as pink and green spheres, respectively. (B) The *hs* TMC1-NT domain explores diverse conformational space in the absence of CIB during equilibrium simulations (S3a). (C) Salt bridges K124:D14 and K112:E80 remain throughout equilibrium simulations. (D) Snapshot of amphipathic helix $\alpha 0$ inserting into the lipid bilayer (S4c). (E) Number of K^+ crossings as a function of time for pores A and B of *hs* TMC1, *hs* TMC1 + *hs* CIB2 + Ca^{2+} , and *hs* TMC1 + *hs* CIB2 in a POPC bilayer at -0.5 V, -0.25 V, and -0.125 V. Insets show top views of pore region with TMC1 (dark purple) and lipids (light gray) in surface representation (protein and lipids partially excluded for clarity). Representative images of K^+ permeating the pore region between $\alpha 4$ and $\alpha 6$ of *hs* TMC1 are shown with protein in surface representation and ions as pink spheres. Conductance values calculated for K^+ crossings. The online version of this article includes source data and the following figure supplement(s) for **Figure 8**. **Figure supplement 1.** Stability of TMC/CIB complexes, monomers, and subdomains. **Figure supplement 2.** BSA in *hs* TMC1/2 and *hs* CIB2/3 complexes. **Figure supplement 3.** Number of K^+ crossings as a function of time for pores A and B of *hs* TMC1 and *hs* TMC2. **Figure supplement 4.** Conduction events in long timescale simulations and summary of predicted conductance values. **Figure supplement 5.** Possible combinations of TMC1, TMC2, CIB2, and CIB3 complexes. **Figure 8**–**video 1.** Side view of the *hs* TMC1 channel pore during a trajectory at -0.5 V (monomer B). K^+ (pink) are seen permeating the pore from one side (S1b); *hs* TMC1 (monomer B) is shown in dark purple and *hs* CIB2 is shown in light sea green. Water molecules, lipids, *hs* TMC1 (monomer A), and protein side chains were omitted for clarity. **Figure 8**–**table supplement 1.** Summary of simulations. **Figure 8**–**table supplement 2.** Ion conduction.

monomers in the POPC bilayer systems. Regardless, in all Anton 2 equilibrium trajectories contacts between CIB2 and the TMC1-IL1 and -NT remained, thus confirming the stability of the complex over a microsecond timescale.

TMC1 and TMC2 complexes with CIB2 and CIB3 are predicted to be cation channels

To further explore the properties of our models of TMC/CIB complexes we took equilibrated TMC1 systems and applied a -0.5 V transmembrane voltage while monitoring ion permeation through the putative pore during 100-ns long trajectories (**Figure 8** [figure supplements 3-4](#), **Figure 8** [table supplement 2](#), **Figure 8** [video 2](#)). In systems with pure POPC bilayers we observed permeation of K^+ in either one or both pores of the TMC1 dimer, with or without CIB2 or CIB3 and with or without bound Ca^{2+} , despite the presence of Cl^- (150 mM KCl). Similar results were obtained for four TMC1 systems simulated at -0.25 V. Variability of the conductance state was attributed to partial blockage by lipids and poor sampling, but in all cases in which a clear conductive state was monitored we observed almost exclusive passage of K^+ with conductance values that reached up to ~ 138 pS for a single pore. While simulations tend to overestimate bulk KCl conductivity and in some cases predictions need to be scaled down (by a factor of ~ 0.6) (Walujkar et al., 2021 [figure supplement 2](#)), the conductance values monitored here are consistent with previous simulations and with the expected conductance of the inner-ear transduction channel (Beurg et al., 2021 [figure supplement 2](#); Walujkar et al., 2021 [figure supplement 2](#)). In contrast, we observed smaller conductance values for TMC1 systems with mixed lipid bilayers (at most ~ 22 pS for a single pore), regardless of the presence of CIB2 or CIB3, with or without bound Ca^{2+} (**Figure 8** [figure supplement 4B](#)). Additional Anton 2 simulations for one of these TMC1 + CIB2 + Ca^{2+} systems with a mixed membrane, equilibrated for 240 ns and then subjected to -0.43 V for 480 ns, revealed a low conductance value of ~ 6.2 pS.

The reduced conductance values observed for mixed-membrane systems were attributed in part to pore blockage by cholesterol and lipids in the initial setup. Interestingly, Anton 2 simulations of TMC1, TMC1 + CIB2, and TMC1 + CIB2 + Ca^{2+} in pure POPC membranes subjected to -0.5 V, -0.25 V, and -0.125 V for 480 ns, 480 ns, and 960 ns, respectively, revealed a trend in which TMC1 exhibits higher conductance with CIB2 bound (**Figure 8E** [figure supplement 2](#), **Figure 8** [table supplement 2](#)). This may begin to explain why CIB2 is essential for normal TMC1 function.

Simulations of TMC2 with an applied transmembrane voltage of -0.5 V revealed a more closed state when compared to our TMC1 model, regardless of membrane composition and presence or absence of CIB2, CIB3 and bound Ca^{2+} (**Figure 8** [figure supplement 4](#)). Conduction of K^+ was observed in most cases, but the maximum conductance did not reach above ~ 42 pS, suggesting that the conformation simulated is less open than the ones explored in simulations of TMC1. We also speculate that this is due to TMC2 having an intrinsic lower single-channel conductance than TMC1, as has been suggested by some experiments (Kim et al., 2013 [figure supplement 2](#)), but not others (Pan et al., 2013 [figure supplement 2](#)). It is also possible that our TMC2 model is not in a fully open conformation, which can only be reached upon mechanical stimulation. Regardless, our simulations unequivocally indicate that TMC1 and TMC2 are cation channels and that these proteins can form stable complexes with CIB2 and CIB3.

Discussion

Previous work on CIB proteins had primarily focused on mammalian auditory hair cells, leaving open the question of whether they play a functional role in vestibular function and across vertebrate species. Recently, Wang et al. have suggested that CIB2 and CIB3 are important for stereocilia maintenance and MET in mouse vestibular hair cells (Wang et al., 2023 [figure supplement 2](#)). This work demonstrated that similar to *Tmc1* and *Tmc2*, *Cib2* is highly expressed in the striolar region while *Cib3* is expressed in the extrastriolar regions, and mice lacking both *Cib2* and *Cib3* had no MET and

deficits in vestibular hair cell bundle structure (Wang et al., 2023 [DOI](#)). Taking a step forward, here we establish that CIB2 and CIB3 are highly conserved regulators of MET in both mouse vestibular hair cells and zebrafish hair cells. We demonstrate that vestibular hair cells in mice and zebrafish lacking CIB2 and CIB3 are not degenerated but have no detectable MET, assessed via FM 1-43 dye uptake, at time points when MET function is well developed in wild-type hair cells. Similar structural-function phenotype has been reported in auditory hair cells in *Tmc1/Tmc2* double mutant mice (Kawashima et al., 2015 [DOI](#)). Besides the known *in vitro* interactions (Giese et al., 2017 [DOI](#); Liang et al., 2021 [DOI](#)), our study provides strong genetic evidence for the direct functional relationship between CIB and TMC proteins in regulating MET both in auditory and vestibular hair cells.

Previous studies have shown that the loss of CIB2 leads to profound hearing loss from birth in both human and mice (Giese et al., 2017 [DOI](#); Liang et al., 2021 [DOI](#); Seco et al., 2016 [DOI](#)). This suggests that the endogenous levels of CIB3 present in auditory hair cells are not sufficient to compensate for CIB2 in hearing. Prior work has also shown that over-expression of CIB3 in mouse auditory outer hair cells can only partially compensate for the loss of CIB2 in MET (Liang et al., 2021 [DOI](#)). However, whether overexpression of CIB3 can rescue impaired hearing thresholds in *Cib2* mutant mice is not known. While CIB2 is also present in vestibular hair cells, compared to auditory hair cells, the expression of CIB3 is several folds higher in vestibular end organs. Indeed, our current data demonstrate that, unlike auditory hair cells, endogenous levels of CIB3 can fully compensate for loss of CIB2 in vestibular hair cells in mice. Together our work on the vestibular system in mice highlights that both CIB2 and CIB3 can play overlapping roles in MET function.

Interestingly, our corresponding work in zebrafish indicates that *Cib3* can also compensate for *Cib2*, but only in specific subsets of hair cells. Recent genetic, morphology and scRNAseq data have revealed that within the zebrafish inner ear, each sensory epithelia (utricle, saccule, cristae) has distinct groups of hair cells (Shi et al., 2023 [DOI](#); Smith et al., 2020 [DOI](#)). In crista of the zebrafish inner ear, which respond to angular acceleration, there are two main groups of hair cells that can be delineated by distinct morphological and genetic features. Morphologically these two group are defined as either short or tall hair cells (**Figure 6A,E** [DOI](#)). Genetic studies have revealed that these two types of hair cells rely on different *Tmc* combinations for MET—short cells rely on *Tmc2a* while tall cells rely on *Tmc2b* and *Tmc1* (Smith et al., 2020 [DOI](#)). More recent mRNA *in situ* studies have validated this *Tmc* requirement and shown that *tmc2a*, *tmc2b*, *tmc1* are expressed in just tall cells, while *tmc2a* is only expressed in short cells (Smith et al., 2023 [DOI](#)). Interestingly, this work also demonstrated that *cib2* is expressed broadly in tall and short hair cells, while *cib3* showed more restricted expression in tall hair cells. Our work on MET in the zebrafish cristae indicates that *Cib2* is required in both short and tall cells, while *Cib3* is required in just tall cells. This expression data set is supported by absence of FM 4-64 labeling from short hair cells when *Cib2* is lost (**Figure 6H** [DOI](#)). In contrast, loss of *Cib3* alone does not impact FM 4-64 labeling in the zebrafish crista, indicating that *Cib2* can compensate for loss of *Cib3* in both short and tall cells (**Figure 6G** [DOI](#)). Currently it is not clear what role different types of hair cells play in zebrafish inner ear epithelia. Our present findings show that zebrafish *cib2* mutants have a severely diminished acoustic startle response, a behavior mediated primarily by the saccule (**Figure 5A** [DOI](#)). These behavioral results suggest that *Cib2* plays an important role in hair cells that detect acoustic stimuli.

In addition to the inner ear, we likewise found that both *Cib2* and *Cib3* play an important role in the zebrafish lateral line. Similar to the zebrafish inner ear and mouse vestibular system, we show that in lateral-line hair cells, *Cib2* can compensate for MET in all hair cells when *Cib3* is lost (**Figure 5B,C** [DOI](#), F,K, **Figure 5** [DOI](#)-figure supplement 2A-C). However, like in the zebrafish inner ear, when *Cib2* is lost, MET is lost from a specific population of lateral-line hair cells. In the lateral line, each neuromast has two cell populations that detect flow in opposing directions (López-Schier and Hudspeth, 2006 [DOI](#); Pujol-Martí and López-Schier, 2013 [DOI](#)). Work in the posterior lateral line has demonstrated that cells that detect anterior flow rely on *Tmc2a/2b* for MET, while cells that detect posterior flow rely on just *Tmc2b* (Chou et al., 2017 [DOI](#)). In our work we find that cells that detect

anterior flow primarily rely on Cib2 for MET (**Figure 6C-D**). Further, this work indicates that Cib3 cannot compensate for loss of Cib2 in these cells. Interestingly, our functional assessment in the zebrafish lateral line and crista suggests that Cib2 is specifically required for mechanosensitive function in populations of hair cells that express Tmc2a. Whether Cib3 is unable to pair with Tmc2a or is simply not expressed along with Tmc2a remains an unanswered question. The significance of this pairing is also unclear. Recent work has shown that the addition of Tmc2a to lateral-line cells that detect anterior flow significantly augments the mechanosensitive responses of these cells (Kindig et al., 2023). Whether Cib2 is required along with Tmc2a for these augmented responses remains unclear.

Our *in vivo* studies of mouse TMC + CIB and zebrafish Tmc + Cib complexes suggest a conserved functional relationship between these two protein families across vertebrates. Structural predictions using AF2 show conserved folds for human and zebrafish proteins, as well as conserved architecture for their protein complexes. Predictions are consistent with previous experimentally validated models for the TMC1 pore (Ballesteros et al., 2018; Pan et al., 2018), with the structure of human CIB3 coupled to mouse TMC1-IL1 (Liang et al., 2021), and with our NMR data validating the interaction between human TMC1 and CIB2/3 proteins. Remarkably, the AF2 models are also consistent with the architecture of the nematode TMC-1 and CALM-1 complex (Jeong et al., 2022), despite low sequence identity (36% between human TMC1 and worm TMC-1 and 51% between human CIB2 and worm CALM-1). This suggests that the TMC + CIB functional relationship may extend beyond vertebrates.

Taken together, the modeling predictions, NMR experiments, and simulations presented here provide a structural framework to interpret and understand seemingly contradictory results. In a previous study (Giese et al., 2017), we showed that the N-termini of TMC1 and TMC2 interact with CIB2, and subsequent work (Liang et al., 2021) presented biochemical data validating the interaction between the TMC1-NT with CIB2. However, X-ray crystallography data demonstrated that the main interaction between TMC1 and CIB3 involved IL1, leaving the role played by CIB2-TMC-NT interactions unaddressed (Liang et al., 2021). Here we show using NMR that both TMC1-NT and TMC1-IL1 bind to CIB2 and CIB3 non-competitively. These interactions are consistent with AF2 models for TMC + CIB complexes where CIB proteins are clamped between TMC1-NT and IL1. Simulations of these models indicate that there is some potential preferential binding of TMC1 and TMC2 to CIB3 over CIB2 (predicted from BSA) and that TMC + CIB interactions are stable and last for microseconds, with biochemical and NMR experiments showing that these interactions are stable at even longer timescales.

Interestingly, the behavior of CIB2 and CIB3 in solution (SEC experiments using 3 mM CaCl₂) is different in the absence of TMC1-IL1. While CIB2 appears as a single peak, CIB3 shows two clear peaks indicating the presence of monomeric and dimeric states, consistent with the crystal structure of an apparent CIB3 dimer (Liang et al., 2021). In the presence of TMC1-IL1, the CIB3 dimeric peak is absent, and the potentially monomeric peak is slightly shifted accounting for the added TMC1-IL1, as observed for CIB2. This result suggests that CIB2, both in the presence and in the absence of TMC1-IL1, is monomeric in solution (**Figure 7**-figure supplement 6). This proposal is consistent with prior conclusions that CIB2 is a monomer (Dal Cortivo et al., 2019), but is in disagreement with other reports indicating that CIB2 is a dimer in solution (Vallone et al., 2018) and our own prior pull-down assays indicating that CIB2 co-immunoprecipitated with differentially tagged CIB2 or CIB3 (Giese et al., 2017; Riazuddin et al., 2012). Moreover, our NMR data (obtained using 3 mM CaCl₂) indicates that TMC1-IL1 + CIB2 is unlikely to directly interact with CIB3. The simplest explanation for these seemingly contradictory results comes from the TMC1 and TMC2 dimeric states (Pan et al., 2018) and our modeling indicating that each TMC monomer interacts with one CIB protein (**Figure 7**-figure supplement 1). Pull-down assays might simply be reflecting the oligomeric state of TMC proteins. In such scenario, and considering that TMC proteins have been suggested to form hetero-oligomers (Pan et al., 2018), there are up to ten different combinations of TMC1/2 and CIB2/3 proteins (**Figure 8**-figure supplement 5) that

might have distinct properties. Such variety of complexes with potentially different conductance values might explain tonotopic changes of conductance along the cochlea, although other explanations exist, including a variable number of TMC proteins around the tip link (Beurg et al., 2018 [DOI](#)) and the direct influence of membrane composition on conductance (Effertz et al., 2017 [DOI](#); Walujkar et al., 2021 [DOI](#)).

How TMC + CIB interactions depend on Ca^{2+} concentration may have important functional implications for adaptation and hair cell mechanotransduction. Structures of CIB3 and worm CALM-1, a CIB2 homologue, both bind divalent ions via EF-hand motifs proximal to their C-termini (Jeong et al., 2022 [DOI](#); Liang et al., 2021 [DOI](#)). Reports on CIB2 affinities for Ca^{2+} are inconsistent, with K_D values that range from 14 μM to 0.5 mM (Blazejczyk et al., 2009 [DOI](#); Vallone et al., 2018 [DOI](#)).

Although qualitative pull-down assays done in the presence or the absence of 5 mM CaCl_2 suggest that the TMC1 and CIB2 interactions are Ca^{2+} -independent (Liang et al., 2021 [DOI](#)), strength and details of the CIB-TMC-IL1 and CIB-TMC-NT contacts might be Ca^{2+} -dependent, especially considering that Ca^{2+} induces changes that lead to exposure of hydrophobic residues involved in binding (Blazejczyk et al., 2009 [DOI](#)).

Our simulations in the presence of a biasing potential unequivocally demonstrate that both TMC1 and TMC2, in the presence or the absence of CIB proteins, with or without bound Ca^{2+} , and in two types of membranes, are cation channels with predicted single-channel conductance values that can reach values that are consistent with those of the transduction channel. Our models emphasize again the role played by the membrane in lining the pore of TMC proteins (Pan et al., 2018 [DOI](#); Walujkar et al., 2021 [DOI](#)), and also indicate that CIB proteins are ideally positioned to connect various parts of cytosolic inter-helix linkers with the helices that form the pore, specially $\alpha 3$ and $\alpha 4$, recently implicated in gating (Akyuz et al., 2022 [DOI](#); Walujkar et al., 2021 [DOI](#)). Last, our modeling also positions the myristoylated N-terminus of CIB2 and CIB3 close enough to the membrane bilayer, which might be yet another way to sense membrane stretch and influence gating. Further studies should help provide a comprehensive view into CIB function in channel assembly, activation, and potentially hair-cell adaption.

Overall, the biochemically supported atomic-resolution structural models and simulations presented here along with our results obtained using animal models strongly suggest that TMC1/2 proteins must function with CIB2/3 in normal hair-cell MET across sensory organs, hair-cell types, and species. This is supported by structural data indicating multiple contact points between TMC and CIB proteins, simulation data predicting that CIB proteins stabilize TMC cytosolic domains and may enhance cation conduction, and *in vitro* and *in vivo* functional data indicating that both TMC1/2 and CIB2/3 proteins are needed for hair-cell MET.

Materials and methods

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 568	Thermo Fisher Scientific	Cat#A-11031; RRID: AB_144696
Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific	A32723
Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific	Cat#A-11034; RRID: AB_2576217
Goat anti-rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 568	Thermo Fisher Scientific	A-11011
Mouse monoclonal anti-FLAG M2	Sigma-Aldrich	Cat#F3165; RRID: AB_259529
Mouse monoclonal anti-FLAG M2-HRP	Thermo Fisher Scientific	Cat#A8592; RRID: AB_439702
Mouse monoclonal anti-V5	Thermo Fisher Scientific	Cat# R960-25; RRID: AB_2556564
Mouse monoclonal anti-V5-HRP	Thermo Fisher Scientific	Cat#R961-25; RRID: AB_2556565
Mouse monoclonal GAPDH	Santa Cruz	sc-32233
Rabbit polyclonal anti-CIB3	Sigma	SAB2103524
Rabbit polyclonal anti-GFP	Invitrogen	A-11122
Rabbit polyclonal anti-GFP-HRP	Thermo Fisher Scientific	Cat#A10260; RRID: AB_2534022
Rabbit polyclonal anti-Myosin VIIa	Proteus Biosciences	#25-6790
Rabbit polyclonal anti-V5	Abcam	Cat#ab9116; RRID: AB_307024
Chemicals		
FM TM 1-43FX	Thermo Fisher Scientific	F35355
FM TM 1-43	Thermo Fisher Scientific	T3163
FM TM 4-64	Thermo Fisher Scientific	T13320
Alexa Fluor TM 488 Phalloidin	Thermo Fisher Scientific	A12379

Alexa Fluor™ 633 Phalloidin	Thermo Fisher Scientific	A22284
16% Paraformaldehyde	Electron Microscopy Sciences	50-980-487
Triton X-100	Polysciences	04605-250
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)	Invitrogen	D1306
Protease inhibitor mixture	Roche	11697498001
Protein A–Sepharose CL-4	Thermo Fisher Scientific	101042
Polyethylenimine	Polysciences	23966
Critical Commercial Assays		
4–20% Tris-Glycine gel	Novex	XV04205PK20
SMARTScribe Reverse Transcriptase kit	Clontech	639537
SYBRgreen technology	Qiagen	330503
Amersham™ ECL™ Prime Western Blotting Detection Reagent	GE Healthcare	RPN2236
QIAshredder	Qiagen	Cat#79656
Trans-Blot® Turbo™ Transfer System	Bio-Rad	Cat#1704156
Trans-Blot® Turbo™ Midi PVDF Transfer Packs	Bio-Rad	Cat#1704157
4–20% Mini-PROTEAN® TGX Stain-Free™ Protein Gels	Bio-Rad	Cat#4568094
4–20% Criterion™ TGX Stain-Free™ Protein Gel	Bio-Rad	Cat#5678094
GFP-Trap Magnetic Agarose	Chromotek	Cat#gtma-20
Anti-v5 agarose affinity gel	Sigma-Aldrich	Cat#A7345
Anti-FLAG® M2 Magnetic Beads	Sigma-Aldrich	Cat#M8823
Nunc™ Lab-Tek™ chambered coverglass	Thermo Fisher Scientific	Cat#155411PK
Lipofectamine™ 3000 Transfection Reagent	Thermo Fisher Scientific	Cat#L3000015
Experimental Models: Organisms/Strains		
<i>Cib2^{tm1a}</i>	EUCOMM	
<i>Cib3^{KO}</i>	This study	N/A
<i>cib2^{idc21}</i> zebrafish	This study	N/A
<i>cib3^{idc22}</i> zebrafish	This study	N/A
Tg(myo6b:GCaMP6s-CAAX) ^{idc1} zebrafish	(Sheets et al., 2017)	ZDB-ALT-170113-3

Mouse strains and husbandry

Cib2^{KO} mice: *Cib2^{tm1a(EUCOMM)Wtsi}* mice were obtained from the EUCOMM repository and maintained on C57BL/6 N background. A gene trap cassette containing lacZ and neomycin resistance genes flanked by FRT sites was inserted downstream of exon 3, with exon 4 flanked by loxP sites.

Cib3^{KO} mice: *Cib3* knockout mice were generated using CRISPR/Cas9 technology. An indel in exon 4 of *Cib3* was introduced leading to a nonsense mutation and a premature stop at the protein level (p.Asp54*). Primers used to genotype *Cib3* mutant mice were F: GTAGGGGCATGGATAACATCTG and R: GTTGAATGCTTGCTCCCCAGG. *Cib3^{KO}* mice were backcrossed using C57Bl6/JN *Cib2^{KO}* mice for several generations. *Cib3* allele was validated at the genomic, RNA messenger, and protein level.

Animals were group-housed with their littermates on a 12:12 h light: dark cycle at 20°C with ad libitum access to food and water. All animal procedures were approved by the Institutional Animal Care and Use Committees (IACUCs) at University of Maryland (protocol #0420002) and at Harvard Medical School (protocol #00001240).

Western blot and qPCR in mice

Mouse heart tissues were homogenized in CHAPS buffer containing 1X protease inhibitors, 1 mM Na orthovanadate, 10 mM Na glycerophosphate, and 10 mM NaF. Equivalent protein concentrations were fractionated on a 4–20% Bis-Tris gel (Invitrogen) and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore). Membranes were blocked and then probed with the rabbit anti-CIB2 (1:500), CIB3 (1:500), and GAPDH (1:500) overnight at 4°C, followed by three washes of 30 min each in 1X TBST. After washing, blots were incubated with horseradish peroxidase-conjugated anti-rabbit antibody (1:1000, Sigma NA934V, Lot # 17640116) for 2 h at room temperature, followed by detection using the ECL Prime Western Blotting System (Thermo Fisher Scientific 32,106).

RNAs from brain were extracted from *Cib2*;*Cib3* mutant and control mice using the Ribopure kit (Ambion). SMARTScribe Reverse Transcriptase kit (Clontech) was used to generate cDNAs, and SYBRgreen technology (Qiagen) was used to perform the qPCR. The following primers were used to amplify *Cib2* and *Cib3*: *Cib2*_exon4_F: CTCTGTGCTCTGCGAATCAG, *Cib2*_exon5_R: GGCCAGCGTCATCTCTAAGT, *Cib3*_exon3_F: TGGTGCCTCTTGACTACACG, *Cib3*_exon5_R: CAGCACCTTCTCACAGACCA. *Hprt* amplification was used to normalize the samples: *hprt*_exon8_F: TGTGTGTTGGATATGCCCTTG, *hprt*_exon9_R: GGCTTTGTATTTGGCTTTTCC.

Quantification of VOR and OKR in mice

Surgical techniques and experimental setup have been previously described (Beraneck and Cullen, 2007). Eye movement data were collected using an infrared video system (ETL-200, ISCAN system). The rotational velocity of the turntable (head velocity) was measured using a MEMS sensor (MPU-9250, SparkFun Electronics). Eye movements during OKR were evoked by sinusoidal rotations of a visual surround (vertical black and white stripes, visual angle width of 5°) placed around the turntable at frequencies 0.2, 0.4, 0.8, 1, and 2 Hz with peak velocities of $\pm 16^\circ/\text{s}$. To record VOR responses, the turntable was rotated at sinusoidal frequencies 0.2, 0.4, 0.8, 1, and 2 Hz with peak velocities of $\pm 16^\circ/\text{s}$ in both light and dark. In the light condition, the visual surround remained stationary, whereas in the dark condition, both the visual surround and turntable rotated in phase. Head and eye movement signals were low-passed filtered at 125 Hz and sampled at 1 kHz. Eye position data were differentiated to obtain velocity traces. Cycles of data with quick phases were excluded from the analysis. Least-square optimization determined the VOR and OKN gains and phases (Beraneck et al., 2008) plotted as mean $\pm$ standard deviation (SD) against all frequencies for all mice. *Cib2*^{+/+}; *Cib3*^{KO/KO} (*n* = 4), *Cib2*^{KO/+}; *Cib3*^{KO/KO} (*n* = 5), and *Cib2*^{KO/KO}; *Cib3*^{KO/KO} (*n* = 4) mice were tested for all frequencies. Two-way ANOVAs and Bonferroni post hoc tests were used to test statistical significance.

Mouse rotarod test

Cib2^{+/+}; *Cib3*^{KO/KO} (*n* = 6), *Cib2*^{KO/+}; *Cib3*^{KO/KO} (*n* = 6), and *Cib2*^{KO/KO}; *Cib3*^{KO/KO} (*n* = 6) mice were used. Motorized rotating rod (Rotarod, IITC Life Science) was programmed to accelerate from 4 rpm to 40 rpm with a ramp of 300 s for each test trial. The time taken for a mouse to fall off the rotarod was recorded. Three trials were performed each day for 5 consecutive days. First day (day 0) was considered the training day.

Quantification of head motion in mice

Head motion was recorded in mice via a miniature motion sensor (MPU-9250, TDK Corporation) attached to the top of the skull. The motion sensor recorded 3-axis linear acceleration (i.e., fore-aft, lateral, and vertical) and 3-axis angular velocity (roll, pitch, and yaw) at 100 Hz sampling rate. The power spectra of the motion signals in all six dimensions were computed using Welch's averaged periodogram with $nfft = 512$ and a Bartlett window (pwelch function, MATLAB, MathWorks).

Circling behavior in mice

Mice were placed in a cylindrical-shape container (27 cm in diameter, 30 cm in height) and their behavior were recorded using a cell-phone camera. The number of rotations made during 120-second recording were counted offline by watching the recorded videos. A rotation is defined as a completed 360-degree rotation of the mouse's heading.

Immunostaining in mice

The cochlear and vestibular sensory epithelia were isolated, fine dissected and permeabilized in 0.25% Triton X-100 for 1 h and blocked with 10% normal goat serum in PBS for 1 h. The tissue samples were probed with primary antibody overnight and after three washes were incubated with the secondary antibody for 45 min at room temperature. Rhodamine phalloidin or Alexa fluor phalloidin 488 were used at a 1:250 dilution for F-actin labeling. Nuclei were stained with DAPI (Molecular Probes). Images were acquired using either LSM 700 laser scanning confocal microscope (Zeiss, Germany) with a 63× 1.4 NA or 100× 1.4 NA oil immersion objectives or Leica SP8 laser scanning confocal microscope with a 100× 1.44 NA objective lens. Stacks of confocal images were acquired with a Z step of 0.05–0.5 μm and processed using ImageJ software (National Institutes of Health). Experiments were repeated at least 3 times, using at least three different animals.

FM 1-43 dye uptake experiments in mice

In the Ahmed lab, cochlear explants from *Cib2* and *Cib3* mutant mice were dissected at postnatal day 5 (P5) and cultured in a glass-bottom petri dish (MatTek, Ashland, MA). They were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS (Life Technologies) for 2 days at 37 °C and 5% CO_2 . Explants were incubated for 10 s with 3 μM FM 1-43, washed three times with Hank's balanced salt solution, and imaged live using a Zeiss LSM 700 scanning confocal microscope.

In the Holt Lab, P60 mice were injected intraperitoneally with 5 mg/g mouse weight of FM 1-43FX fixable form as previously described (Meyers et al., 2003 [DOI](#)). Tissue was collected after ~24 h and fixed for 1 h at 25 °C in 4%PFA/1X PBS protected from light. Tissue was then transferred into 120 mM EDTA pH 7.4 for 48–72 h protected from light. Then tissue was transferred to 1XPBS, dissected, incubated in 1:1000 dilution of Phalloidin (Invitrogen), washed three times in 1XPBS and mounted under glass coverslips for microscopy. Imaging was completed using a 20× objective on a Zeiss 800 confocal microscope with laser intensity, detector gain, and post processing set equally for each sample.

ABR measurements in mice

Hearing thresholds of *Cib2* and *Cib3* mutant mice ($n = 5$ –10 each genotype) were evaluated by recording ABRs. All ABR recordings, including broadband clicks and tone-burst stimuli at three frequencies (8, 16, and 32 kHz), were performed using an auditory-evoked potential RZ6-based auditory workstation (Tucker-Davis Technologies) with high frequency transducer RA4PA Medusa PreAmps. Maximum sound intensity tested was 100 dB SPL. TDT system III hardware and BioSigRZ software (Tucker Davis Technology) were used for stimulus presentation and response averaging.

Zebrafish strains and husbandry

Zebrafish (*Danio rerio*) were grown at 28°C using standard methods. Zebrafish were maintained in a Tübingen or TAB background. Larvae were raised in E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, and 0.33 mM MgSO₄, buffered in HEPES, pH 7.2). Zebrafish work at the National Institute of Health (NIH) was approved by the Animal Use Committee at the NIH under animal study protocol #1362-13. All experiments were performed on larvae at 5-6 days post fertilization (dpf). At this age sex determination is not possible. Neuromasts (L1-L5) in the primary posterior lateral line were used for our analyses. For Ca²⁺ imaging the following transgenic line was used: *Tg(myo6b:memGCaMP6s)^{idc1}* (Jiang et al., 2017 [DOI](#)).

Cib2^{idc21} and *cib3^{idc22}* mutants were generated in-house using CRISPR-Cas9 technology as previously described (Varshney et al., 2016 [DOI](#)). Exon 3 and exon 6 were targeted for *cib2* and *cib3* respectively. Guides for *cib2* and *cib3* are as follows: 5'-GGAGGAGCTTACACCAG(AGG)-3' and 5'-GGAAATCCTCCAGAGAAAGG(CGG)-3'. Founder fish were identified using fragment analysis of fluorescent PCR products (Varshney et al., 2016 [DOI](#)). Founder fish containing a 10 bp insertion in *cib2* 5'-GCTTACACCA(CCTTTGGTAG)GAGGAGGTTA-3' resulting in a stop codon in the third EF-hand domain, at amino acid 148, was selected for analysis (Figure 5 [DOI](#)-figure supplement 1). In addition, a founder fish containing a 4 bp deletion in *cib3* 5'-TGGCCGCTT(TTCT)CTGGAGG-3' resulting in a stop codon in the third EF-hand domain, at amino acid 159, was selected for analysis (Figure 5 [DOI](#)-figure supplement 1). After behavior or imaging analyses, genotypes were confirmed using standard PCR and sequencing. The following primers were used for genotyping: *cib2_FWD* 5'-ACTGAAGCAACTCCTTTTGTGG-3' and *cib2_REV* 5'-GAACCATGCATTTTACAGC-3' and *cib3_FWD* 5'-ATAGTCTCTCCATCTATAGACT-3' and *cib3_REV* 5'-TTTTAGGGTGAAATAAACAGA-3'. Crosses of *cib2^{+/-}; cib3^{+/-}* X *cib2^{+/-}; cib3^{+/-}* or *cib2^{-/-}; cib3^{+/-}* X *cib2^{+/-}; cib3^{+/-}* adults were used to propagate larvae for our analyses. All controls or siblings are double heterozygous animals. Larvae were chosen at random, except for *cib2;cib3* homozygous mutants which, similar to other zebrafish mutants with hearing and balance defects, had no swim bladder and no acoustic startle response.

Labeling zebrafish hair cells with FM 1-43, FM 4-64, and FM 1-43FX

To label lateral-line hair cells with FM 1-43, larvae were immersed in 1 µM FM 1-43 (ThermoFisher, T3163) in E3 for 30 s, and then washed 3 times in E3. After washing, larvae were mounted in 1% low melt agarose containing an anesthetic, 0.02% tricaine methanesulfonate (Syndel, ANADA 200-226). FM 1-43 labeled hair cells were then imaged on an A1 Nikon upright laser-scanning confocal microscope with a 60× 1.0 NA water objective. FIJI was used to process image Z-stacks. To estimate the number of hair cells per neuromast, laser scanning DIC images were used to count the number of hair bundles per neuromast. To quantify FM 1-43 label, a 5 µm circular region of interest (ROI) was placed on the soma of each hair cell with a hair bundle in the DIC image. These ROIs were used to quantify the mean FM 1-43 intensity per hair cell. These intensity values were averaged to calculate the average FM 1-43 intensity per neuromast.

FM 4-64 (ThermoFisher, T13320) was used to label hair cells in the crista within the zebrafish inner ear. For this labeling, *Tg(myo6b:memGCaMP6s)^{idc1}* larvae were mounted on their side on Sylgard chamber in E3 embryo media containing 0.02% MESAB. Approximately 2-3 nL of 10 µM FM 4-64 in 0.1 M KCl were injected into the otic capsule as previously described (Smith et al., 2020 [DOI](#)). FM 4-64 labeled hair cells of the medial crista were then imaged on an A1 Nikon upright laser-scanning confocal microscope with a 25× 1.10 NA or 60× 1.0 NA water objective. FIJI was used to process image Z-stacks. To assess tall versus short hair cells in each crista *memGCaMP6s* label was used.

To correlate hair bundle orientation in lateral line hair cells with intact mechanotransduction, larvae were immersed in 3 µM FM 1-43FX (ThermoFisher, F25255) in E3 for 30 s, and then washed 3 times in E3. After washing, larvae were fixed with 4% paraformaldehyde in PBS at 4°C overnight.

After fixation, larvae were washed 4×5 min in 0.1% Tween in PBS (PBST). Larvae were then placed in blocking solution (2% goat serum, 1% bovine serum albumin, 2% fish skin gelatin in PBS + 0.3% triton) for 2 h at room temperature. After block Alexa Fluor 633 Phalloidin (ThermoFisher, A22284) was added at 1:100 in PBST. Larvae were incubated for 4 h at room temperature and then washed 4×5 min in PBST. Larvae were mounted on glass slides with Prolong Gold (ThermoFisher Scientific) using No. 1.5 coverslips. To image hair bundle orientation and FM 1-43FX label in lateral-line hair cells, a Zeiss LSM780 confocal microscope with Airyscan was used. Hair cells were imaged using a 63×1.3 NA oil objective and processed with an Airyscan processing factor of 7.0. For analysis, Z-stacks were max-projected in FIJI. Images were background subtracted using a rolling ball correction. Hair cells with a FM 1-43FX intensity more than twice the background intensity were scored as FM 1-43FX positive.

Zebrafish Ca^{2+} imaging and behavior

For imaging of mechanosensitive Ca^{2+} signals in neuromast hair bundles, *Tg(myo6b:memGCaMP6s)* animals were mounted on their side on Sylgard chamber in E3 embryo media containing 0.2% MESAB (Lukasz and Kindt, 2018). Larvae were then pinned and a solution containing α -bungarotoxin (125 μM , Tocris) was injected into the heart cavity for paralysis during imaging. After paralysis, larvae were immersed in extracellular imaging solution (in mM: 140 NaCl, 2 KCl, 2 CaCl_2 , 1 MgCl_2 and 10 HEPES, pH 7.3, OSM 310 $\pm$ 10). To image Ca^{2+} signals in the hair bundles we used a Bruker Swept-Field confocal system equipped with a Rolera EM-C2 CCD camera (QImaging, Surrey, Canada) and a Nikon CFI Fluor 60 $\times$ 1.0 NA water immersion objective as previously described (Q. Zhang et al., 2018). The system includes a band-pass 488/561 nm filter set (59904-ET, Chroma) and is controlled using Prairie View software (Bruker Corporation). A piezoelectric motor (PICMA P-882.11-888.11 series, PI Instruments) attached to the objective and used to acquire 5-plane Z-stacks every 0.5 μm using a 35 μm slit at a 50-Hz frame rate for a 10-Hz volume rate. A fluid-jet composed of a glass capillary attached to a pressure clamp system (HSPC-2-SB, ALA) was used to deliver a 500-ms anterior and posterior step stimulation to deflect hair bundles along their axis of sensitivity.

To quantify GCaMP6s fluorescent intensity changes during stimulation, we registered the Z-stacks and then average projected the Z-stacks into a single plane. We then loaded the projected images into FIJI and used the Time Series Analyzer V3 plugin to create circular ROIs with a ~ 1.7 μm diameter. ROIs were placed on the center of each individual bundle. We then measured and plotted change in the mean intensity ($\Delta F/F_0$) within the region during the recording period. The mean intensity within each ROI was computed for each hair bundle. A hair bundle with a signal magnitude (peak value of intensity change upon stimulation) above 10% $\Delta F/F_0$ was considered mechanosensitive. To create the AVG hair bundle GCaMP6s ($\Delta F/F_0$) per neuromast plot in **Figure 5** -figure supplement 2C, only traces from mechanosensitive cells were averaged.

A Zantiks MWP behavioral system was used to examine startle responses. The Zantiks system tracked and monitored behavioral responses via a built-in infrared camera at 30 frames per second. A 12-well plate was used to house larvae during behavioral analysis. Each well was filled with E3 and 1 larva. All fish were acclimated in the plate within the Zantiks chamber in dark for 15 min before each test. To induce startle, an integrated stepper motor was used to drive a vibration-induced startle response. A vibrational stimulus that triggered a maximal % of animals startling in controls without any tracking artifacts (due to the vibration) was used for our analyses. Each larva was presented with the vibrational stimulus 5 times with 100 s between trials. During the tracking and stimulation, a Cisco router connected to the Zantiks system was used to relay x, y coordinates of each larva every frame. To qualify as a startle response, a distance above 4 pixels or ~ 1.9 mm was required within 2 frames after stimulus onset. Animals were excluded from our analysis if no tracking data was recorded for the animal.

Zebrafish Statistics

All data shown are mean $\pm$ standard error of the mean (SEM). All experiments were performed on at least 4 animals and 8 neuromasts and 4 cristae. Behavior analysis and Ca^{2+} imaging was repeated at least twice on two independent days. All statistical analysis was performed using GraphPad Prism 8.0. A D'Agostino and Pearson test was used to test data for normality. A one-way ANOVA with a Dunnett's correction for multiple comparisons was used to compare differences between siblings and controls regarding the % of mechanosensitive hair cells, number of hair cells, % of FM 1-43 positive hair cells, average FM 1-43 label. For proportion of animals startling and comparisons for FM 1-43FX label, a Kruskal-Wallis test with a Dunnett's correction for multiple comparisons was used.

Expression and purification of bacterially expressed *hs* CIB2, CIB3, TMC1-IL1, and TMC1-NT

DNA sequences encoding for *hs* CIB2, CIB3, TMC1-IL1, and TMC1-NT were subcloned into *NdeI* and *XhoI* sites of the pET21a vector. All DNA constructs were sequence verified. Protein fragments were expressed in *Escherichia coli* BL21 Rosetta (DE3) (Novagen) cells, which were cultured in LB or TB media, induced at $\text{OD}_{600} \sim 0.4\text{--}0.6$ with 1 mM IPTG and grown at 20°C or 30°C for ~ 16 h. Protein fragments for NMR were produced using the same cells cultured in M9 minimal media supplemented with MEM vitamin solution (Gibco) and 1g/L $^{15}\text{NH}_4\text{Cl}$ (Cambridge Isotopes) to produce uniformly labeled $[\text{U-}^{15}\text{N}]$ protein. Cells were lysed by sonication in denaturing buffer (20 mM Tris HCl, pH 7.5, 6 M guanidine hydrochloride, 10 mM CaCl_2 , and 20 mM imidazole) or non-denaturing buffer (20 mM Tris HCl, pH 7.5, and 20 mM imidazole for *hs* TMC1-NT). The cleared lysates were loaded onto Ni-Sepharose (GE Healthcare) and eluted with denaturing buffer or non-denaturing buffer supplemented with 500 mM imidazole. Denatured proteins were refolded using MWCO 2,000 membranes (Spectra/Por 7) in dialysis buffer (400 mM Arg-HCl, 20 mM Tris-HCl, pH 7.5, 150 mM KCl, 50 mM NaCl, 2 mM CaCl_2 , 2 mM DTT) for ~ 16 hr. Before starting refolding reactions (when applicable), elution solutions were diluted to ~ 0.5 mg/mL using the denaturing buffer, and then reduced by using 2 mM DTT in the diluted sample. Refolded and soluble proteins were concentrated using Vivaspinn 20 or Amicon 15 centrifugal concentrators (10 kDa, 5 kDa, or 3 kDa molecular weight cutoff) and further purified on Superdex S75 columns (GE Healthcare) in SEC buffer (50 mM HEPES, pH 7.5, 126 mM KCl, 10 mM NaCl, 3 mM CaCl_2 , 5 mM DTT). Proteins were concentrated to ~ 0.5 mL for nuclear magnetic resonance (NMR) experiments.

NMR

Purified NMR samples were at concentrations of ~ 1.4 mM, ~ 0.2 mM, ~ 0.8 mM, and ~ 0.2 mM for CIB2, CIB3, CIB2 + TMC1-IL1, and CIB3 + TMC1-IL1, respectively, in SEC buffer (50 mM HEPES, pH 7.5, 126 mM KCl, 10 mM NaCl, 3 mM CaCl_2 , 5 mM DTT), supplemented with 10% (v/v) D_2O and 0.2 mM 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) for field-frequency locking and chemical shift referencing. Two-dimensional ^1H - ^{15}N TROSY-HSQC spectra (Nietlispach, 2005 [\[1\]](#)) were recorded for each sample at 25°C on a Bruker Avance III HD 800 MHz spectrometer equipped with a 5 mm TXI cryoprobe with z-gradients. Data were collected using 20 to 24 scans, 2,048 x 280 datapoints, and spectral widths of 17482.518 x 4054.298 Hz in the ^1H and ^{15}N dimensions, respectively. Data were processed and visualized using NMRFX (Norris et al., 2016 [\[2\]](#)).

Simulated Systems

Models for monomeric *hs* TMC1 (residues 84 to 760; NCBI ID: NP_619636.2) and *hs* TMC2 (148 to 863; NCBI ID: NP_542789.2) in complex with full-length *hs* CIB2 (NCBI ID: NP_006374.1) and *hs* CIB3 (NCBI ID: NP_473454.1), as well as truncated dimeric models of *hs* TMC1 (residues 260 to 760; NCBI ID: NP_619636.2) and *hs* TMC2 (residues 324 to 863; NCBI ID: NP_542789.2) were generated using AF2 (Evans et al., 2021 [\[3\]](#); Jumper et al., 2021 [\[4\]](#)). The TMC2 C-terminal end (residues 818 to 863)

was truncated to match the C-terminal end of TMC1. Using VMD (Humphrey et al., 1996 [DOI](#)), a model of dimeric *hs* TMC1 + *hs* CIB3-Ca²⁺ was constructed by aligning the monomeric *hs* TMC1 + *hs* CIB3 complex to the transmembrane region of the truncated dimeric *hs* TMC1 model. Coordinates for Ca²⁺ ions were obtained by aligning the crystal structure of Mg²⁺-bound *hs* CIB3 in complex with a *hs* TMC1-IL1 peptide (PDB code: 6WUD) (Liang et al., 2021 [DOI](#)) to the AF2 model of monomeric *hs* TMC1 + *hs* CIB3, then manually changing Mg²⁺ for Ca²⁺. This model was minimized for 1,000 steps in vacuum using NAMD (Phillips et al., 2005 [DOI](#)) and the CHARMM36 force field (Best et al., 2012 [DOI](#); Huang and Mackerell, 2014 [DOI](#); Vanommeslaeghe and Mackerell, 2015 [DOI](#)) and served as a reference model for aligning other combinations of *hs* TMC1 or *hs* TMC2 with *hs* CIB2 or *hs* CIB3. All models were either embedded into pure POPC or mixed membrane lipid bilayers using CHARMM-GUI (Jo et al., 2009 [DOI](#), 2008 [DOI](#), 2007 [DOI](#); Lee et al., 2019) as summarized in Table supplement 1. The mixed membrane mimicked stereocilia membrane composition (Zhao et al., 2012 [DOI](#)). The protein-lipid system was then solvated using TIP3P explicit water and neutralized with 0.150 M KCl, representative of endolymph ionic concentration (Bosher and Warren, 1978 [DOI](#)).

Simulations using NAMD

All-atom MD simulations were performed and analyzed using NAMD 2.13 (Phillips et al., 2005 [DOI](#)) and the CHARMM36 force field with CMAP corrections (Best et al., 2012 [DOI](#); Huang and Mackerell, 2014 [DOI](#); Vanommeslaeghe and Mackerell, 2015 [DOI](#)). For all systems, equilibrium simulations consisted of 2,000 steps of minimization, 0.5 ns of dynamics with everything excluding lipid tails fixed/constrained, 0.5 ns of dynamics with harmonic constraints applied to the protein (1 kcal mol⁻¹ Å⁻²), 1 ns of free dynamics in the *NpT* ensemble ($\gamma = 1$ ps⁻¹), and up to 100 ns of free dynamics in the *NpT* ensemble ($\gamma = 0.1$ ps⁻¹) prior to non-equilibrium production runs. A piston period of 200 fs and a damping timescale of 50 fs was used for pressure control and atomic coordinates were saved every 5 ps. All simulations were performed at 310 K and 1 atmosphere of pressure by using the Langevin thermostat and Nosé-Hoover Langevin pressure control. A 12-Å cutoff distance was employed with a force-based switching function starting at 10 Å. Periodic boundary conditions and the PME method were used to calculate long-range electrostatic interactions with a grid density greater than 1 Å⁻³. An integration timestep of 2 fs with electrostatic interactions computed every other timestep was utilized for every simulation in tandem with the SHAKE algorithm for the constraint of hydrogen atoms. A constant electric field was applied to all atoms in non-equilibrium simulations with transmembrane potential (Gumbart et al., 2012 [DOI](#)).

Simulations using Anton 2

We used Anton 2, a massively parallel special purpose machine for MD simulations (Shaw et al., 2014 [DOI](#)), to run simulations of *hs* TMC1 + *hs* CIB2-Ca²⁺ in POPC (simulations S1d-g; Table supplement 1), *hs* TMC1 + *hs* CIB2 in POPC (simulations S2d-2g; Table supplement 1), *hs* TMC1 in POPC (simulations S3d-g; Table supplement 1), and *hs* TMC1 + *hs* CIB2 in a mixed membrane (simulations S4c-d; Table supplement 1). Coordinates of the pre-equilibrated systems were converted to an Anton 2 compatible dms file format using the NAMDtoDMS.py script provided by the Pittsburgh Supercomputing Center. Simulations used CHARMM36 forcefields and were performed in the *NpT* ensemble (310 K, 1 atm) using the multigrator integration framework (Lippert et al., 2013 [DOI](#)) with a 2.5 fs integration timestep and saved every 120 ps. The MTK barostat and Nosé-Hoover thermostat was updated every 480 and 24 steps, respectively. Semi-isotropic pressure coupling was used. Long range electrostatic interactions were calculated using u-series electrostatics with a 64 ξ 64 ξ 64 grid. Van der Waals interactions were calculated with a cutoff of 12 Å. Transmembrane voltages were generated by applying a constant electric field to all atoms (Gumbart et al., 2012 [DOI](#)).

Simulation analysis procedures and tools

BSA was computed in VMD by measuring SASA for both TMC and CIB monomers, then subtracting SASA for the complex. BSA for interacting molecules A and B were computed as $BSA_{AB} = 1/2(SASA_A + SASA_B - SASA_{AB})$. RMSD values were computed in VMD using Ca atoms and initial models as references, with alignments carried out using the corresponding subdomains when indicated. VMD was used to analyze trajectories, render molecular images, and create videos. Xmgrace and gnuplot were used to generate plots. Sequences for CIB and TMC isoforms from various species were obtained from the NCBI protein database. Sequences were aligned utilizing the MUSCLE algorithm within Geneious (Kearse et al., 2012 [DOI](#)). Aligned sequences were loaded into JalView (Waterhouse et al., 2009 [DOI](#)) and colored based on a sequence identity threshold of 45%. Ionic currents were computed by counting the number of crossing, with conductance values estimated as in Walujkar et al., 2021 [DOI](#).

Resource Availability

Lead Contact

Further information and requests for resources and reagents should be directed to the Lead Contacts, Drs. Marcos Sotomayor (sotomayor@uchicago.edu) and Zubair M. Ahmed (zmahmed@som.umaryland.edu).

Materials availability

Materials generated in this study, including strains, plasmids and clones, are freely available from the Lead Contact upon request.

Data and code availability

This study did not generate any unique datasets or code.

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Additional information Author contributions

Z.M.A., K.S.K., and M.S. conceived the studies. A.P.J.G. carried out all audiometric recordings in mice, immunolocalization studies, cochlear and vestibular explant cultures, FM 1-43 uptake experiment on mouse tissues, RT-qPCRs, exploratory testing, *Cib2* and *Cib3* mouse phenotyping and genotyping, cloning of CIB2 and CIB3 in pET21a vectors. K.S.K. conducted zebrafish FM 1-43, FM 4-64, FM 1-43FX labeling, Ca^{2+} imaging and immunostaining. A.J.B. created the *cib2* and *cib3*

zebrafish mutants. R.A.R. did the zebrafish behavioral studies. W-H.W. did the cloning and mutagenesis of CIB and TMC constructs, protein expression and purification for NMR experiments, as well as model building and molecular dynamics simulations. J.S.M. did NMR experiments and NMR data processing. E.M.R. performed FM 1-43 uptake experiments in mice. J.M.L. and S.W. did model building. M.P.F. supervised NMR experiments and project. M.S. supervised project and assisted with cloning, strategies for protein refolding, model building, and simulations. H.H.V.C. and O.A.Z. performed VOR, OKN measurements and vestibular function evaluation of mice. J.R.H., analyzed the mouse FM 1-43 experiments data, provided supervision and resources. S.R., and Z.M.A., supervised the project, reviewed and interpreted the results, provided resources. All authors contributed to data analysis. A.P.J.G., W-H. W., K.S.K., K.C., M.S. and Z.M.A. wrote the manuscript. All authors edited the manuscript and approved for submission.

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Reviewer #1 (Public Review):

Revised Public Review

This reviewed preprint is essentially three papers combined into one-one paper focused on the role of CIB2/CIB3 in vestibular hair cells, one on the role of CIB2/CIB3 in zebrafish, and one on structural modeling of a CIB2/3 and TMC1/2 complex. The authors try to combine the three parts with the overarching theme of demonstrating that CIB2/3 play a functionally conserved role across species and hair cell types. It is important to note that many of the basic results from the mouse have already been reported by other groups in Liang et al. (2021) and Wang et al. (2023).

That said, their demonstration of the importance of CIB2 and CIB3 in zebrafish hair cell function is novel. The results largely coincide with what is seen in the mouse—they are both important, with stimulus-dependent Ca²⁺ entry reduced more in cib2 KOs than in cib3 KOs, and the cib2;cib3 showing the greatest impact. Interestingly, cib2 is uniquely localized in and important for specific hair cell types in the neuromast and crista.

The last part of the manuscript also offers significant new findings. Here structural studies (AlphaFold 2 modeling, NMR structure determination, and molecular dynamics simulations) brings us closer to the structure of the mammalian TMCs, alone and in complex with the CIB proteins. Moreover, the structural work supports the assignment of the TMC pore to alpha helices 4-7.

In summary, while this reviewed preprint has some data that replicate data from publications from other labs, it provides a comprehensive look at the CIB family in hair cells, especially in vestibular hair cells.

<https://doi.org/10.7554/eLife.89719.2.sa2>

Reviewer #2 (Public Review):

The paper by Giese and coworkers is quite an intense reading. The manuscript is packed with data pertaining to very different aspects of MET apparatus function, scales, and events. I have to praise the team that combined molecular genetics, biochemistry, NMR, microscopy,

functional physiology, in-vivo tests for vestibulo-ocular reflexes, and other tests for vestibular dysfunction with molecular modeling and simulations. The authors nicely show the way CIBs are associated with TMCs to form functional MET channels. The authors clarify the specificity of associations and elucidate the functional effects of the absence of specific CIBs and their partial redundancy.

Comments on revised version:

I appreciate the author's effort to address my comments. The revised paper 'Complexes of vertebrate TMC1/2 and CIB2/3 proteins form hair-cell mechanotransduction cation channels' by Giese and coworkers is definitely cleaner but remains a compendium of related but very uneven parts. By saying 'uneven,' I mean that the grounding of the experimental and computational parts is different, and the firmness of conclusions, respectively, is not matched.

My conclusion is that this is a great collaborative project. However, in its present form, different components pull the emphasis in several directions with little cross-talk. It is worth splitting into two papers.

<https://doi.org/10.7554/eLife.89719.2.sa1>

Author response:

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

Public Reviews:

Reviewer #1 (Public Review):

This reviewed preprint is a bit of Frankenstein monster, as it crams together three quite different sets of data. It is essentially three papers combined into one-one paper focused on the role of CIB2/CIB3 in VHCs, one on the role of CIB2/CIB3 in zebrafish, and one on structural modeling of a CIB2/3 and TMC1/2 complex. The authors try to combine the three parts with the overarching theme of demonstrating that CIB2/3 play a functionally conserved role across species and hair cell types, but given the previous work on these proteins, especially Liang et al. (2021) and Wang et al. (2023), this argument doesn't work very well. My sense is that the way the manuscript is written now, the sum is less than the individual parts, and the authors should consider whether the work is better split into three separate papers.

We appreciate the frank evaluation of our work and point out that combining structural with functional data from mouse and zebrafish offers a comprehensive view of the role played by TMC1/TMC2 and CIB2/3 complexes in hair-cell mechanotransduction. We believe that readers will benefit from this comprehensive analyses.

The most important shortcoming is the novelty of the work presented here. In line 89 of the introduction the authors state "However, whether CIB2/3 can function and interact with TMC1/2 proteins across sensory organs, hair-cell types, and species is still unclear." They make a similar statement in the first sentence of the discussion and generally use this claim throughout the paper as motivation for why they performed the experiments. Given the data presented in the Liang et al. (2021) and Wang et al. (2023 papers), however, this statement is not well supported. Those papers clearly demonstrate a role for CIB2/CIB3 in auditory and vestibular cells in mice. Moreover, there is also data in Riazuddin et al. (2012) paper that demonstrates the importance of CIB2 in zebrafish and Drosophila. I think the authors are really stretching to describe the data in the

manuscript as novel. Conceptually, it reads more as solidifying knowledge that was already sketched out in the field in past studies.

We note that work on mouse and fish CIB knockouts in our laboratories started over a decade ago and that our discoveries are contemporary to those recently presented by Liang et al., 2021 and Wang et al., 2023, which we acknowledge, cite, and give credit as appropriate. We also note that work on fish knockouts and on fish *Cib3* is completely novel. Nevertheless, the abstract text “Whether these interactions are functionally relevant across mechanosensory organs and vertebrate species is unclear” has been replaced by “These interactions have been proposed to be functionally relevant across mechanosensory organs and vertebrate species.”; and the introduction text “However, whether CIB2/3 can function and interact with TMC1/2 proteins across sensory organs, hair-cell types, and species is still unclear” has been replaced by “However, additional evidence showing that CIB2/3 can function and interact with TMC1/2 proteins across sensory organs, hair-cell types, and species is still needed.”. The work by Wang et al., 2023 is immediately discussed after the first sentence in the discussion section and the work by Liang et al., 2021 is also cited in the same paragraph. We believe that changes in abstract and introduction along with other changes outlined below put our work in proper context.

There is one exception, however, and that is the last part of the manuscript. Here structural studies (AlphaFold 2 modeling, NMR structure determination, and molecular dynamics simulations) bring us closer to the structure of the mammalian TMCs, alone and in complex with the CIB proteins. Moreover, the structural work supports the assignment of the TMC pore to alpha helices 4-7.

Thanks for the positive evaluation of this work.

Reviewer #2 (Public Review):

The paper 'Complexes of vertebrate TMC1/2 and CIB2/3 proteins form hair-cell mechanotransduction cation channels' by Giese and coworkers is quite an intense reading. The manuscript is packed with data pertaining to very different aspects of MET apparatus function, scales, and events. I have to praise the team that combined molecular genetics, biochemistry, NMR, microscopy, functional physiology, in-vivo tests for vestibulo-ocular reflexes, and other tests for vestibular dysfunction with molecular modeling and simulations. The authors nicely show the way CIBs are associated with TMCs to form functional MET channels. The authors clarify the specificity of associations and elucidate the functional effects of the absence of specific CIBs and their partial redundancy.

We appreciate the positive evaluation of our work and agree with the reviewer in that the combination of data obtained using various techniques *in vivo* and *in silico* provide a unique view on the role played by CIB2 and CIB3 in hair-cell mechanotransduction.

Reviewer #3 (Public Review):

This study demonstrates that from fish to mammals CIB2/3 is required for hearing, revealing the high degree of conservation of CIB2/3 function in vertebrate sensory hair cells. The modeling data reveal how CIB2/3 may affect the conductance of the TMC1/2 channels that mediate mechanotransduction, which is the process of converting mechanical energy into an electrical signal in sensory receptors. This work will likely impact future studies of how mechanotransduction varies in different hair cell types.

One caveat is that the experiments with the mouse mutants are confirmatory in nature with regard to a previous study by Wang et al., and the authors use lower resolution

tools in terms of function and morphological changes. Another is that the modeling data is not supported by electrophysiological experiments, however, as mentioned above, future experiments may address this weakness.

We thank the reviewer for providing positive feedback and for highlighting caveats that can and will be addressed by future experiments.

Reviewer #1 (Recommendations For The Authors):

Lines 100-101. Please temper this statement, as FM1-43 is only a partial proxy for MET.

The original text has been modified to: “In contrast to auditory hair cells, we found that the vestibular hair cells in *Cib2KO/KO* mice apparently have MET. We assessed MET via uptake of FM 1-43 (Figure 1A), a styryl dye that mostly permeates into hair cells through functional MET channels (Meyers et al., 2003), indicating that there may be another CIB protein playing a functionally redundant role.”

Lines 111-113. These data do not fully match up with the Kawashima et al. (2011) data. Please discuss.

We have modified the text to better report the data: “*Tmc2* expression increases during development but remains below *Tmc1* levels in both type 1 and type 2 hair cells upon maturation (Figure 1C).”

*Lines 125-126. The comparison in 2A-B is not described correctly for the control. The strain displayed is *Cib2*^{+/+};*Cib3*^{KO/KO} (not wild-type). Show the *Cib2*^{+/+};*Cib3*^{+/+} if you are going to refer to it (and is this truly *Cib2*^{+/+};*Cib3*^{+/+} from a cross or just the background strain?).*

Thanks for pointing this out. To avoid confusion, we have revised the sentence as follow: “We first characterized hearing function in *Cib3KO/KO* and control littermate mice at P16 by measuring auditory-evoked brainstem responses (ABRs). Normal ABR waveforms and thresholds were observed in *Cib3KO/KO* indicating normal hearing.”

*Lines 137-140. Did you expect anything different? This is a trivial result, given the profound loss of hearing in the *Cib2*^{KO/KO} mice.*

We did not expect anything different and have deleted the sentence: “Furthermore, endogenous CIB3 is unable to compensate for CIB2 loss in the auditory hair cells, perhaps due to extremely low expression level of CIB3 in these cells and the lack of compensatory overexpression of CIB3 in the cochlea of *Cib2KO/KO* mice (Giese et al., 2017).”

*Lines 194-196. But what about *Cib2*^{KO/KO}; Isn't the conclusion that the vestibular system needs either CIB2 or CIB3?*

Yes, either CIB2 or CIB3 can maintain normal vestibular function. A prior study by Michel et al., 2017, has evaluated and reported intact vestibular function in *Cib2KO/KO* mice.

Lines 212-214. Yes. This is a stronger conclusion than the one earlier.

We have revised the sentence as follow: “Taken together, these results support compulsory but functionally redundant roles for CIB2 and CIB3 in the vestibular hair cell MET complex.”

Lines 265-267. I'm not sure that I would state this conclusion here given that you then argue against it in the next paragraph.

We have modified this statement to make the conclusions clearer and more consistent between the two paragraphs. The modified text reads: "Thus, taken together the results of our FM 1-43 labeling analysis are consistent with a requirement for both Cib2 and Cib3 to ensure normal MET in all lateral-line hair cells."

Line 277. I would be more precise and say something like "and sufficiently fewer hair cells responded to mechanical stimuli and admitted Ca²⁺..."

We have modified the text as requested: "We quantified the number of hair bundles per neuromast with mechanosensitive Ca²⁺ responses, and found that compared to controls, significantly fewer cells were mechanosensitive in *cib2* and *cib2;cib3* mutants (Figure 5-figure supplement 2A, control: 92.2 ± 2.5 ; *cib2*: 49.9 ± 5.8 , *cib2;cib3*: 19.0 ± 6.6 , $p > 0.0001$)."

Line 278 and elsewhere. It doesn't make sense to have three significant digits in the error. I would say either "92.2 {plus minus} 2.5" or "92 {plus minus} 2."

Edited as requested.

Lines 357-358. Move the reference to the figure to the previous sentence, leaving the " (Liang et al., 2021) juxtaposed to its reference (crystal structure). Otherwise, the reader will look for crystal structures in Figure 7-figure supplements 1-5.

Text has been edited as requested: "The intracellular domain linking helices a2 and a3, denoted here as IL1, adopts a helix-loop-helix with the two helices running parallel to each other and differing in length (Figure 7-figure supplements 1-5). This is the same fold observed in its crystal structure in complex with CIB3 (Liang et al., 2021), which validated the modeling approach."

Line 450. What other ions were present besides K⁺? I assume Cl⁻ or some other anion.

What about Na⁺ or Ca²⁺? It's hard to evaluate this sentence without that information.

Systems have 150 mM KCl and CIB-bound Ca²⁺ when indicated (no Na⁺ or free Ca²⁺). This is now pointed out when the models are described first: "These models were embedded in either pure POPC or stereocilia-like mixed composition bilayers and solvated (150 mM KCl) to ...". The sentence mentioned by the reviewer has also been modified: "In systems with pure POPC bilayers we observed permeation of K⁺ in either one or both pores of the TMC1 dimer, with or without CIB2 or CIB3 and with or without bound Ca²⁺, despite the presence of Cl⁻ (150 mM KCl)."

Lines 470-472. These results suggest that the maximum conductance of TMC1 > TMC2. How do these results compare with the Holt and Fettiplace data?

Thanks for pointing this out. A comparison would be appropriate and has been added: "We also speculate that this is due to TMC2 having an intrinsic lower singlechannel conductance than TMC1, as has been suggested by some experiments (Kim et al., 2013), but not others (Pan et al., 2013). It is also possible that our TMC2 model is not in a fully open conformation, which can only be reached upon mechanical stimulation."

Line 563. Yes, the simulations only allow you to say that the interaction is stable for at least microseconds. However, the gel filtration experiments suggest that the interaction is stable for much longer. Please comment.

Thank you for pointing this out. We agree with this statement and modified the text accordingly: “Simulations of these models indicate that there is some potential preferential binding of TMC1 and TMC2 to CIB3 over CIB2 (predicted from BSA) and that TMC + CIB interactions are stable and last for microseconds, with biochemical and NMR experiments showing that these interactions are stable at even longer timescales.”

Figure 3. Please use consistent (and sufficiently large to be readable) font size.

Figure has been updated.

Figure 4. Magnification is too low to say much about bundle structure.

The reviewer is right – we cannot evaluate bundle structure with the images shown in Figure 4. Our goal was to determine if the vestibular hair cells had been degenerated in the absence of CIB2/3 and Figure 4 panel A data reveals intact hair cells. We changed the text “High-resolution confocal imaging did not reveal any obvious vestibular hair cell loss and hair bundles looked indistinguishable from control in *Cib2KO/KO*; *Cib3KO/KO* mice (Figure 4A).” to “High-resolution confocal imaging did not reveal any obvious vestibular hair cell loss in *Cib2KO/KO*; *Cib3KO/KO* mice (Figure 4A).” to avoid any confusions.

Reviewer #2 (Recommendations For The Authors):

Some datasets presented here can be published separately. Although I understand that the field is developing fast and there is no time to sort and fit the data by category or scale, everything needs to be published together and quickly.

*I have no real questions about the data on the functional association of CIB2 and 3 with TMC 1 and 2 in mouse hair cells as well as association preferences between their homologs in zebrafish. The authors have shown a clear differentiation of association preferences for CIB2 and CIB3 and the ability to substitute for each other in cochlear and vestibular hair cells. The importance of CIB2 for hearing and CIB3 for vestibular function is well documented. The absence of the startle response in *cib2/3* negative zebrafish is a slight variation from what was observed in mice where CIB2 is sufficient for hearing. The data look very solid and show an overall structural and functional conservation of these complexes throughout vertebrates. The presented models look plausible, but of course, there is a chance that they will be corrected/improved in the future.*

Thanks for appreciating the significance of our study.

Regarding NMR, there is indeed a large number of TROSY peaks of uniformly labeled CIB2 undergoing shifts with sequential additions of the loop and the N-terminal TMC peptides. Something is going on. The authors may consider a special publication on this topic when at least partial peak assignments are established.

We are continuing our NMR studies of CIB and TMC interactions and plan to have follow up studies.

After reading the manuscript, I may suggest four topics for additional discussion.

(1) Maybe it is obvious for people working in the field, but for the general reader, the simulations performed with and without Ca^{2+} come out of the blue, with no explanation. The authors did not mention clearly that CIB proteins have at least two functional EF-hand (EF-hand-like) motifs that likely bind Ca^{2+} and thereby modulate the MET channel.

This is a good point. We have modified the introductory text to include: “CIB2 belongs to a family of four closely related proteins (CIB1-4) that have partial functional redundancy and similar structural domains, with at least two $\text{Ca}^{2+}/\text{Mg}^{2+}$ -binding EF-hand motifs that are highly conserved for CIB2/3 (Huang et al., 2012).”

If the data on affinities for Ca^{2+} , as well as Ca^{2+} -dependent propensity for dimerization and association with TMC exist, they should be mentioned for CIB2 and CIB3 and discussed.

To address this, we have added the following text to the discussion: “How TMC + CIB interactions depend on Ca^{2+} concentration may have important functional implications for adaptation and hair cell mechanotransduction. Structures of CIB3 and worm CALM-1, a CIB2 homologue, both bind divalent ions via EF-hand motifs proximal to their C-termini (Jeong et al., 2022; Liang et al., 2021). Reports on CIB2 affinities for Ca^{2+} are inconsistent, with K_D values that range from 14 μM to 0.5 mM (Blazejczyk et al., 2009; Vallone et al., 2018). Although qualitative pull-down assays done in the presence or the absence of 5 mM CaCl_2 suggest that the TMC1 and CIB2 interactions are Ca^{2+} -independent (Liang et al., 2021), strength and details of the CIB-TMC-IL1 and CIB-TMCNT contacts might be Ca^{2+} -dependent, especially considering that Ca^{2+} induces changes that lead to exposure of hydrophobic residues involved in binding (Blazejczyk et al., 2009).”

Also, it is not clearly mentioned in the figure legends whether the size-exclusion experiments or TROSY NMR were performed in the presence of (saturating) Ca^{2+} or not. If the presence of Ca^{2+} is not important, it must be explained.

Size exclusion chromatography and NMR experiments were performed in the presence of 3 mM CaCl_2 . We have indicated this in appropriate figure captions as requested, and also mentioned it in the discussion text: “Interestingly, the behavior of CIB2 and CIB3 in solution (SEC experiments using 3 mM CaCl_2) is different in the absence of TMC1-IL1.” and “Moreover, our NMR data (obtained using 3 mM CaCl_2) indicates that TMC1-IL1 + CIB2 is unlikely to directly interact with CIB3.”

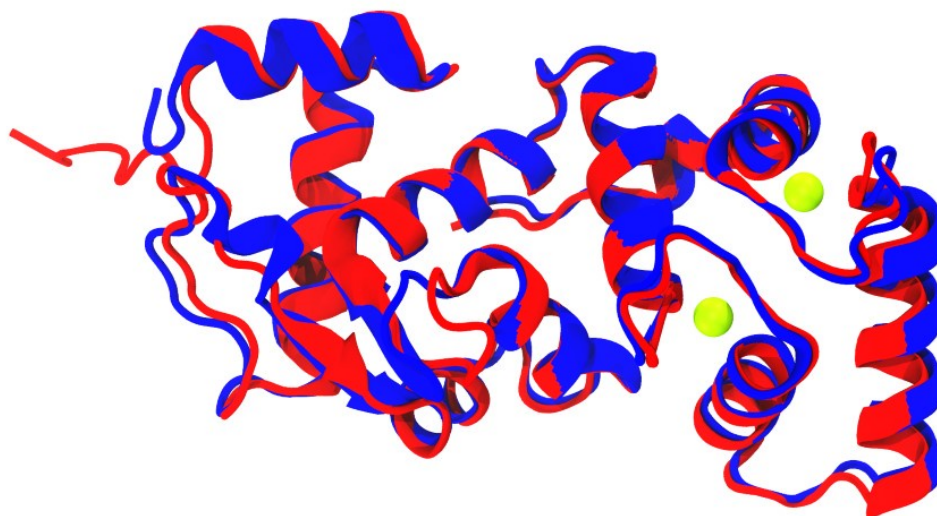
*(2) Speaking about the conservation of TMC-CIB structure and function, it would be important to compare it to the *C. elegans* TMC-CALM-1 structures. Is CALM-1, which binds Ca^{2+} near its C-terminus, homologous or similar to CIBs?*

This is an important point. To address it, we have added the following text in the discussion: “Remarkably, the AF2 models are also consistent with the architecture of the nematode TMC-1 and CALM-1 complex (Jeong et al., 2022), despite low sequence identity (36% between human TMC1 and worm TMC-1 and 51% between human CIB2 and worm CALM-1). This suggests that the TMC + CIB functional relationship may extend beyond vertebrates.” We also added: “How TMC + CIB interactions depend on Ca^{2+} concentration may have important functional implications for adaptation and hair cell mechanotransduction. Structures of CIB3 and worm CALM-1, a CIB2 homologue, both bind divalent ions via EF-hand motifs proximal to their C-termini (Jeong et al., 2022; Liang et al., 2021).”

Additionally, superposition of CALM-1 (in blue) from the TMC-1 complex structure (PDB code: 7usx; Jeong et al., 2022) with one and our initial human CIB2 AF2 models (in red) show similar folds, notably in the EF-hand motifs of CALM-1 and CIB2 (Author response image 1).

Author response image 1.

Superposition of CALM-1 structure (blue; Jeong et al., 2022) and AlphaFold 2 model of CIB2 (red). Calcium ions are shown as green spheres.



(1) Based on simulations, CIBs stabilize the cytoplasmic surfaces of the dimerized TMCs.

The double CIB2/3 knock-out, on the other hand, clearly destabilizes the morphology of stereocilia and leads to partial degeneration. One question is whether the tip link in the double null forms normally and whether there is a vestige of MET current in the beginning. The second question is whether the stabilization of the TMC's intracellular surface has a functional meaning. I understand that not complete knock-outs, but rather partial loss-of-function mutants may help answer this question. The reader would be impatient to learn what process most critically depends on the presence of CIBs: channel assembly, activation, conduction, or adaptation. Any thoughts about it?

These are all interesting questions, although further investigations would be needed to understand CIB's role on channel assembly, activation, conduction, and adaption. We have added to the discussion text: "Further studies should help provide a comprehensive view into CIB function in channel assembly, activation, and potentially hair-cell adaption."

(2) *The authors rely on the permeation of FM dyes as a criterion for normal MET channel formation. What do they know about the permeation path a 600-800 Da hydrophobic dye may travel through? Is it the open (conductive) or non-conductive channel? Do ions and FM dyes permeate simultaneously or can this be a different mode of action for TMCs that relates them to TMEM lipid scramblases? Any insight from simulations?*

We are working on follow-up papers focused on elucidating the permeation mechanisms of aminoglycosides and small molecules (such as FM dyes) through TMCs as well as its potential scramblase activity.

Reviewer #3 (Recommendations For The Authors):

Introduction:

The rationale and context for determining whether Cib2 and Cib3 proteins are essential for mechanotransduction in zebrafish hair cells is completely lacking in the introduction. All background information about what is known about the MET complex in sensory hair cells focuses on work done with mouse cochlear hair cells without regard to other species. This is especially surprising as the third author uses zebrafish as an animal model and makes major contributions to this study, addressing the primary question posed in the introduction. Instead, the authors relegate this important information to the results section. Moreover, not mentioning the Jeong 2022 study when discussing the Liang 2021 findings is odd considering that the primary question is centered on CIB2 and TMC1/2 in other species.

Thank you for pointing this out. We now discuss and reference relevant background on the MET complex in zebrafish hair cells in the introduction. We added: “In zebrafish, Tmcs, Lhfp15, Tmie, and Pcdh15 are also essential for sensory transduction, suggesting that these molecules form the core MET complex in all vertebrate hair cells (Chen et al., 2020; Erickson et al., 2019, 2017; Ernest et al., 2000; Gleason et al., 2009; Gopal et al., 2015; Maeda et al., 2017, 2014; Pacentine and Nicolson, 2019; Phillips et al., 2011; Seiler et al., 2004; Söllner et al., 2004).”. We also added: “In zebrafish, knockdown of Cib2 diminishes both the acoustic startle response and mechanosensitive responses of lateral-line hair cells (Riazuddin et al., 2012).”

Discussion:

The claim that mouse vestibular hair cells in the double KO are structurally normal is not well supported by the images in Fig. 4A and is at odds with the findings by Wang et al., 2023. More discussion about the discrepancy of these results (instead of glossing over it) is warranted. The zebrafish image of the hair bundles in the zebrafish cib2/3 double knockout also appear abnormal, i.e. somewhat thinner. These results are consistent with Wang et al., 2023. Is it the case that neither images (mouse and fish) are representative? Unfortunately, the neuromast hair bundles in the double mutant are not shown, so it is difficult to draw a conclusion.

The reviewer is right – we cannot evaluate mouse hair-cell bundle structure with the images shown in Figure 4. Our goal was to determine if the vestibular hair cells had been degenerated in the absence of CIB2/3 and Figure 4 panel A data reveals intact hair cells. We changed the text “High-resolution confocal imaging did not reveal any obvious vestibular hair cell loss and hair bundles looked indistinguishable from control in Cib2KO/KO;Cib3KO/KO mice (Figure 4A).” to “High-resolution confocal imaging did not reveal any obvious vestibular hair cell loss in Cib2KO/KO;Cib3KO/KO mice (Figure 4A).” to avoid any confusions. In addition, we have changed the discussion as follows: “We demonstrate that vestibular hair cells in mice and zebrafish lacking CIB2 and CIB3 are not degenerated but have no detectable MET, assessed via FM 1-43 dye uptake, at time points when MET function is well developed in wild-type hair cells.”

In the discussion, the authors mention that Shi et al showed differential expression with cib2/3 in tall versus short hair cells of zebrafish cristae. However, there is no in situ data in the Shi study for cib2 and cib3. Instead, Shi et al show in situs for zp1d1a and cabp5b that mark these cell types in the lateral crista. The text is slightly misleading and should be changed to reflect that UMAP data support this conclusion.

We have removed reference to cib2/3 zebrafish differential expression from our discussion. It is true that this differential expression has only been inferred by UMAP and not *in situ* data.

It should be noted that the acoustic startle reflex is mediated by the saccule in zebrafish, which does not possess layers of short and tall hair cells, but rather only has one layer of

hair cells. Whether saccular hair cells can be regarded as strictly 'short' hair cell types remains to be determined. In this paragraph of the discussion, the authors are confounding their interpretation by not being careful about which endorgan they are discussing (line 521). In fact, there is a general error in the manuscript in referring to vestibular organs without specifying what is shown. The cristae in zebrafish do not participate in behavioral reflexes until 25 dpf and they are not known to synapse onto the Mauthner cell, which mediates startle reflexes.

Thank you for pointing out these issues. We now state in the results that the startle reflex in zebrafish relies primarily on the saccule. In the discussion we now focus mainly on short and tall hair cells of the crista. We also outline again in the discussion that the saccule is required for acoustic startle and the crista are for angular acceleration.

Minor points:

Lines 298-302: The Zhu reference is not correct (wrong Zhu author). The statement on the functional reliance on Tmc2a versus Tmc1/2b should be referenced with Smith et al., 2020 and the correct Zhu 2021 study from the McDermott lab. Otherwise, the basis for the roles of the Tmcs in the cartoon in panel 6E is not clear.

Thanks for pointing out this oversight. We have updated the reference.

Line 548 should use numbers to make the multiple points, otherwise, this sentence is long and awkward.

The sentence has been re-arranged to make it shorter and to address another point raised by referees: “Structural predictions using AF2 show conserved folds for human and zebrafish proteins, as well as conserved architecture for their protein complexes. Predictions are consistent with previous experimentally validated models for the TMC1 pore (Ballesteros et al., 2018; Pan et al., 2018), with the structure of human CIB3 coupled to mouse TMC1-IL1 (Liang et al., 2021), and with our NMR data validating the interaction between human TMC1 and CIB2/3 proteins. Remarkably, the AF2 models are also consistent with the architecture of the nematode TMC-1 and CALM-1 complex (Jeong et al., 2022), despite low sequence identity (36% between human TMC1 and worm TMC-1 and 51% between human CIB2 and worm CALM-1). This suggests that the TMC + CIB functional relationship may extend beyond vertebrates.”

Suggested improvements to the figures:

In general, some of the panels are so close together that keys or text for one panel look like they might belong to another. Increasing the white space would improve this issue.

Figure 3 has been adjusted as requested, Figure 7 has been split into two (Figure 7 and Figure 8) to make them more readable and to move data from the supplement to the main text as requested below.

Fig1A. The control versus the KO images look so different that this figure fails to make the point that FM labeling is unaffected. The authors should consider substituting a better image for the control. It is not ideal to start off on a weak point in the first panel of the paper.

We agree and have updated Figure 1 accordingly.

Fig1C. It is critical to state the stage here. Also P12?

scRNA-seq data are extracted from Matthew Kelley's work and are a combination of P1, P12 and P100 utricular hair cells as following: Utricular hair cells were isolated by flow cytometry from 12- and 100-day old mice. Gene expression was then measured with scRNA-seq using the 10x platform. The data were then combined with a previously published single cell data set (samples from GSE71982) containing utricular hair cells isolated at P1. This dataset shows gene expression in immature vs mature utricular hair cells. The immature hair cells consist of a mixture of type I and type II cells.

Fig1D. This schematic is confusing. The WT and KO labels are misplaced and the difference between gene and protein diagrams is not apparent. Maybe using a different bar diagram for the protein or at least adding 'aa' to the protein diagrams would be helpful.

Sorry for the confusion. We have revised panel 1D to address these concerns.

Fig1E. Would be good to add 'mRNA' below the graph.

Done. We have added "mRNA fold change on the Y-axis" label.

Fig2C and D. Why use such a late-stage P18 for the immunohistochemistry?

Data presented in panel 2C are from P5 explants kept 2 days *in vitro*. For panel 2D, P18 is relevant since ABR were performed at P16 and hair cell degeneration in CIB2 mutants as previously described occurs around P18-P21.

Fig3A. Why isn't the cib2^{-/-} genotype shown?

Data on cib2^{-/-} mutant mice have already been published and no vestibular deficits have been found. See Giese *et al.*, 2017 and Michel *et al.*, 2017

Fig3F. Does this pertain to the open field testing? It would make sense for this panel to be associated with those first panels.

Figure 3 has been updated as requested.

Fig4A. Which vestibular end organ? Are these ampullary cells? (Same question for 4B.) The statement in the text about 'indistinguishable' hair bundles is not supported by these panels. There appears to be an obvious difference here--the hair bundles look splayed in the double KO. Either the magnification of the images is not the same or the base of the bundles is wider in the double KO as well. This morphology appears to be at odds with results reported by Wang et al., 2023.

The vestibular end organs shown in Figure 4A are ampullae. Magnifications are consistent across all the panels. While reviewer might be right regarding the hair bundle morphology, SEM data would be the best approach to address this point. Unfortunately, we currently do not have such data and we believe that only vestibular hair loss can be addressed using IF images. Thus, we are only commenting on the absence of obvious vestibular haircell loss in the double KO mutants.

Fig4C. To support the claim that extrastriolar hair cells in the Cib3^{-/-} mice are less labeled with FM dye it would be necessary to at least indicate the two zones but also to quantify the fluorescence. One can imagine that labeling is quite variable due to differences in IP injection.

The two zones have been outlined in Figure 4C as requested.

Fig5. Strangely the authors dedicate a third of Figure 1 to describing the mouse KO of Cib3, yet no information is given about the zebrafish CRISPR alleles generated for this study. There is nothing in the results text or in this figure. At least one schematic could be added to introduce the fish alleles and another panel of gEAR information about cib2 and cib3 expression to help explain the neuromast data as was done in Fig1C.

We have added a supplemental figure (Figure 5-figure Supplement 1) that outlines where the zebrafish *cib2* and *cib3* mutations are located. We also state in the results additional information regarding these lesions. In addition, we provide context for examining *cib2/3* in zebrafish hair cells by referencing published data from inner ear and lateral line scRNAseq data in the results section.

Absolutely nitpicky here, but the arrow in 5H may be confused for a mechanical stimulus.

The arrow in 5H has been changed to a dashed line.

Why not include the data from the supplemental figure at the end of this figure?

The calcium imaging data in the supplement could be included in the main figure but it would make for a massive figure. In eLife supplements can be viewed quite easily online, next to the main figures.

Fig6. The ampullary hair bundles look thinner in 6I. Is this also the case for double KO neuromast bundles? Such data support the findings of Wang et al., 2023.

We did not quantify the width of the hair bundles in the crista or neuromast. It is possible that the bundles are indeed thinner similar to Wang et al 2023.

Fig7A. IL1 should be indicated in this panel.

IL1 has been indicated, as suggested.

Fig7 supp 12. Color coding of the subunits would be appreciated here.

Done as requested.

Fig7. Overall the supplemental data for Figure 7 is quite extensive and the significance of this data is underappreciated. The authors could consider pushing panel C to supplemental as it is a second method to confirm the modeling interactions and instead highlight the dimer models which are more relevant than the monomer structures. Also, I find the additional alpha 0 helix quite interesting because it is not seen in the C. elegans cryoEM structure. Panel G should be given more importance instead of positioned deep into the figure next to the salt bridges in F. Overall, the novelty and significance of the modeling data deserves more importance in the paper.

We thank the reviewer for these helpful suggestions. The amphipathic alpha 0 helix is present in the *C. elegans* cryo-EM structure, although it is named differently in their paper (Jeong et al., 2022). We have now clarified this in the text: “Our new models feature an additional amphipathic helix, which we denote α_0 , extending almost parallel to the expected plane of the membrane bilayer without crossing towards the extracellular side (as observed for a mostly hydrophobic α_0 in OSCA channels and labeled as H3 in the worm TMC-1

structure) ...”. In addition, we have modified Figure 7 and highlighted panel G in a separate Figure 8 as requested.

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