Alterations of the CIB2 calcium- and integrin-binding protein cause Usher syndrome type 1J and nonsyndromic deafness DFNB48

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Sensorineural hearing loss is genetically heterogeneous. Here, we report that mutations in CIB2, which encodes a calcium- and integrin-binding protein, are associated with nonsyndromic deafness (DFNB48) and Usher syndrome type 1J (USH1J). One mutation in CIB2 is a prevalent cause of deafness DFNB48 in Pakistan; other CIB2 mutations contribute to deafness elsewhere in the world. In mice, CIB2 is localized to the mechanosensory stereocilia of inner ear hair cells and to retinal photoreceptor and pigmented epithelium cells. Consistent with molecular modeling predictions of calcium binding, CIB2 significantly decreased the ATP-induced calcium responses in heterologous cells, whereas mutations in deafness DFNB48 altered CIB2 effects on calcium responses. Furthermore, in zebrafish and Drosophila melanogaster, CIB2 is essential for the function and proper development of hair cells and retinal photoreceptor cells. We also show that CIB2 is a new member of the vertebrate Usher interactome.

We previously mapped to chromosome 15q23-q25.1 a locus associated with type 1 Usher syndrome (USH1H) that segregated in two families and a locus associated with autosomal recessive nonsyndromic hearing impairment (ARNSHI) (DFNB48) that segregated in five families1,2. Subsequently, we identified 52 additional families with DFNB48 (Supplementary Figs. 1 and 2). Here, we report that, in affected subjects in 54 Pakistani families with DFNB48, we found a homozygous mutation (c.272T>C, p.Phe91Ser) of CIB2 (Figs. 1 and 2a and Supplementary Fig. 3), and, in two families with DFNB48 (DEM4025 and DEM4225), we identified a c.297C>G (p.Cys99Trp) CIB2 mutation that cosegregated with deafness (Figs. 1 and 2a). Hence, mutation of CIB2 is one of the major causes of ARNSHI within the Pakistani population (Supplementary Tables 1 and 2). In addition, a c.368T>C transition in CIB2 (p.Ile123Thr) cosegregated with ARNSHI in a Turkish family with DFNB48, family 802 (Figs. 1 and 2a). SNPs linked to CIB2 were genotyped in unrelated affected individuals homozygous for the c.272T>C and c.297C>G mutations, and the flanking haplotypes were consistent with a founder effect for both alleles (Supplementary Tables 3 and 4).

The CIB2 gene lies distal to the critical interval of the USH1H locus that was defined by linkage analysis in family PKDF125 (ref. 2). As expected, no mutations in exons of CIB2 were found in affected members of family PKDF125. However, affected individuals in another family with USH1, PKDF117 (Fig. 1c), were found to be homozygous for the c.192G>C (p.Glu64Asp) mutation in CIB2. This new locus is designated USH1J. Thus, USH1J and deafness DFNB48 are caused by allelic mutations. The four recessive mutations of CIB2 cosegregate with deafness or deafblindness, whereas carriers have normal hearing. No carriers of the c.192G>C and c.368T>C mutations were found in 676 and 724

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proteins containing three or four EF-hand domains that change conformation upon binding of calcium and presumably mediate intracellular calcium signaling. Human CIB2 encodes three alternatively spliced isoforms, all of which are affected by the four mutations identified in USH1J and deafness DFNB48 (Fig. 2a). The CIB1 protein is 38% identical and 59% similar to CIB2, and its crystal and nuclear magnetic resonance (NMR) structures,7,8 were used to model the effects of mutations in USH1J and deafness DFNB48 (Fig. 2b,c). Three of the affected residues, which are conserved, Glu64, Phe91 and Cys99 (Supplementary Fig. 4), are in a region implicated in interaction with the C-terminal unstructured, negatively charged tail of β integrin.7,8 The substitutions at these residues may weaken the interaction of CIB2 with integrin (Fig. 2b,c, Supplementary Fig. 5 and Supplementary Table 5), affecting integrin activation9 and possibly the efficiency of calcium sequestration by CIB2, as a result of potentially subtle changes in subcellular localization. In the absence of integrin, Arg33 forms a salt bridge with Glu64 (Fig. 2b).

Figure 1 Pedigrees of families with USH1J or DFNB48. One family with USH1J and 4 of 57 families with nonsyndromic sensorineural hearing impairment (NSHI) DFNB48 segregating CIB2-mutant alleles. Filled symbols represent affected individuals, and a double horizontal line indicates a consanguineous marriage. Haplotypes for selected individuals of families PKDF356 and PKDF282 indicate the smallest linkage interval. The proximal breakpoint (arrowhead) is defined by affected individual VI:3 of family PKDF356 at marker D15S975 (78.13 Mb). The distal breakpoint (arrowhead) is defined by unaffected individual V:5 (PKDF282) at D15S975 (78.56 Mb). CIB2-mutant alleles (c.272T>C, p.Phe91Ser; c.297C>G, p.Cys99Tyr; c.297C>G, p.Ile123Thr) cosegregate with the NSHI phenotype. The USH1 phenotype of family PKDF117 cosegregates with the c.192G>C (p.Glu64Asp) mutation of CIB2. These four recessive mutations cosegregate with deafness or deaf-blindness, whereas carriers have normal hearing A, B and C represent the alleles for each marker genotyped.

Figure 2 CIB2 isoforms, molecular models and functional effects of mutations. (a) Human CIB2 has six exons encoding three isoforms. Non-coding segments, sequences encoding EF-hand domains and other coding regions of exons are denoted by gray, blue and black boxes, respectively. The mutation and protein alteration identified in USH1J are shown in red, and the changes identified in DFNB48 are shown in black. (b) Molecular models using for a template the Protein Data Bank (PDB) 1X05 crystal structure of Ca2+-CIB1. (c) Model of CIB2 using a template for the NMR structure of CIB1 bound to αβ integrin peptide2. In b and c, the backbone ribbon is colored blue (N terminus) to red (C terminus), and two Ca2+ ions are represented by blue spheres. (d) Calcium responses in COS-7 cells transfected with five different DsRed-tagged CIB2 constructs. Data are normalized to the average response of mock-transfected cells and are shown as mean ± s.e.m. ***P < 0.0001; **P < 0.01; *P < 0.05. The p.Cys99Tyr alteration abolished the ability of CIB2 to decrease ATP-induced calcium release from the cell, whereas the p.Ile123Thr alteration enhanced this ability.
integrin, Arg33 and Glu64 do not form a salt bridge as a result of the different conformation of CIB2 (Fig. 2c). The p.Glu64Asp alteration might change the energetic cost of accommodating the integrin C-terminal tail, thereby affecting binding affinity or kinetics. Phe91 lines the effector-binding pocket, and the p.Phe91Ser alteration might therefore disrupt effector binding. Similarly, substitution at Trp99 may alter the effector-binding pocket or calcium binding by the second EF-hand domain. In contrast, the p.Ile123Thr alteration is located within the second CIB2 EF-hand domain neighboring Thr122, a Ca²⁺-coordinating residue. Our molecular modeling predicts that the p.Ile123Thr alteration increases the affinity of calcium binding (Fig. 2b,c).

To experimentally explore the effects of these CIB2 mutations on intracellular calcium signaling, we used ratiometric Ca²⁺ imaging to measure ATP-induced inositol triphosphate (IP₃)-dependent calcium responses¹⁰ in transiently transfected COS-7 cells. Cells expressing wild-type CIB2 had a 40% decrease in ATP-induced Ca²⁺ release compared to cells transfected with empty vector (Fig. 2d and Supplementary Fig. 6). The inhibitory effect of CIB2 on calcium responses could be due to its calcium-buffering ability⁶ or, similar to CIB1 and CaBP1, could be due to CIB2 interaction with IP₃ receptors³⁰–¹³. None of the four missense mutations resulted in notable changes in CIB2 distribution (data not shown). However, the p.Cys99Trp alteration abolished the ability of CIB2 to decrease Ca²⁺ release, whereas the p.Ile123Thr alteration enhanced this inhibition (Fig. 2d). These observations are consistent with modeling predictions of the effects of CIB2 mutations on the calcium-binding affinity of CIB2.

CIB2 is widely expressed in human and mouse tissues, including in the inner ear and retina (Supplementary Fig. 7). Transcriptome analysis showed a 57-fold enrichment of Cib2 mRNA in mouse cochlear hair cells at postnatal day (P) 7 compared to supporting cells (SHIELD; see URLs). Cib2 immunoreactivity was first observed at P2 in the organ of Corti and vestibular organs and was limited to supporting cells in the developing organ of Corti up until P8 (Supplementary Fig. 8a,b). Cib2 was observed in the cytoplasm of adult supporting cells, in the inner hair cell (IHC) and outer hair cell (OHC) cuticular plate and along the length of stereocilia (Fig. 3a–d and Supplementary Fig. 8a–k). Cib2 staining was often more intense at shorter row stereocilia tips than in the neighboring stereocilia of a longer row (Supplementary Table 6),
where it may be involved in calcium signaling that regulates mechanoelectrical transduction\(^4\). In P25 vestibular hair cells, Cib2 was also localized along the length of stereocilia and was concentrated in patches toward the tips of stereocilia (Fig. 3e,f). Gene gun transfection of auditory and vestibular hair cells with a CIB2-GFP expression vector resulted in targeting and concentration of CIB2-GFP in stereocilia tips (Fig. 3g–j). Notably, CIB2-GFP was also more concentrated at the tips of shorter row stereocilia.

Many members of the USH interactome bind to myosin VIIa and whirlin\(^{15,16}\). We show that Cib2 can multimerize and that it interacts with whirlin, which is localized at the tips of stereocilia\(^7\), and myosin VIIa (Fig. 4 and Supplementary Fig. 9). No interaction of CIB2 was found with the other reported USH proteins. Thus CIB2 is a member of the USH interactome (Supplementary Fig. 10). To explore whether myosin VIIa or whirlin are necessary for CIB2 localization at hair cell stereocilia tips, we immunostained organs of Corti from homozygous shaker-1 (Myo7a\(^{46,26}\)) and whirlin (Whrniwi) mice using antibody to CIB2. We observed no mislocalization of Cib2 in stereocilia of homozygous Myo7a\(^{-/-}\) or whrn\(^{-/-}\) mutant mice (Supplementary Figs. 8i–p and 11), indicating that, in vivo, myosin VIIa and whirlin are not required for localization of Cib2 in mouse inner ear hair cell stereocilia.

We next probed the function of Cib2 in the sensory cells of the ear and eye, which we studied in zebrafish, where cib2 (NM_200706.1) is expressed throughout development (Supplementary Fig. 12a). Using specific cib2-targeting morpholinos, we knocked down cib2 in embryos (Supplementary Figs. 12b and 13). We categorized the phenotypes at 72 hours post-fertilization (h.p.f.) into class 1 (normal appearance), class 2 (tail defect), class 3 (tail defect, microphthalmia and blood pooling) and class 4 (hydropigmentation, microphthalmia, tail defect and delayed development) (Fig. 5a,b). Approximately 80\% of 5-d-old morphants did not respond to acoustic stimuli or were unable to remain upright while swimming (Fig. 5c). Scanning electron microscopy (SEM) revealed a marked decrease in the number of hair cell patches (neuromasts) in morphants (Fig. 5d–k). However, among class 1 and 2 morphants, we found some neuromasts with hair cell bundles (Fig. 5f–i and Supplementary Fig. 14). To assess the functional status of neuromast hair cells in the lateral lines, we briefly exposed larvae to FM1–43, a styryl pyridinium dye that enters the hair cells via partially open MET channels at rest\(^{18–21}\), or its fixable analog, AM1–43. Controls showed prominent fluorescent neuromasts at the head and lateral line regions (Fig. 5i). Morphants had few or no fluorescent neuromasts at 96 h.p.f. (Fig. 5i). We measured the microphonic potentials of these neuromasts, and, consistent with FM1–43 dye uptake, we observed a reduction in extracellular receptor potentials in morphant embryos relative to scrambled morpholino–injected controls (Fig. 5m–o), which could be a result of nonfunctional or degenerating MET components in lateral-line hair cells. Thus, Cib2 function is essential for development, maintenance and/or function of the mechanosensory hair cells in zebrafish.

In the mammalian inner ear, an optimal intracellular Ca\(^{2+}\) concentration is critical for MET, adaptation, frequency tuning, hair bundle twitching, outer hair cell electromotility and afferent synaptic transmission\(^{22–32}\). Ca\(^{2+}\) concentrations in stereocilia depend on mobile calcium buffers, a mitochondria belt beneath the cuticular plate, and PMCA, a Ca\(^{2+}\)–ATPase\(^{14,22,33–35}\). Hair cell bundles of both cochlear and vestibular organs differentially express various calcium-binding proteins, including calmodulin, calretinin, parvalbumin and calbindin-D28K\(^{33,36–42}\), and mobile buffers that help maintain an optimal Ca\(^{2+}\) concentration. On the basis of stereocilia tip localization of Cib2, one may hypothesize that Cib2 temporarily sequesters calcium entering the stereocilia through MET channels until this
calcium exits the stereocilia through PMCA\textsuperscript{35,43–46} or is taken up by mitochondria beneath the cuticular plate. Another plausible function of Cib2, which is not mutually exclusive, is the maintenance of calcium homeostasis in the hair cell body, which, in turn, may modulate OHC electromotility\textsuperscript{28,29,47}. Furthermore, Cib2 is also concentrated in the cuticular plate region of hair cells, where an ATP-gated IP\textsubscript{3}-dependent intracellular Ca\textsuperscript{2+} store is located\textsuperscript{12}. Similar to CaBP1, Cib2 may interact directly with IP\textsubscript{3} receptors\textsuperscript{10}, modulating purinergic responses in OHCs\textsuperscript{12}. The analysis of hair cell physiology in a Cib2-mutant mice would help clarify and distinguish between some of these hypotheses.

To gain insight into the function of Cib2 in the mammalian eye, we determined the localization of Cib2 in the mouse retina. Cib2 immunoreactivity was observed in inner and outer segments of photoreceptor cells, as well as in the retinal pigmented epithelium (RPE). Diffuse immunoreactivity was also observed in the inner (IPL) and outer (OPL) plexiform layers and in the ganglion cell layer (Supplementary Fig. 15).

The Drosophila genome encodes one CIB-related gene, CG9236, which codes for a protein similar to human CIB2 (59% identical and 71% similar). CG9236 (referred to here as cib2) is expressed in several tissues, including the adult eye\textsuperscript{48}. Calcium levels control many aspects of Drosophila phototransduction\textsuperscript{49,50}. To further assess Cib2 function, we measured phototransduction activity with electoretino-grams (ERGs) following cib2-knockdown through RNA interference (RNAi). cib2\textsuperscript{RNAi} flies showed significantly reduced photoreceptor amplitude (Fig. 6a,b) and impaired responses to flicker stimuli at high frequencies (Fig. 6c,d). cib2\textsuperscript{RNAi} flies failed to reliably follow individual pulses by ~40 Hz, whereas controls only exhibited this behavior by ~70 Hz (Fig. 6e). The response amplitudes of cib2\textsuperscript{RNAi} flies to individual flicker stimuli also approached noise levels at lower frequencies than those of controls (Fig. 6f). Finally, cib2\textsuperscript{RNAi} flies failed to sustain an adequate photoresponse during prolonged stimulation, even at a low frequency of 1.7 Hz (Fig. 6e,f). Collectively, these data indicate that cib2 in Drosophila is necessary to achieve a strong, sustained photoresponse and to track fast light stimuli, phenotypes consistent with transiently elevated intracellular Ca\textsuperscript{2+} concentrations\textsuperscript{49,50}.

Because calcium dysregulation is associated with retinitis pigmentosa and light-induced retinal degeneration\textsuperscript{50}, we analyzed photoreceptor morphology in cib2\textsuperscript{RNAi} flies. Control and cib2\textsuperscript{RNAi} flies showed no obvious eye dysmorphism when raised under 12-h light:12-h dark conditions (Fig. 6g). However, cib2\textsuperscript{RNAi} flies exhibited significant photoreceptor degeneration when raised under constant light for 5 d (Fig. 6g). Thus, cib2 is required for proper phototransduction and prevention of light-dependent retinal degeneration. These physiological and morphological phenotypes are consistent with the presence of an elevated intracellular Ca\textsuperscript{2+} concentration\textsuperscript{49,50}, which inhibits the phospholipase C activity that normally activates cation-permeable TRP channels opening in response to light\textsuperscript{51}. High levels of intracellular calcium are also known to inactivate TRP channels and thereby reduce the photoreceptor response\textsuperscript{52}. Several previous studies have indicated that proper calcium regulation and phototransduction are necessary to maintain photoreceptor integrity, and defects in calcium regulation render individuals particularly sensitive to light-induced photoreceptor degeneration\textsuperscript{49,50}.

In summary, CIB2 mutations underlie USH1J and nonsyndromic deafness DFNB48. Because Cib2 is concentrated in stereocilia and interacts with myosin VIIa and whirlin and Cib2 morphants have reduced hair cell microphonic potential, we speculate that human CIB2 participates in calcium regulation in the mechanotransduction process. Our studies reveal that, as in humans, Drosophila Cib2 is critical for proper photoreceptor maintenance and function and that CIB2 has conserved roles in calcium homeostasis.

URLs. NHLBI Exome Sequencing Project variant database, http://evs.gs.washington.edu/EVS/; SHIELD, https://shield.hms.harvard.
eduviewgene.html?gene=Cib2; Primer3, http://frodo.wi.mit.edu/; 1000 Genome Project, browser.1000genomes.org/; Polyphen-2, http://genetics.bwh.harvard.edu/pph2/index.shtml; MutationTaster, http://www.mutationtaster.org/; Project HOPE, http://www.cmbi.ru.nl/hope/modeling; Y1000 Genome Project, browser.1000genomes.org/; Polyphen-2, manuscript. All authors contributed to the final version of the manuscript. Z.M.A. wrote the manuscript; G.I.F., E.K.B., T.C., I.A.B. and S.M.L. edited the work at the University of Kentucky, and T.B.F. supervised the work at the

**METHODS**

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

Z.M.A., Saima Riazzuddin and T.B.F. conceived and designed the study. Saima Riazzuddin and Z.M.A. performed linkage, RT-PCR and mutational analyses, cloned isoforms and provided bioinformatic evaluations. I.A.B. and S.L. contributed to the final version of the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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Two homology models of the CIB2 protein were constructed. The SWISS-MODEL server was used for modeling, and energy minimization constructs for mouse whirlin have been described previously. Informed consent was obtained from adult subjects and the parents of minor subjects. The degree of hearing impairment was assessed by pure tone air conduction audiometry at frequencies ranging from 250 to 8,000 Hz. Vestibular function was evaluated by tandem gait and Romberg testing. Funduscopic and ERG examinations were performed by an ophthalmologist on 14 affected individuals from families with deafness DFNB48 and two affected individuals from the family with USH1 (PKDF117) to detect the absence or presence of frank retinopathy. Peripheral blood samples or buccal swabs for genomic DNA extraction were collected from participating subjects.

Genetic linkage and mutation analysis studies. Using genomic DNA from affected members of two families with USH1 and five families with DFNB48 (Supplementary Table 2), we sequenced ~100 bp of adjacent intron sequence flanking all exons of the 16 candidate genes. CIB2-directed primer sequences are listed in Supplementary Table 7. Methods for direct sequencing and mutational analyses were described previously. Control DNA samples from ancestry-matched Pakistani, Turkish, Cornell Human Diversity and Caucasian populations were sequenced for CIB2 mutations.

Molecular modeling. Two homology models of the CIB2 protein were constructed; the templates were the high-resolution crystal structure of human CIB1 (PDB 1X0S) and the solution structure of a Ca2+-CIB1 complex with the cytoplasmic domain of the integrin cß1b subunit (PDB 2L5M) (Fig. 2b). The SWISS-MODEL server was used for modeling, and energy minimization and analysis were performed with Yasara (see URLs). We also used the Project HOPE web server to further determine the effects of missense mutations on the structure of CIB2 (ref. 55).

Calcium imaging. COS-7 cells were transfected with various DsRed-tagged constructs using Lipofectamine 2000 (Invitrogen) and 3–4 μg of DNA per ~1 ml of serum-free Opti-MEM medium (Invitrogen). After 20–28 h, cells were loaded with 18 μM ratiometric Ca2+ indicator, Fura-2 AM (Molecular Probes), for 1–1.5 h at room temperature. Fura-2 fluorescence was observed in L-15 medium at room temperature with sequential 340- and 380-nm illuminations at a rate of 0.78–0.81 image pairs per second. The 340- to 380-nm fluorescence (F340/F380) ratio images were calculated, and pixel values were converted to intracellular Ca2+ concentration using the calibration curve obtained with the Fura-2 Calibration kit (Molecular Probes). Calcium responses were evoked by application of 1 μM ATP for 50 s through a puff pipette of ~1 μm in diameter that was situated ~25 μm from the cells (Supplementary Fig. 6). The number of dishes used for every construct was 4 or greater, and the number of transfected cells for every construct was over 40.

Qualitative and relative quantitative RT-PCR analysis. For PCR-based expression analyses, we used cDNA panels (Clontech) produced from tissues obtained from 19- to 69-year-old humans and from 8- to 12-week-old mice. Gene-specific primers were used for exons 1 and 5 of Cib2. For relative quantitative analysis of Cib2, the PCR primer-binding sites were located in exons 4 and 5, and the TaqMan probe spanned the junction of exons 4 and 5.

Expression constructs encoding fluorescently tagged protein. We used PCR-ready adult human eye cDNA (Clontech) to clone the full-length isoform of CIB2 into vectors encoding GFP and tdTomato (Clontech) tags (Supplementary Fig. 16). Mouse full-length Cib2 cDNA was PCR amplified from P1 to P5 inner ear cDNA. For expression plasmids, both strands of the cDNA inserts were verified with Sanger sequencing. Full-length and deletion constructs for mouse whirlin have been described previously. Antibody validation. To validate antibody to CIB2 (H00010518-A01, Abnova), we performed a colocalization assay using COS-7 cells transfected with constructs expressing CIB2-GFP, DsRed-CIB1, GFP-CIB3 and GFP-CIB4 (Supplementary Fig. 16). COS-7 cells were transfected by electroporation (Neon, Invitrogen) and were incubated overnight at 37 °C with 5% CO2. Cells were then fixed with 4% paraformaldehyde (EM) for 20 min, permeabilized for 15 min in 0.2% Triton X-100 and blocked by incubation in 2% BSA and 5% normal goat serum for 30 min. All solutions were made with 1× PBS. COS-7 cells were incubated for 2 h with antibody to CIB2 diluted with blocking solution to a concentration of ~5 μg/ml. After washes, Alexa Fluor 568-conjugated goat antibody to rabbit IgG (Molecular Probes) diluted 1:500 was incubated with cells for 20 min at room temperature. Samples were mounted using ProLong Gold Antifade Reagent (Molecular Probes), and images were captured on an LSM780 confocal microscope equipped with a 63×1.4 numerical aperture (N.A.) objective (Zeiss Microimaging).

Immunostaining. C57BL/6j, shaker-2 (Myo15ash2/sh2) and whirler (Whrnwt/wh) mutant mice were handled according to the US NIH protocol 2635. Inner ears were dissected and immunostained as described with slight modifications. Tissue was fixed in 4% paraformaldehyde in 1× PBS (with Ca2+ and Mg2+) overnight at 4 °C. All other reagents, including antibody to CIB2 used at a 1:200 dilution and Alexa Fluor 488–conjugated IgG (Invitrogen), were diluted in 1× PBS supplemented with 2 mM EDTA. Samples were mounted with ProLong Gold Antifade Reagent and imaged using an LSM780 system. The sensory epithelium of the retina was dissected from adult CD1 mice, stained with the antibody to CIB2 and imaged using an LSM700 system. The fluorescence intensity of CIB2 labeling at the stereocilia tips of P13 and P31 C57BL/6j, P11 Whrnwt/wh and P31 Myo7a4626/4626 mice was measured using ImageJ software. The region of interest covered the tips of either the first (tallest) or second row of stereocilia from individual stereocilia bundles of inner hair cells. The integrated intensity of fluorescence was measured within these regions at the focal planes where stereocilia tips were in best focus. The values obtained were then divided by the number of stereocilia within the corresponding region to determine the labeling intensity per stereocilium. The amounts of CIB2 labeling at different stereocilia rows were compared using an unpaired t test.

Helios gene gun transfection. P2 to P3 organ of Corti and vestibular sensory epithelial explants of C57BL/6j, shaker-2 and whirler mutant mice were cultured for 1 d in DMEM supplemented with 7% FBS (Invitrogen) at 37 °C with 5% CO2 and were transfected with construct encoding CIB2-GFP using a Helios gene gun as described. After 24–48 h, cultures were fixed in 4% paraformaldehyde overnight at 4 °C and stained with rhodamine phalloidin as described. Then, samples were mounted with ProLong Gold Reagent and imaged using an LSM780 system.

Coinmunoprecipitation assays. HEK 293 cells (ATCC) were maintained using DMEM supplemented with 10% FBS, glutamine and penicillin-streptomycin (Invitrogen). Cells were plated at 80% confluency for 24 h at 37 °C in 5% CO2. On the day of transfection, 10 μg of each RNA was transfected into cells using the Fugene HD kit (Promega). After 48 h, cells were homogenized with a sonicator (Fisher Scientific) at intensity setting 2 for 10 s in buffer A (50 mM Tris-HCl, pH 7.5, 100 mM NaCl and 1% NP-40) containing a protease inhibitor mixture (Roche). Protein A–Sepharose CL-4B beads were incubated for 4 h with 5 μg of antibody to GFP and were washed three times with PBS containing 0.1% Triton X-100. Lysates were incubated for 16 h with the beads and were centrifuged at 10,000g for 3 min. Beads were washed with buffer A three times and boiled in 2× SDS sample buffer.

Zebrafish morpholino injections and fluorescent staining. Embryos were injected with a specific translation-blocking morpholino to zebrafish chd2 (7.5 ng; Supplementary Table 7), a splice-junction site-specific morpholino (10 ng) or a control morpholino (scrambled sequence) at the one-cell stage as described. Fluorescent labeling of lateral-line neuromasts with 3 μM AM1-43 was performed as described.
Five-day-old cib2 morphants and control larvae were placed in 10-cm diameter dishes and allowed to acclimate for 10 min before being tested. The startle reflex was recorded on video using a digital camera after larvae were stimulated with a series of taps on the edge of the dish, as previously described.58

Zebrafish imaging by SEM. cib2 morphants and control, mock-transfected larvae were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer supplemented with 2 mM CaCl2 for 1–2 h at room temperature. Specimens were dehydrated in a graded series of ethanol baths, dried at the critical point from liquid CO2, sputter coated with platinum (5.0 nm, controlled by a film-thickness monitor) and observed with a field-emission SEM (S-4800).

Recording of microphonic potential in zebrafish. Zebrafish larvae were anesthetized using 0.01% MS-222 (Tricaine, Sigma) dissolved in a normal bath solution containing 120 mM NaCl, 2 mM KCl, 10 mM HEPES, 2 mM CaCl2 and 0.7 mM NaH2PO4 at pH 7.3, supplemented with 150 mM tetrodotoxin (Sigma) to reduce muscle twitching. Larvae were secured on glass-bottom dishes with nylon fibers. Microphonic potentials were recorded at room temperature (22 °C) using borosilicate glass electrodes with 5–6 MΩ resistance that were placed near the apical edges of the lateral-line neuromasts. We recorded from posterior neuromasts that had seemingly healthy hair cells. Kinocilia tufts were deflected with a stiff glass probe driven by a piezoelectric actuator (PA 8/12, Piezosystem Jena) with sinusoidal stimuli of 2-μm peak-to-peak amplitude at 100 Hz. Microphonic potentials were recorded with an Axopatch 200B amplifier (Molecular Devices) in a current-clamp mode, further amplified by 10× (SIM983, Stanford Research) and low-pass filtered at 1–2 kHz. Each recording represents an average of 1,000 responses.

Drosophila genetics and morphogenesis studies. Transgenic lines carrying a UAS-driven hairpin RNA interference (RNAi) construct for CG9236 (ref. 59) on chromosomes 2 and 3 were obtained from the Vienna Drosophila RNAi Center (VDRC). These flies were crossed to a recombinant chromosome carrying three transgenes—pWIZ, upstream activating sequence (UAS)-Dicer2 and long-GMR-GAL4. pWIZ is an RNAi line that has diminished expression of the white gene, thus reducing autofluorescent pigmentation40; Dicer2 increases the efficiency of RNAi-mediated knockdown40; and long-GMR-GAL4 drives UAS-dependent gene expression specifically from a 25-W fluorescent light bulb for 5 d. Photoreceptor morphology was monitored in live flies using a water immersion, cornea neutralization epifluorescence procedure that has previously been described.62 We verified photoreceptor morphology using 2-μm plastic sections of dissected retinas that were counterstained with toluidine blue (Sigma) as previously described.63

Ten to 12 flies were examined per experimental group, and light-dependent degeneration assays were conducted 3 times.

ERG recordings in Drosophila. Flies were immobilized with CO2, mounted on a coverslip with pink dental wax (Electron Microscopy Sciences) and adapted to the dark. The recording electrode (a cotton wick containing 0.9% NaCl connected to a silver wire) was positioned on the surface of the eye, and the indifferent electrode (silver wire) was placed in the abdomen. Recordings were acquired at 10,000 Hz. White light stimuli (with an intensity of 1.70 × 10−14 photons per cm2 per second) were delivered through an optical fiber connected to a light-emitting diode (LED). Data were analyzed in MATLAB (MathWorks). All data were smoothed with (filter(ones(1,windowsize)/windowsize,1,data)), with windowsize = 1,000 measurements for 5-s stimuli, windowsize = 100 measurements for 300-ms stimuli and windowsize = 10% of cycle length for frequency measurements. To establish the amplitude, the absolute voltage difference between baseline and maximal responses was averaged over approximately 20 pulses per fly. To establish the response amplitude to individual flicker frequencies, each cycle’s maximum response and following minimum response were established, and the absolute value of their difference was averaged for pulses 11–23. Sustainability was tested with a train of 150 300-ms pulses at 1.7 Hz. Noise levels are given on the basis of baseline data.

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