Supporting Information

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SI Materials and Methods

Animals. All experimental procedures were conducted according to the policies of the Institutional Animal Care and Use Committee of Oregon Health & Science University. The stable transgenic lines *Tg(myo6b:R-GECO$_1$)* and *Tg(myo6b:Tmc2a$^{1–260}$-GFP-CAAX)* were generated by injecting a construct based on the Tol2/Gateway system (1) that contained a 6-kb minimal promoter of *myo6b* (2) driving expression specifically in hair cells.

Construction of the Ear cDNA Library. Juvenile zebrafish [25–50 d postfertilization (dpf)] were killed in E3 medium with a lethal overdose of the anesthetic tricaine methanesulfonate (Sigma). About 1,500 ears were collected to isolate 1.5 μg of RNA using an RNeasy kit (Qiagen). The cDNA library (constructed by Dualsystems Biotech AG) was generated using a pPR3-N vector, resulting in an N-terminal tag of NubG on each prey protein. The average insert size is 1.71 kb tagged with sequence encoding a NubG fragment at the 5′ end and 95% of the clones contain inserts larger than 250 bp.

Expression of Pcdh15a Bait. Truncated cDNAs of both *pcdh15aCD1* and *pcdh15aCD3* (encoding a short stretch of the extracellular domain plus transmembrane domains plus full-length cytosolic domains) were inserted into the pBT3-SUC vector used to generate pBT-CD1 and pBT-CD3 for screening. Both pBT-CD1 and pBT-CD3 were transformed into the yeast strain NMY51 (*MATa his3Δ200 trp1-901 leu2-3, 112 ade2 LYS2::(lexAop)$_4$-HIS3 ura3::(lexAop)$_8$-lacZ ade2::(lexAop)$_8$-ADE2 GAL4*) (Dualsystems Biotech). NMY51 transformants were selected on SD-Leu plates. pOst1-NubI and pPR3-N vectors were used as positive and negative controls for functional validation of pBT-CD1 and pBT-CD3 vectors.

Screening of Library for Interactors. All screening steps were followed the manufacturer's manual with proper controls and validations (DUALmembrane kit; Biotech). Ear-pPR3-NubG-x library DNA (20 μg) was transformed into NMY51 containing either pBT-CD1 or pBT-CD3, respectively. The double transformants were plated on SD-LeuTrp to calculate transformation efficiency and SD-LeuTrpHisAde plus 5 mM 3-amino-1,2,4-triazole (3-AT) to select positive interactors. A total of 3×10^7 colonies was screened, and about 500 colonies were recovered. Small amounts of positive colonies were used for colony PCR using the forward primer, 5′-ggaatccctggtggtccatac, and the backward primer, 5′-gcg tcc caa aac ctt ctc aag c. PCR products larger than 300 bp were sequenced. Nearly 100 colonies were identified with inserts larger than 300 bp, which represented 24 independent genes. Six vectors that, when cotransformed into yeast, showed comparable growth to the positive control vector on SD-LHWA plate were picked as candidates. For CD1, we identified *tmc2a*, *tmed9*, *sdcbp*, and *cldnb* as strong interactors. For CD3, we identified strong interactions with *pkar2aa* and *tmem35*.

RT-PCR and in Situ Hybridization. For 5 dpf larval tissues, two ears or five neuromasts were dissected or collected under anesthesia using a glass pipette. Extracted tissues were then placed into PCR tubes with RNase OUT (Genemate). The extracts were vigorously pipetted using a 200-μL pipette tip. Equal amounts of lysate were pipetted into Ecodry RT-PCR tubes for RT-PCRs using the primers listed above. For RT-PCR of adult tissues, organs were collected from two killed fish, and RNA was extracted using RNeasy columns (Qiagen). The Ecodry kit (Clontech) was used to generate cDNA via reverse transcription. The following primers were used for RT-PCR: *tmc1* forward, GTCTCCATTCCTCCCTCCTT; *tmc1* reverse, GGGGTTTGAGGCATAACTGA; *tmc2a* forward, CGGAACAACAAAGACAGCAC; *tmc2a* reverse, TCTTTCTGCAGATGCACACC; tmc2b forward, GTGATGAAGGACCTGGAGGA; *tmc2b* reverse, TCTGAACATGAACCCCCTTC.

For in situ hybridization of *tmc2a* and *tmc2b* transcripts, DIG-labeled sense and antisense RNA probes were synthesized from PCR fragments containing T3 and T7 RNA polymerase sites generated using the following primers (gene-specific sequence underlined): tmc2a-T3-F CATTAACCCTCACTAAAGGGAA TGCAGAAAGATGTGTCACTCGCATC, tmc2a-T7-R TAATACGACTCACTATAGGGTTGGGGCTACCGTATGTATGTGATATTC; tmc2b-T3-F CATTAACCCTCACTAAAGGGAAACCAGGAAATCCTCGTGGACCTC, tmc2b-T7-R TAATACGACTCACTATAGGGGAAGACAACTGTTTCGACTTCATCAGTG. For *tmc1*, DIG-labeled, sense and antisense RNA probes were synthesized from linearized pCR4 plasmid containing a *tmc1* insert generated using ACTTGGAGCAGGCAAGAAAA and TGACAACTGGCTGACAAAATG forward and reverse primers. In situ hybridization using sense probes yielded no specific staining pattern.

Antibodies for Coimmunoprecipitation and Immunohistochemistry. We chose peptides from mouse TMC1 and TMC2 sequences that scored highly on the Hopp and Woods hydrophilicity scale (3) and the Welling antigenicity scale (4). Antibodies for TMC1 and TMC2 were made (by Genemed Synthesis) by injecting rabbits with mouse TMC peptides [TMC1: KLPRRESLRPKRKRTR[C] (residues 24–39), [C]DEETRKAREKERRRRLRRG (residues 53–71), CKPWKMEKKIEVLKEAKKF (residues 102–120), and [C]NATAKGQKAANLDLKKKMK (residues 714–732); TMC2: [C]RPGSSRKKQMEHGSYHKGLQ (residues 49–68), [C]QGRKKDRRTSLKEQR (residues 80–94), SPKKEREALRKEAGKQLRKPRS[C] (residues 95–117), and [C]SRANAQLRKKIQALREVEKNHK) (residues 765–732)]. Peptide-specific antibodies were affinity purified, and then pooled. Each pooled antibody selectively recognized the appropriate TMC (Fig. S4). PCDH15-CD3 was detected using PB811 (Ulrich Mueller, The Scripps Research Institute, San Diego). Zebrafish anti-Pcdh15a is a custom made monoclonal antibody against an N-terminal fragment (1–324 aa; Abmart).

For the pulldown experiments of Fig. 2*A*, HEK 293T cells (10-cm dish) were transfected with 2 μg of each plasmid using Effectene (Qiagen). After 48 h, cells were harvested and washed with PBS, lysed on ice in 1 mL of PBS containing protease inhibitors (Sigma) using a probe sonicator. Membranes were sedimented at 100,000 × *g* for 30 min; the pellet was washed with 1 mL of PBS/protease inhibitors with sonication and resedimented. Membranes were solubilized using 1 mL of radioimmunoprecipitation assay (RIPA) buffer/protease inhibitors and sonication; detergent-insoluble material was removed by centrifugation at 100,000 × *g* for 15 min and 0.45-μm filtration.

An aliquot (200 μL) of the extract was trichloroacetic acid precipitated and resuspended in 200 μL of SDS/PAGE sample buffer and analyzed for totals. For immunoprecipitations (IPs), 200 μL of the extract was incubated with 0.5 mg of Dynabeads MyOne Tosylactivated beads (coupled with 12.5 μg of anti-TMC1, anti-TMC2, or rabbit IgG), and 100 μg of uncoupled rabbit IgG in RIPA buffer overnight (o/n) at 4 °C. Beads were washed three times with RIPA buffer, and proteins eluted at 70 °C for 30 min in

100 μL of sample buffer. For SDS/PAGE analysis, 2.5 μL was used for TMC1 or TMC2 and 25 μL for PCDH15. For immunoblotting, proteins were transferred to Immobilon P (Millipore); blotting was carried out with TMC1, TMC2, or PCDH15 antibodies.

The chimera experiments of Fig. 2 *B* and *C* used 2 μg of cadherin or chimera plasmid and 0.4 μg of Tmc1 plasmid. After 48 h, cells were harvested and washed with PBS, lysed on ice in 1 mL of PBS containing protease inhibitors (Sigma) using a probe sonicator. Nuclei and unbroken cells were sedimented at 5,000 × *g*, for 10 min. Three rounds of lysis were performed, and the liberated membrane material was sedimented at 100,000 × *g* for 30 min; the pellet was washed with 1 mL of PBS/protease inhibitors with sonication and resedimented. Membranes were solubilized using 1.5 mL of RIPA buffer/protease inhibitors and sonication; detergent-insoluble material was removed by centrifugation at 100,000 × *g* for 15 min. For totals, 50 μL of the extract was reduced in 100 μL total volume SDS/PAGE sample buffer; 20 μL was analyzed. For IPs, 1,450 μL of the extract plus 100 μg of uncoupled rabbit IgG was incubated with 0.5 mg of Dynabeads MyOne Tosylactivated beads (coupled with 12.5 μg of anti-TMC1), in an equal volume of PBS containing 0.5% BSA and 0.05% Tween 20 for 1 h at room temperature. Beads were washed three times with RIPA buffer, and proteins eluted in 100 μL of 2% (wt/vol) SDS. SDS/PAGE sample buffer was added to 150 μL total volume. For SDS/PAGE analysis, 20 μL was used for each Western.

To minimize IgG heavy-chain interference, a light-chain–specific HRP-conjugated secondary was used (Jackson ImmunoResearch). Blots were imaged using ECL prime (GE) and a Fuji LAS3000 camera.

For immunolabeling of HEK 293T cells, after transfection with AcGFP-TMC1 and PCDH15-CD3, the cells were fixed for 1 h with 4% (wt/vol) formaldehyde in DMEM containing 100 mM Hepes, pH 7.4. Cells were permeabilized for 10 min in 0.25% Triton X-100 in PBS, and then blocked with 1 mg/mL BSA, 2% donkey serum, and 5 mM EGTA in PBS. Cells were incubated with 1:400 (~1 μg/mL) anti-PCDH15 antibody PB811 for 1 h in block, washed, and then incubated with 2 μg/mL donkey anti-rabbit conjugated with Alexa 568 in block. After washing, cells were mounted with hard-set mounting medium containing DAPI. GFP, Alexa 568, and DAPI fluorescence were examined using an Olympus FV-1000 confocal microscope.

For immunolabeling of zebrafish hair bundles, larvae (4 dpf; $n > 5$ for each condition) were fixed with 4% (wt/vol) paraformaldehyde in PBST o/n at 4 °C, rinsed with PBST (0.1% Tween 20), and then permeabilized with 0.5% Triton X-100 (in PBST) o/n at 4 °C. Specimens were incubated in blocking solution (PBS/1% BSA plus 1% DMSO plus 2% goat serum) for 2 h. The primary antibody was diluted 1:200 (Pcdh15a; Abmart) in blocking solution, and specimens were incubated o/n at 4 °C. Alexa Fluor 647 goat anti-mouse IgG (H+L) (Life Technologies) secondary antibody was used at a 1:100 dilution (incubation for 2 h in PBST). Rhodamine–phalloidin was used at 1:50 (Life Technologies). For confocal images of live or fixed zebrafish specimens, samples were imaged on an upright Zeiss Imager.M1 LSM 700 microscope with a 63×, 0.95 N.A. Achroplan lens.

Quantification of the distribution of Pcdh15a immunolabeling was achieved using the *Histogram* function of *ZEN* imaging software (Carl Zeiss) to determine the area containing Pcdh15a-positive pixels that were above background. Phalloidin and antibody labeling was used to outline the region of interest for each hair bundle. The intensity of phalloidin was equally variable among GFP-positive and GFP-negative hair cells ($n \geq 10$ for each condition, $P > 0.5$, Student *t* test). Immature bundles under 4 μm tall were excluded from analysis.

1. Kwan KM, et al. (2007) The Tol2kit: A multisite gateway-based construction kit for Tol2 transposon transgenesis constructs. *Dev Dyn* 236(11):3088–3099.
2. Obholzer N, et al. (2008) Vesicular glutamate transporter 3 is required for synaptic transmission in zebrafish hair cells. *J Neurosci* 28(9):2110–2118.
3. Hopp TP, Woods KR (1981) Prediction of protein antigenic determinants from amino acid sequences. *Proc Natl Acad Sci USA* 78(6):3824–3828.
4. Welling GW, Weijer WJ, van der Zee R, Welling-Wester S (1985) Prediction of sequential antigenic regions in proteins. *FEBS Lett* 188(2):215–218.

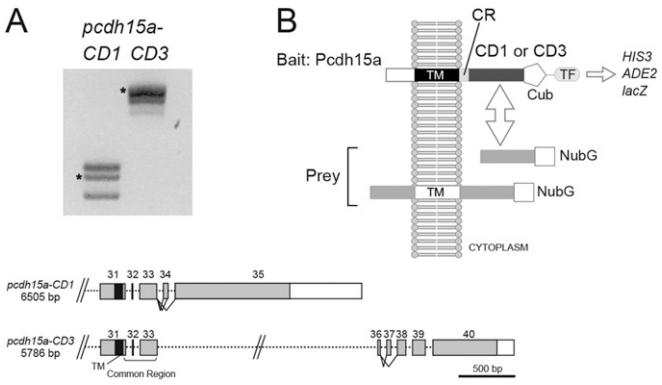

Fig. S1. Zebrafish splice variants of pcdh15a and the split-ubiquitin two-hybrid screening method. (*A*) RT-PCR products encoding the intracellular domains of the CD1 and CD3 splice variants of *pcdh15a* in zebrafish. The asterisks indicate the amplicons used in the screen. Additional shorter splice variants (lower bands) were identified and found to be the product of alternative splicing of either exon 34 (CD1) or exon 37 (CD3) as indicated in lower bar diagram. (*B*) Diagram of the split-ubiquitin screen using a truncated version of Pcdh15a-CD1 or Pcdh15a-CD3 that includes the single-pass transmembrane domain. Both isoforms were tagged with the C-terminal half of a ubiquitin moiety (Cub) at the C terminus. In total, 1×10^7 library clones were tagged at the 5′ end with sequence encoding the N-terminal half of a ubiquitin moiety (NubG). Binding of prey reconstitutes the ubiquitin tag (NubG + Cub = ubiquitin), which can then be cleaved by cellular proteases, thereby releasing a transcriptional activator (TF) of reporter genes: ADE2 (phosphoribosylaminoimidazole carboxylase), HIS3 (imidazoleglycerol-phosphate dehydratase), and lacZ (β-galactosidase).

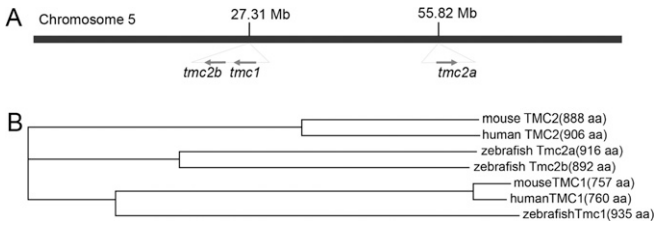

Fig. S2. The chromosomal locations and phylogenetic relationships of the zebrafish *tmc1/2* genes. (*A*) In the Ensembl database (Zv9), zebrafish *tmc1* (si:dkey-229d2.1) is located on chromosome 5 at 27.29 Mb, directly adjacent to *tmc2b* (ENSDARG00000030311.60) at 27.26 Mb, whereas *tmc2a* (ENSDARG00000033104.7) is located on the same chromosome at position 55.81 Mb. (*B*) Phylogenetic relationships of the zebrafish, mouse, and human orthologs using *ClustalW2* (neighbor joining tree without distance corrections). GenBank accession numbers are as follows: mTMC1, NP_083229.1; mTMC2, NP_619596; hTMC1, NP_619636.2; hTMC2, NP_542789; the accession numbers of the zebrafish genes are as follows: *tmc1*, KM115406; *tmc2a*, KM115407; *tmc2b*, KM115408.

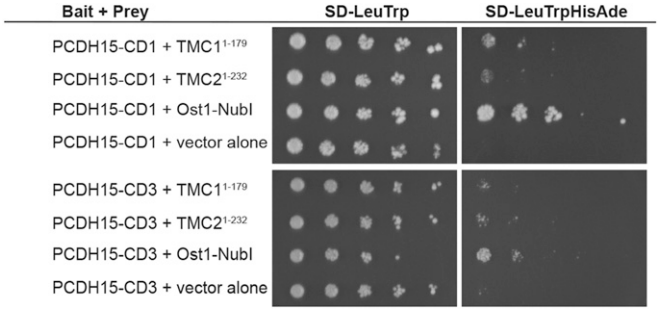

Fig. S3. Interaction of the N termini of *Mus musculus* TMC1 (1–179 aa) and TMC2 (1–232 aa) with either mouse PCDH15 isoform (membrane domain and cytoplasmic tail) using the split-ubiquitin two-hybrid assay.

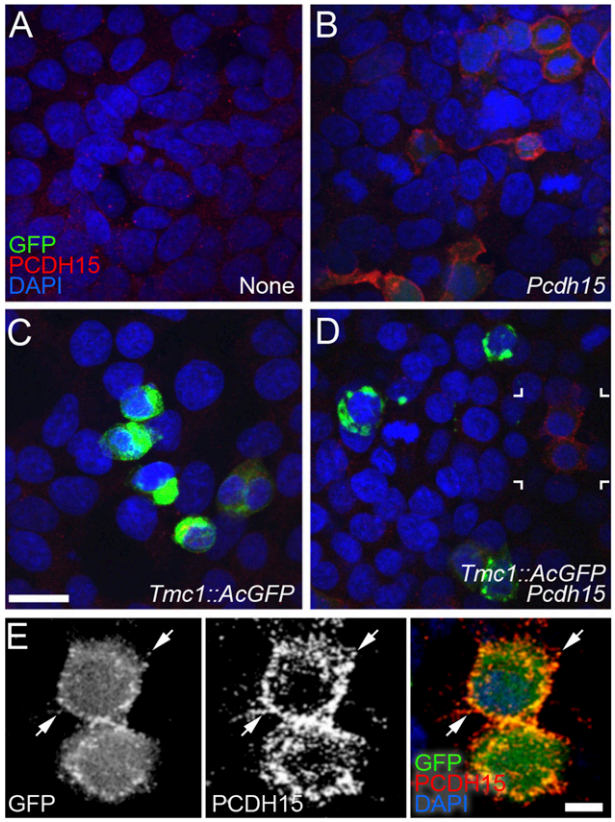

Fig. S4. Expression of PCDH15-CD3 and TMC1-AcGFP in HEK 293T cells. (*A*) No plasmids. (*B*) *Pcdh15* plasmid alone. (*C*) *Tmc1::GFP* plasmid alone. (*D*) Co-transfection with *Pcdh15* and *Tmc1::GFP* plasmids. PCDH15 staining was reduced. Following transfection of HEK cells with a mixture of TMC1 and PCDH15 plasmids, most cells had only one of the two proteins detectably expressed. For *A*–*D*, all imaging settings were identical. (*E*) Magnified version of double-transfected cells indicated by marks in *D*. (*Left*) TMC1 immunolabeling. (*Center*) PCDH15 immunolabeling. (*Right*) Merge with DAPI stain of nuclei. The arrows indicate the presence of both proteins at the plasma membrane. Coexpression of TMC1 and PCDH15 in HEK cells; more colocalization is apparent in the cytoplasm. Only a small subset of cells, including the two depicted, had clear colocalization. (Scale bar: 5 μm.)

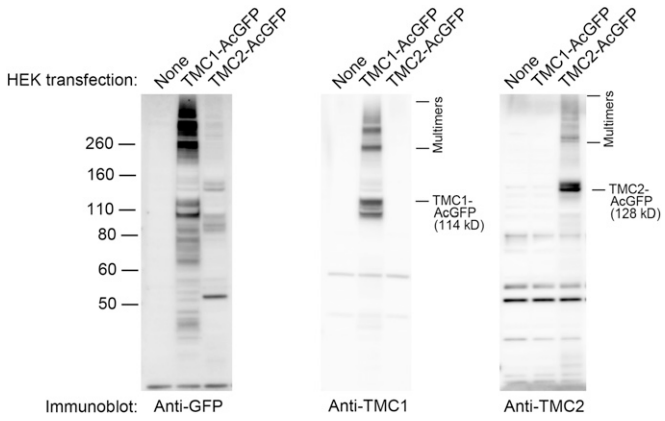

Fig. S5. Specificity of the TMC antibodies. HEK 293T cells were transfected with the indicated plasmids; whole-cell lysates were separated by SDS/PAGE and immunoblotted with the indicated antibodies. The anti-GFP blot (*Left*) shows that total TMC1 expression was much greater than total TMC2 expression. The TMC1 antibody recognized TMC1 but not TMC2, with minimal background (*Center*); the TMC2 antibody was absolutely selective for TMC2 over TMC1 but also reacted with several nonspecific bands (*Right*). Each antibody recognized bands in the correct size range for TMC monomers (indicated on blot), but also high–molecular-weight bands that were likely SDS-resistant multimers.

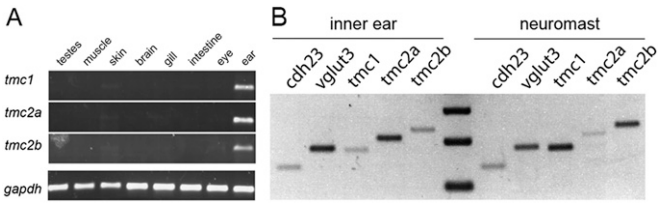

Fig. S6. Expression of *tmc1, tmc2a*, and *tmc2b* transcripts in adult and larval tissues. (*A*) RT-PCR revealed products for all three genes in the inner ear. Lanes: 1, testes; 2, muscle; 3, skin; 4, brain; 5, gills; 6, intestine; 7, eye; 8, ear. cDNA for *gapdh* was used as a positive control. (*B*) RT-PCR of cDNA isolated from larval inner ears and lateral-line neuromasts. Control reactions include amplification of hair cell-specific *cdh23* and *vglut3* cDNA.

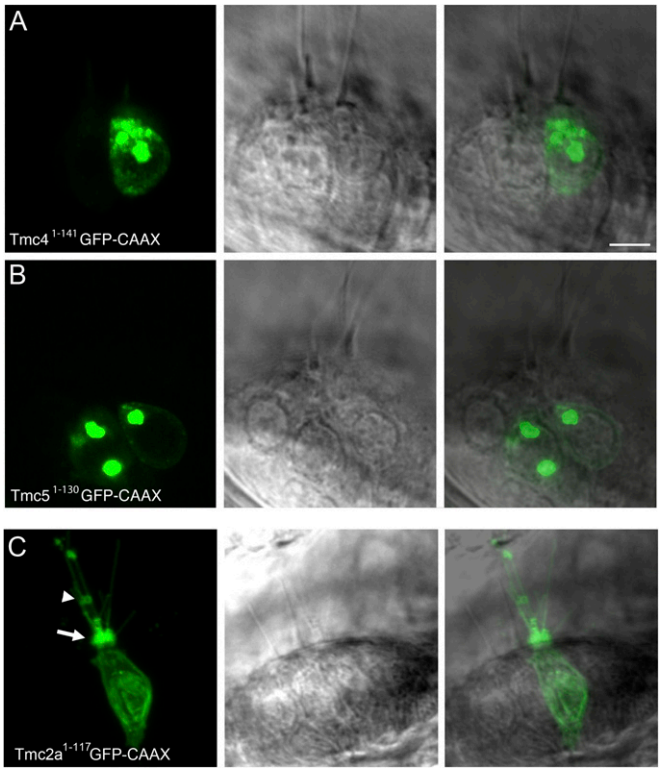

Fig. S7. N-terminal fragments of distantly related Tmcs accumulate within the cell body of hair cells. (*A*–*C*) Live images of ampullary hair cells. (*A*) Tmc4 (1–141 aa) and (*B*) Tmc5 (1–130 aa) were tagged with GFP-CAAX and mosaically expressed in hair cells using the *myo6b* promoter. In both low and high expressing hair cells, GFP signal is associated either with intracellular membrane compartments or the basolateral membrane and mostly excluded from hair bundles. Some faint bundle label is seen in the cell of *A* ($n \geq 24$ hair cells for each fragment). (*C*) Example of morphological defects in a hair cell expressing high levels of Tmc2a$^{1-117}$-GFP-CAAX. The hair bundle appears amorphous (arrow), and blebbing of the plasma membrane (arrowhead) and unusually long apical processes are evident. In contrast to Tmc4 and Tmc5, large brightly labeled internal compartments were not observed. Cells with morphological defects were excluded from our immunohistochemical and physiological experiments. (Scale bar: 5 μm.)

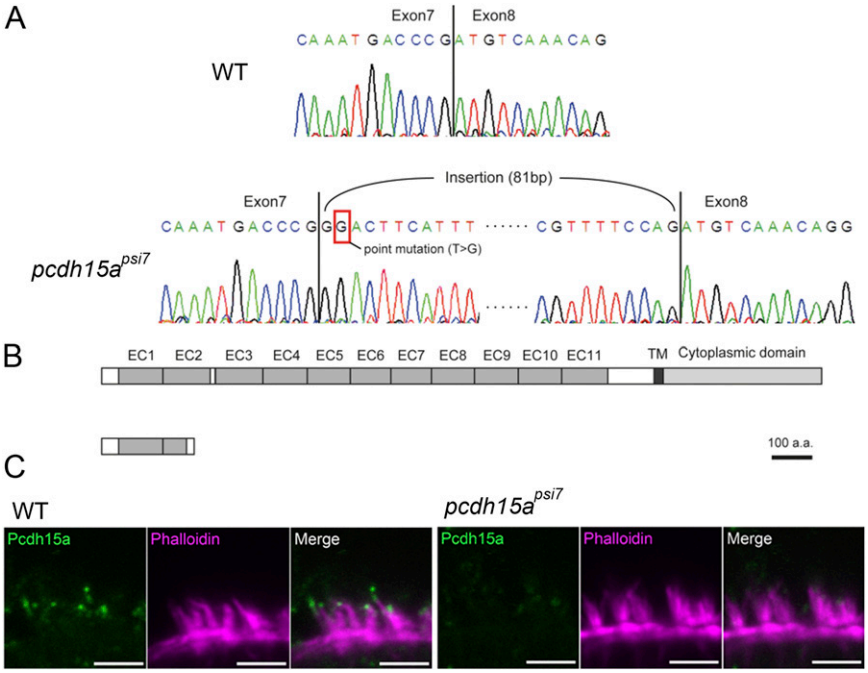

Fig. S8. Immunolabeling of Pcdh15a in wild-type siblings and *pcdh15a*^{psi7} mutants. (*A*) Sequence of the *psi7* allele, which encodes splice site mutation causing a frameshift following exon 7. (*B*) The predicted product is a short N-terminal fragment of Pcdh15a. (*C*) Immunolabeling with a monoclonal antibody against Pcdh15a. Actin filaments were labeled with rhodamine–phalloidin. (*Right*) Note the splaying of stereocilia, which is typical for mutants expressing strong alleles of *pcdh15a* (1). (Scale bar: 5 μm.)

1. Seiler C, et al. (2005) Duplicated genes with split functions: Independent roles of protocadherin15 orthologues in zebrafish hearing and vision. *Development* 132(3):615–623.

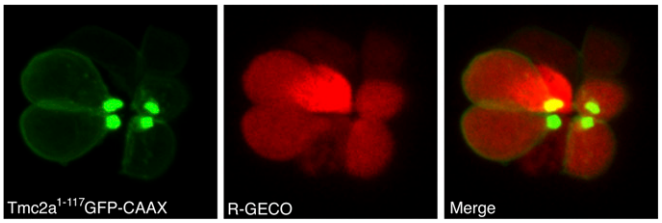

Fig. S9. Example of the Red-GECO$_1$ indicator in lateral-line hair cells. Top-down view of the mosaic expression of Tmc2a$^{1–117}$-GFP-CAAX in hair cells expressing R-GECO$_1$ at 3 dpf. In this example, four out of eight cells are GFP-CAAX positive. Note the four bright bundles near the center of the rosette. Most of the GFP signal is concentrated at the plasma membrane. Mature GFP-negative cells served as controls, allowing direct comparison of cells within the same neuromast.

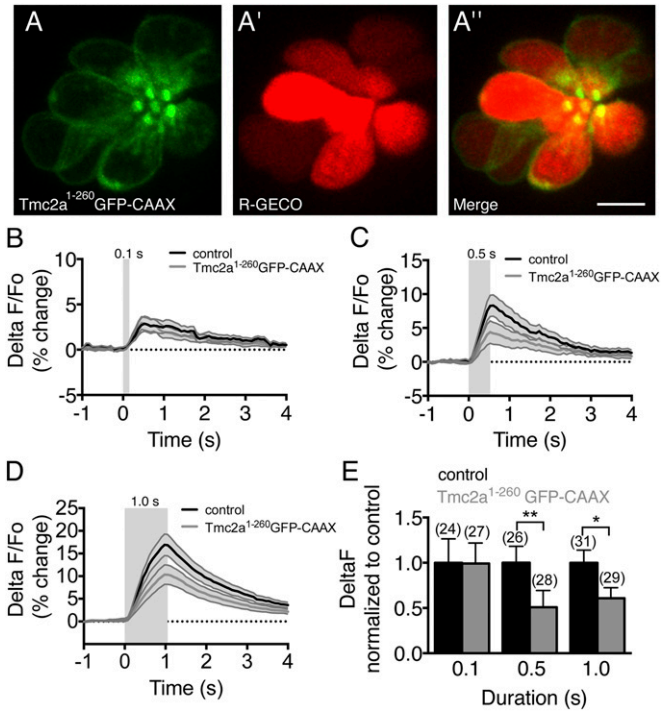

Fig. S10. Decreased mechanosensitive responses of hair cells stably expressing Tmc2a$^{1–260}$-GFP-CAAX at 3 dpf. (*A–A″*) Example of expression of the 260-aa N-terminal fragment in lateral-line hair cells. The bright oval-shaped puncta of exogenous Tmc2a fragments are enriched in apical hair bundles at the center of the rosette, although more label is present in the soma in comparison with the 117-aa fragment (Fig. S9). (Scale bar: 5 μm.) (*B–D*) Calcium transients in lateral-line hair cells in response to 0.1-, 0.5-, and 1.0-s deflections. (*E*) Summary of responses normalized to transgenic R-GECO$_1$ control hair cells.