Supporting Information

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SI Materials and Methods

Antibodies. Affinity-purified rabbit antibodies PB205 and PB206 (Covance) were generated against peptide (LPGQEGQAPSGFEDLERGR) corresponding to mouse myosin VIIa (MYO7A) isoform 1-amino acids 956 to 974 (GenBank: EDL16329.1). A mouse monoclonal anti-MYO7A antibody directed against amino acids 11 to 70 (sc-74516) was purchased from Santa Cruz Biotechnology. The specificity of all three MYO7A antibodies was validated by Western blots of protein extracts of COS7 cells expressing GFP-tagged MYO7A and of protein extracts from inner ear and testis tissues (Fig. S1) and by immunocytochemistry on COS7 cells expressing GFP-MYO7A (Fig. S2 *A–C*). Affinity-purified rabbit antibodies PB852 and PB906 were generated against peptides RQGTYANPKEWGRAPLR and AEPAHSEVSTDSGHD corresponding to human sans (GenBank: NP_775748.2) amino acids 257 to 273 and 290 to 304, respectively. The specificity of these two antibodies was validated by Western blots of protein extracts of COS7 cells expressing GFP-tagged sans (Fig. S1), by immunocytochemistry on COS7 cells expressing GFP-tagged sans (Fig. S2 *D* and *E*) and by the lack of labeling in the sans-deficient *Jackson shaker* (*Ush1g*^{js}) mice hair cells (Fig. S3*E*). Rabbit anti-CDH23 antibodies PB240 and PB264 were previously described (1). H3 rabbit anti-harmonin antibody (2) was obtained from Ulrich Muller (The Scripps Research Institute, La Jolla, CA). Anti-GFP antibody was purchased from Millipore, and secondary fluorescent antibodies and the mouse monoclonal anti-Flag M2 antibody were purchased from Sigma.

Western Blots. For protein extraction from P9 rat inner ear and testis, tissues were collected on-dry-ice, dounce-homogenized on ice, and sonicated in RIPA buffer (10% glycerol, 1% Nonidet P-40, 0.4% deoxycholate, 0.05% SDS, 150 mm NaCl, 5 mm EGTA, and 5 mm EDTA in 50 mm Tris, pH 7.4) with protease inhibitor mixture (Roche). The homogenates was cleared by centrifugation at 14,000 × *g* for 15 min at 4 °C, then diluted in 2× SDS loading buffer, heated at 95 °C for 5 min, then resolved by SDS/PAGE on 4% to 20% Tris glycine gels (Invitrogen) and transferred to polyvinylidene difluoride membranes. Immunoblotting was performed using peroxidase-coupled secondary antibodies (GE Healthcare) and chemiluminescence (Amersham Biosciences; ECL Plus). Protein extracts of 0.6 inner ear or 0.025 testis were loaded per lane. For Western blots of COS7 cells protein extracts, cells were lysed in RIPA buffer 24 hours after transfection with GFP-tagged sans or MYO7A.

Immunocytochemistry. Inner-ear tissues were dissected from mice, rats, and guinea pigs killed in accordance with National Institutes of Health guidelines under National Institute on Deafness and Other Communication Disorders animal protocol 1215–08. Immunofluorescence staining of MYO7A, sans, harmonin, and CDH23 was performed on whole-mount preparations of finely dissected organ of Corti and vestibular organs, as previously described (3). To minimize the effects of epitope masking, we fixed the tissue with low concentrations of fixative (2–3% paraformaldehyde) and incubated the tissues in the antibody solutions for over 12 h. Fluorescence confocal images were obtained with a Nikon microscope equipped with a 100× 1.45 numerical aperture objective and a Yokogawa spinning-disk confocal unit (PerkinElmer). For each immunofluorescence labeling and quantification, we used at least three cells from at least three different tissue preparations. All image analyses including fluorescence intensity quantification, Mander's fluorescence overlap coefficient (*R*), and Pearson's correlation coefficient (*Rr*) calculations (4) were performed with the National Institutes of Health ImageJ software. *R* values range between zero and 1, with 1 indicating high colocalization and zero indicating low colocalization. *Rr* values range between +1 and −1, with a value of +1 indicating a perfect correlation, −1 indicating perfect exclusion, and 0 indicating random localization (also see http://www.macbiophotonics.ca/imagej/colour_analysis.htm). To match the intensities of the each color channel in the final color images, the level and γ-settings were adjusted equally throughout entire images using Photoshop CS5 software (Adobe Systems).

cDNA Expression Vectors. pCMV6 expression vector containing cDNA clone encoding C-terminally in-tandem FLAG (DYKDDDDK) and Myc (EQKLISEEDL) tagged full-length human sans (nucleotides 184–1569 of sequence NM_173477.2), clone# RC218255, was purchased from OriGene. Full-length human SANS cDNA sequence has been amplified and cloned into pEGFP-C1 and pmCherry-C1 vectors (Clontech) within EcoRI and BamHI restriction sites, and the final constructs sequenced. EGFP-tagged full-length MYO7A and harmonin b1-encoding constructs were gifts from Peter J. Gillespie (Oregon Hearing Research Center, Portland, OR) and Ulrich Muller (The Scripps Research Institute, La Jolla, CA), respectively. Harmonin b1 cDNA was amplified and subcloned into pmCherry-C1 vector. Selected clones were verified by full-length cDNA sequencing. The EGFP-MYO10 encoding construct was a gift from Richard Cheney (University of North Carolina, Chapel Hill, NC), EGFP-MYO15A encoding construct was a gift from Thomas Friedman (National Institutes of Health, Bethesda, MD), and the EGFP-MYO3A-ΔK construct was previously described (3).

Cultures and Transfection of Rat Inner-Ear Tissue. After CO_2 anesthesia, postnatal day 0 (P0) to P4 rat pups were killed in accordance with National Institutes of Health guidelines, and their temporal bones were isolated and placed in L-15 medium (Invitrogen). Inner-ear explants were generated and transfected with several constructs using a Helios gene gun, as previously described (5), and incubated for up to 48 h. Samples were washed with PBS, fixed in 4% paraformaldehyde for 30 min, and permeabilized with 0.5% Triton X-100 in PBS containing alexa fluor 568 phalloidin for 30 min before washing and mounting on a microscope slide with the antifade medium (Sigma). The efficiency of transfection ranged from zero to four hair cells per tissue explant, and we examined at least three transfected cells for each condition.

Culture and Transfection of COS7 Cells. COS-7 cells were plated on coverslips and maintained at 37 °C in DMEM supplemented with 10% FBS. Cultures were transfected using GeneJuice solution (Novagen) according to the manufacturer's directions. Cells were fixed 24 h after transfection for 20 min in 4% PFA in PBS, permeabilized for 10 min in 0.5% Triton X-100 in PBS, processed for immunocytochemistry, and mounted on a microscope slide with antifade. Fluorescence colocalization evaluation has been performed on at least 10 transfected cells per experiment.

BAPTA Treatment for Tip-Link Disruption. Cochleae were dissected and perfused with 10 mM Hepes-buffered Ca^{2+} and Mg^{+}-free Hank's Buffered Salt Solution (HBHBSS) to allow solution to sufficiently substitute for endolymph. Samples were then perfused with HBHBSS supplied with 5 mM BAPTA [1,2-bis

(*o*-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid] and incubated for 2 min at room temperature. Samples were then recovered for 15 min at 37 °C in HBHBSS medium supplied with 3 mM $CaCl_2$ and 1 mM $MgCl_2$. Specimens were then fixed in 3% PFA and processed for immunofluorescence.

Mutant Mice and Genotyping. The *Jackson shaker* (*Ush1g*^{js}) mice carrying deleterious mutation 681insG were obtained from The Jackson Laboratory stock, no. 0007823. Primers *Js-Geno-F*: 5′-AGCTGGCCGAGCGCTCCGACACTC-3′ and *Js-Geno-R*: 5′-TCCGAGTGAGCAGGCTCGGCAGCCAG-3′ were used for allele PCR amplification (amplicon size of wild-type allele was 385 pb) and sequencing. *Shaker-1* mice (original) carrying transversion G1505C destroying BsrfI restriction site and causing Arg502Pro nonconservative substitution in Myo7a were obtained from The Jackson Laboratory stock, no. 000271. Primers *Shaker1-Geno-F*: 5′-TCAGCGTAGGAGTTGGACTTGATAG-3′ and *Shaker1-Geno-R*: 5′-AGTTCAAGGCTAGCCTGATCA-ATAGAG-3′ were used for allele PCR amplification. PCR products were then submitted to BsrfI digestion. *Deaf circler* mice (*Ush1c*$^{dfcr/J}$) carrying a 12.8-kb genomic deletion encompassing exons encoding for coil-coiled 1 and 2 domains and the proline-serine-threonine–rich domain were obtained from The Jackson Laboratory stock, no. 004771. These mice were genotyped by multiplex PCR using primers *in11-F*: 5′-GAGCCG-TAAGTCCAGAATGC-3′ and *inD-R*: 5′-TCATCAGAGGCT-GCTGACAC-3′ for mutant allele amplification; amplicon size was 282 bp and primers *in11-F* and *ex12-R*: 5′-TGCTCCTG-GAGGATCTTGTT-3′ for wild-type allele amplification; amplicon size was 234 bp.

Scanning Electron Microscopy. Freshly dissected cochleae were processed for scanning electron microscopy by the OTOTO (osmium-thiocarbohydrazide-osmium-thiocarbohydrazide-osmium) method, as previously described (6) and viewed on a Hitachi S-4800 (Hitachi) operating at 5 kV.

1. Kazmierczak P, et al. (2007) Cadherin 23 and protocadherin 15 interact to form tip-link filaments in sensory hair cells. *Nature* 449:87–91.
2. Grillet N, et al. (2009) Harmonin mutations cause mechanotransduction defects in cochlear hair cells. *Neuron* 62:375–387.
3. Schneider ME, et al. (2006) A new compartment at stereocilia tips defined by spatial and temporal patterns of myosin IIIa expression. *J Neurosci* 26:10243–10252.
4. Zinchuk V, Zinchuk O (2008) Quantitative colocalization analysis of confocal fluorescence microscopy images. *Curr Protoc Cell Biol* Chapter 4:Unit 4:19.
5. Rzadzinska AK, Schneider ME, Davies C, Riordan GP, Kachar B (2004) An actin molecular treadmill and myosins maintain stereocilia functional architecture and selfrenewal. *J Cell Biol* 164:887–897.
6. Manor U, et al. (2011) Regulation of stereocilia length by myosin XVa and whirlin depends on the actin-regulatory protein Eps8. *Curr Biol* 21:167–172.

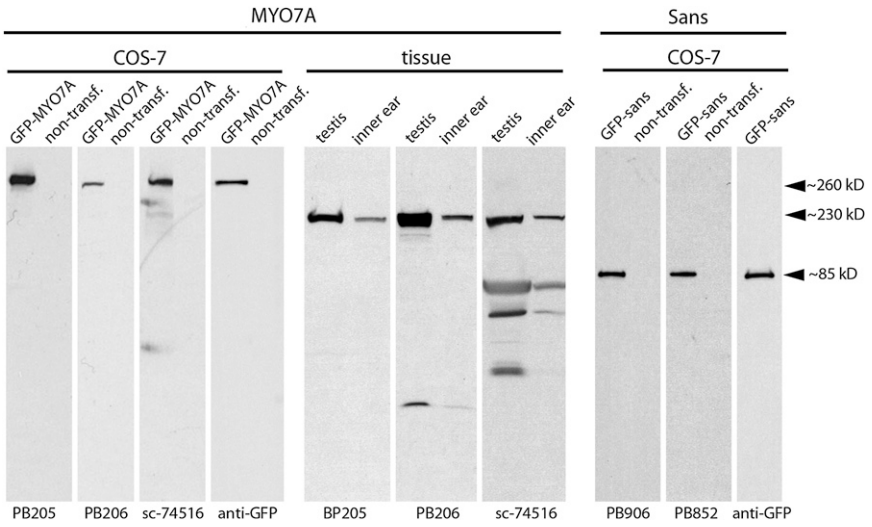

Fig. S1. Validation of the specificity of MYO7A and sans antibodies by Western blot. (*Left*) Western blots of protein extracts of COS7 cells expressing GFP-MYO7A and nonexpressing controls (nontransf.) using the MYO7A antibodies PB205 (dilution: 1/1,000), PB206 (dilution: 1/2,500), and sc-74516 (dilution: 1/500), as well as the anti-GFP antibody (1/5,000). All four antibodies detect specifically a ~260-kDa band in GFP-MYO7A–expressing COS7 cells protein extract corresponding to the predicted size of GFP-tagged MYO7A. (*Center*) Western blots of testis and inner ear tissues using MYO7A antibodies. Rabbit polyclonal PB205 and PB206 antibodies detect specifically a ~230-kDa band in testis and inner-ear protein extracts corresponding to the predicted size of the long native form of MYO7A. Similarly, monoclonal antibody sc-74516 recognizes a major ~230-kDa MYO7A band in both inner ear and testis, but also highlights other smaller bands. (*Right*) Western blots of protein extracts of COS7 cell expressing GFP-sans and nonexpressing controls using sans antibodies PB906 (dilution: 1/1,000) and PB852 (dilution: 1/1,000), as well as the anti-GFP antibody (dilution: 1/5,000). All three antibodies recognize specifically a ~85-kDa band corresponding to the predicted size of GFP-sans.

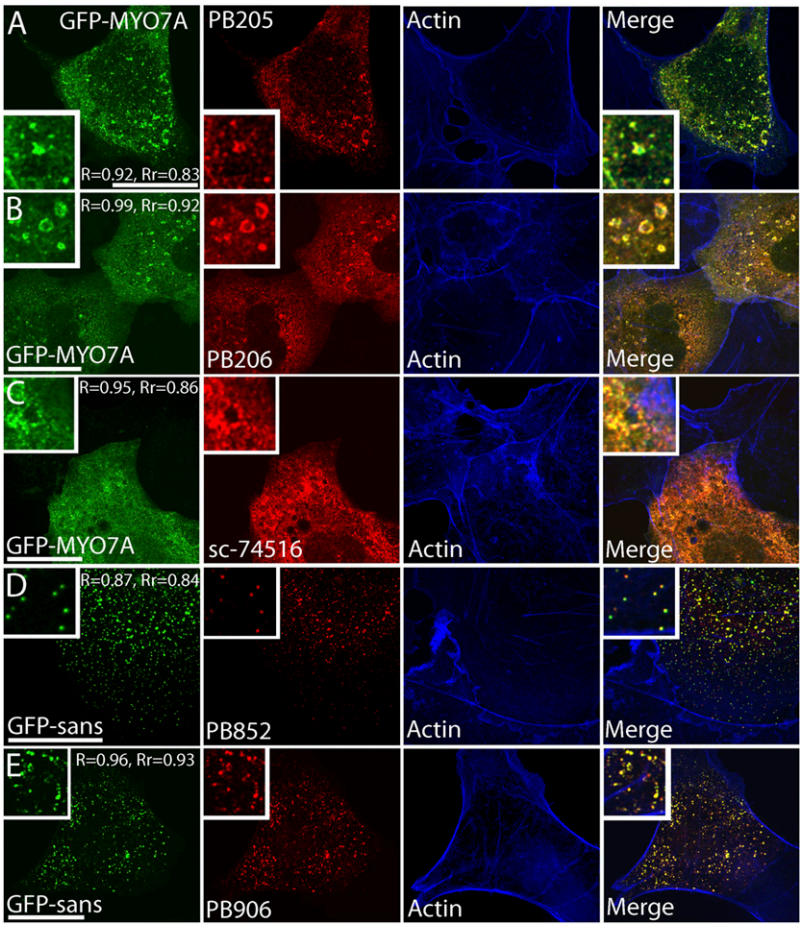

Fig. S2. Validation of the specificity of MYO7A and sans antibodies by immunocytochemistry. Immunofluorescence on COS7 cells expressing GFP-MYO7A (green in *A*–*C*) and GFP-sans (green in *D* and *E*) using immunopurified anti-MYO7A antibodies PB205 (red in *A*), PB206 (red in *B*), the monoclonal antibody sc-74516 (red in *C*), and anti-sans antibodies PB852 (red in *D*), and PB906 (red in *E*). All five antibodies show specificity in recognizing their respective antigens in transfected cells, with no background staining in nontransfected cells counterstained in blue for actin. The calculated Mander's (*R*) and Pearson's (*Rr*) coefficients are shown for each image.

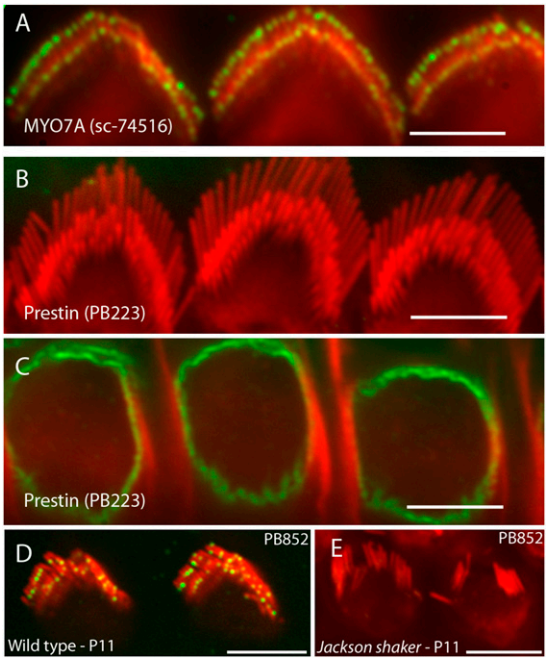

Fig. S3. Validation of the specificity of localization of sc-74516 and PB852 antibodies by whole-mount immunofluorescence on the inner-ear organ of Corti. (*A*) MYO7A mouse monoclonal antibody sc-74516 shows pattern of immunoreactivity compatible with upper tip-link density (UTLD) localization in P15 rat outer-hair cells, similarly to PB205 and PB206 (Fig. 1). However, the nonrelated prestin antibody PB223 does not show any immunoreactivity in stereocilia (*B*), but an intense fluorescence in the basolateral plasma membrane of outer hair cells (*C*). (*D* and *E*) Immunofluorescence images of postnatal day 11 wild-type (*D*) and sans-deficient *Jackson shaker* (*Ush1g*^{js}) outer hair-cell stereocilia (*E*) using antibody PB852. (Scale bars: 5 μm.)

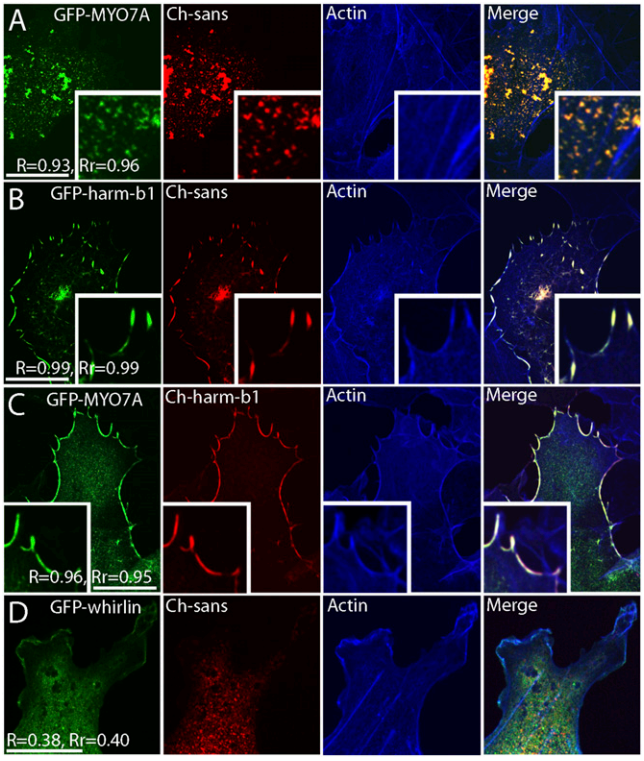

Fig. S4. Bipartite interactions of MYO7A, sans, and harmonin in COS7 cells. (*A*) GFP-MYO7A (green) colocalizes strongly (*R* = 0.93, *Rr* = 0.96) with Cherry-sans (red) in clusters not related to the cytoplasmic actin network (counterstained with phalloidin and shown in blue). (*B*) Cherry-sans (red) colocalizes with GFP-harmonin plaques (green) associated with the actin at the cell edge (*R* = 0.99, *Rr* = 0.99). (*C*) GFP-MYO7A colocalizes with Cherry-harmonin-b (red) plaques (*R* = 0.96, *Rr* = 0.95). GFP-whirlin colocalizes partially and randomly with Cherry-sans, as indicated by the low values of *R* and *Rr* (*R* = 0.38, Rr = 0.40). (Scale bars: 5 μm.)

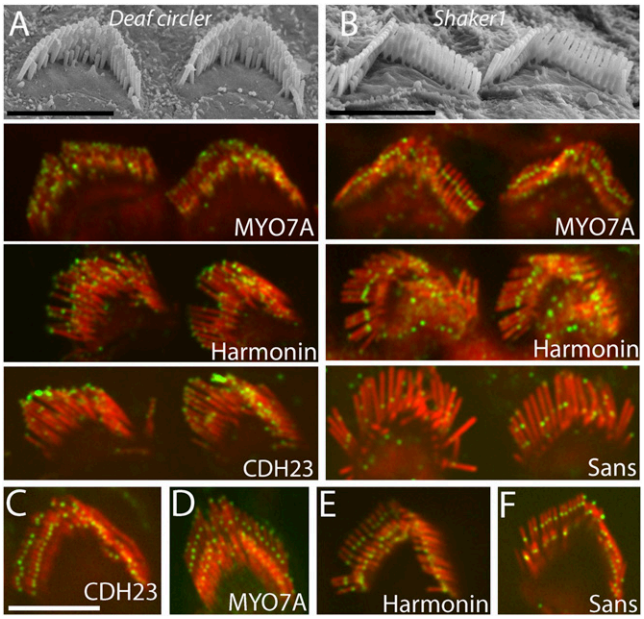

Fig. S5. Distribution of UTLD components in *deaf circler* (*Ush1c*^{dfcr}) and *shaker-1* (*Myo7a*^{sh1}) mutants and after BAPTA treatment. (*A*) Scanning electron micrograph of an outer hair-cell stereocilia bundle of P12-P13 *deaf circler* mice. The bundles are essentially composed of two rows of stereocilia with the occasional presence of a partial third row. MYO7A, harmonin, and CDH23 labeling is dispersed with a number of fluorescent puncta appearing at stereocilia tips. (*B*) Scanning electron micrograph of an outer hair-cell stereocilia bundle of P12-P13 *shaker-1* mouse. The bundles are composed of two rows of stereocilia. In these hair cells, MYO7A, harmonin, and sans immunofluorescence show localization at the UTLD. (*C*–*F*) Immunofluorescence confocal images of P17 rat outer hair cells stained for CDH23 (*C*), MYO7A (*D*), harmonin (*E*), and sans (*F*) processed after BAPTA treatment for 2 min, and recovery for 15 min at 37 °C show preservation of the UTLD immunofluorescence puncta. Occasionally, relatively weak fluorescence puncta for MYO7A and sans can be found at stereocilia tips. (Scale bars: 5 μm.)

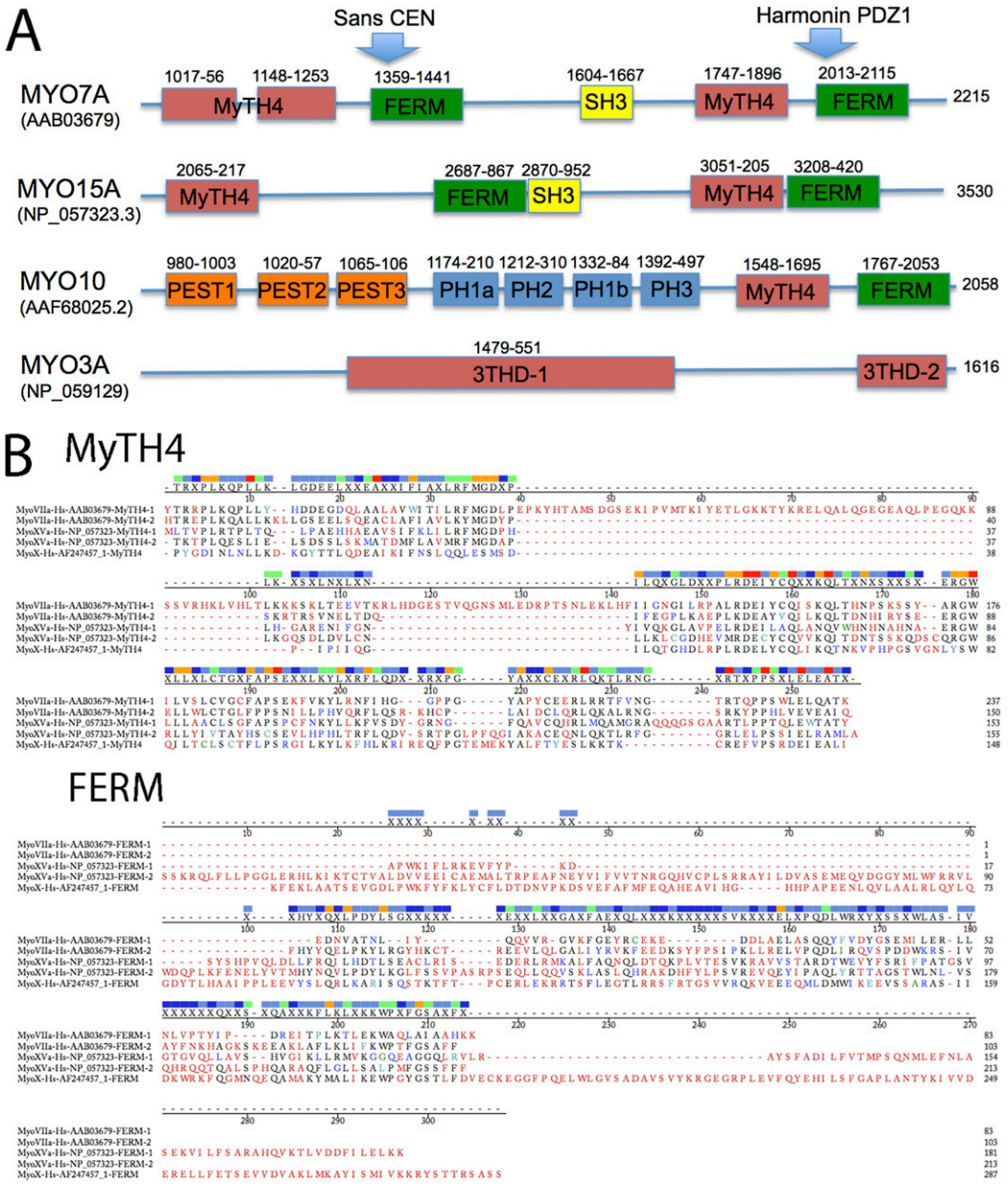

Fig. S6. Tail domain composition and MyTH4-FERM amino acid sequence variability for stereocilia myosins. (*A*) C-terminal tail domains and their respective amino acid position for MYO7A (GenBank: AAB03679), MYO15A (NP_057323.3), MYO10 (AAF68025.2), and MYO3A (NP_059129). (*B*) Sequence alignment for MyTH4 and FERM domains of MYO7A, MYO15A, and MYO10 using Clustal W Method (DNASTAR, MegAlign Lasergene). Intermyosin sequence divergence in these two domains is very high. Additionally, the length of MyTH4-FERM intercalating sequences is highly variable between myosins, and between modules within the same myosin.