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Usher type 1G protein sans is a critical component of the tip-link complex, a structure controlling actin polymerization in stereocilia

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The mechanotransducer channels of auditory hair cells are gated by tip-links, oblique filaments that interconnect the stereocilia of the hair bundle. Tip-links stretch from the tips of stereocilia in the short and middle rows to the sides of neighboring, taller stereocilia. They are made of cadherin-23 and protocadherin-15, products of the Usher syndrome type 1 genes USH1D and USH1F, respectively. In this study we address the role of sans, a putative scaffold protein and product of the USH1G gene. In Ush1g−/− mice, the cohesion of stereocilia is disrupted, and both the amplitude and the sensitivity of the transduction currents are reduced. In Ush1gfl/flMyo15-cre−/− mice, the loss of sans occurs postnatally and the stereocilia remain cohesive. In these mice, there is a decrease in the amplitude of the total transducer current with no loss in sensitivity, and the tips of the stereocilia in the short and middle rows lose their prolate shape, features that can be attributed to the loss of tip-links. Furthermore, stereocilia from these rows undergo a dramatic reduction in length, suggesting that the mechanotransduction machinery has a positive effect on F-actin polymerization. Sans interacts with the cytoplasmic domains of cadherin-23 and protocadherin-15 in vitro and is absent from the hair bundle in mice defective for either of the two cadherins. Because sans localizes mainly to the tips of short- and middle-row stereocilia in vivo, we conclude that it belongs to a molecular complex at the lower end of the tip-link and plays a critical role in the maintenance of this link.

Results and Discussion
Mechanoelectrical Transduction Currents Are Defective in Cochlear and Vestibular Hair Cells of Ush1g−/− Mice. The 460-amino-acid putative scaffolding protein sans contains a series of three ankyrin repeats and a sterile α-motif (SAM) domain, with an intervening a 95-aa central region (CENT) and a C-terminal.

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PDZ domain-binding consensus motif. The previously described Ush1g mutant mice (js/js mice), which display profound deafness and a balance defect, have nucleotide insertions (19) that may result in a protein truncated just upstream or at the beginning of the SAM domain. These mutants may therefore not be appropriate to explore the consequences of the absence of sans in the inner ear. To produce bona fide snai-snull (Ush1g−/−) mice, we first engineered Ush1gfl/fl mice by targeting exon 2 (Fig. S1A). These mice were then crossed with PGK-cre mice (20).

Sound processing in the cochlea involves two types of sensory cells: specifically, the inner hair cells (IHCs), which are the genuine sensory cells that release neurotransmitters and induce action potentials in afferent neurons, and the outer hair cells (OHCs), which locally amplify the sound-induced motion of the sensory epithelium. Exploration of the auditory functions of the sensory epithelium. Furthermore, the inner hair cells (IHCs) and the outer hair cells (OHCs) of the vestibule from P1 Ush1g−/− mice. Notably, not all of the UHC hair bundles were fragmented, and MET current recordings were performed on selected cells that displayed morphologically intact hair bundles. The maximal amplitudes of the MET current in Ush1g−/− mice (88 ± 12 pA) were 30% of those in Ush1g+/+ UHCs (295 ± 30 pA) (unpaired t test, P < 10−4) (Fig. S3). Both MET current abnormalities could result from the morphologically abnormal hair bundles in these mice (Fig. S3).

In contrast, residual MET current could not be detected in IHCs and OHCs from P5 cadherin-23-null (Cdhh3−/−) mice (24) that lack tip-links (Fig. S4). We then analyzed the MET currents in utricular hair cells (UHCS) of the vestibule from P1 Ush1g−/− mice. The fact that we found residual MET currents in the vestibular and cochlear Ush1g−/− hair cells at P1 and P5, respectively, indicates that sans plays a role in the MET machinery.

The maximal amplitudes of MET currents were markedly reduced in IHCs and OHCs from P5 Ush1g−/− mice (51 ± 9 pA in IHCs, 91 ± 24 pA in OHCs) compared with Ush1g+/+ control mice (471 ± 40 pA in IHCs, 696 ± 50 pA in OHCs; unpaired t test, P < 10−4 for both comparisons), and the average sensitivity of the MET currents to hair bundle displacement was decreased by ~50% in both IHCs and OHCs as well (unpaired t test, P < 10−4) (Fig. S3).
**Ush1g<sup>fl/fl</sup>Myo15-cre<sup>+/−</sup> Mice Are Profoundly Deaf and Develop a Balance Defect.** To circumvent the early morphogenetic defect present in the hair bundles of Ush1g<sup>fl/fl</sup> mice, we engineered Ush1g<sup>fl/fl</sup>Myo15-cre<sup>−/−</sup> mutant mice, in which Ush1g was deleted postnatally. These mice were obtained by crossing Ush1g<sup>fl/fl</sup> mice with Myo15-cre<sup>+/−</sup> mice in which cre recombinase gene expression was driven by the Myo5Sr promoter (Fig. S1B). The presence of the Myo15-cre allele leads to the sequential inactivation of a lacZ reporter gene from the basal to the apical region of the cochlea, starting at P0 at the base (Fig. S1C). ABRs, DPOAEs, and CM were analyzed in Ush1g<sup>fl/fl</sup>Myo15-cre<sup>+/−</sup> mice at P13, P15, and P21 (Fig. S2). At P13, these mice lacked identifiable ABR waves for all sound frequencies, even at the highest intensity tested (105 dB SPL). This indicates that IHCs are impaired because even a complete loss of OHC function cannot account for more than a 60−dB threshold elevation (25). In the P13 mutant mice, the round-window CM in response to sound stimulation could not be detected either, thus indicating that the high-frequency OHCs from the basal end of the cochlea were severely impaired. Moreover, the DPOAE amplitudes at 15 kHz did not significantly differ from background noise at all sound intensities tested, thus indicating that the OHC functional defect extends to cells tuned to this lower frequency. DPOAE amplitudes at 10 kHz were, however, still close to those obtained in Ush1g<sup>fl/fl</sup> P13 mice in response to 70 dB SPL stimuli, indicating partly preserved OHC function at this lower frequency (Fig. S2A). From P15 onward, neither CMs nor DPOAEs could be recorded in Ush1g<sup>fl/fl</sup>Myo15-cre<sup>+/−</sup> mice at all sound intensities and frequencies tested, indicating that the activity of all OHCs was now severely impaired (Fig. S2B). Notably, these mice also displayed a progressive vestibular dysfunction starting at P21, as assessed by their circling behavior and abnormal vestibular tests (26).

**Amplitude of MET Currents Is Reduced in IHCs from Ush1g<sup>fl/fl</sup>Myo15-cre<sup>−/−</sup> Mice.** We studied the MET currents in hair cells from Ush1g<sup>fl/fl</sup>Myo15-cre<sup>−/−</sup> mice. Recordings were performed in Ush1g<sup>fl/fl</sup>Myo15-cre<sup>+/−</sup> and Ush1g<sup>fl/fl</sup> mice at positions located in the apical end (at ∼35% the total length of the cochlea from its apex) and in the middle (∼55% the total length) of the cochlea. At P7, at both cochlear sites, MET currents in IHCs and OHCs of Ush1g<sup>fl/fl</sup>Myo15-cre<sup>−/−</sup> mice were indistinguishable from those of Ush1g<sup>fl/fl</sup> mice (Fig. S5). In contrast, P8 Ush1g<sup>fl/fl</sup>Myo15-cre<sup>−/−</sup> IHCs displayed a decrease in the maximal amplitude of their MET current. At the apex and middle of the cochlea, the maximal amplitudes in Ush1g<sup>fl/fl</sup>Myo15-cre<sup>+/−</sup> mice (346 ± 71 pA and 84 ± 44 pA, respectively) were on average 60% and 13% those of IHCs from Ush1g<sup>fl/fl</sup> mice (582 ± 52 pA and 652 ± 44 pA; unpaired t test, P = 0.015 and P < 10<sup>−3</sup>), respectively (Fig. 1B). The P<sub>0.1</sub>(X) curves of Ush1g<sup>fl/fl</sup>Myo15-cre<sup>+/−</sup> and Ush1g<sup>fl/fl</sup> IHCs at both cochlear sites could, however, be superimposed, thus indicating that the MET sensitivity to hair bundle displacement is preserved. We then investigated the characteristics of MET current adaptation, the process that resets the sensitivity of the MET channel close to its normal resting value during sustained stimuli. No changes were observed in the extent of adaptation or the kinetics when analyzed in IHCs from apical cochlear regions (Fig. S6). Therefore, the lack of sans in IHCs affects only the amplitude of the MET current without affecting the sensitivity of the hair bundle to displacement or adaptation. This suggests that the MET machinery functions normally in some of the stereocilia, but ∼40% and 87% of the MET complexes are entirely nonfunctional at the apex and at the mid region of the cochlea, respectively. The role of sans in the MET process thus markedly differs from that of harmonin-b, which is involved in the extent of adaptation but does not play a significant role in the amplitude of the MET current (22). No difference in the OHC MET currents could be detected between Ush1g<sup>fl/fl</sup>Myo15-cre<sup>+/−</sup> and Ush1g<sup>fl/fl</sup> P8 mice (Fig. S7).
modulated (28). Tip-link tension may also enhance actin polymerization by increasing MET channel opening probability, and hence the influx of Ca\(^{2+}\) ions at the stereocilia tip may modulate actin polymerization (27). The rapid [in about 2 d, the time period for the renewal of stereociliary actin (29)] and total disappearance/collapse of the small and medium stereocilia rows contrasts with the maintenance of stereocilia of reduced size in mice defective for myosin XV, whirlin, or espin (30, 31) and

Fig. 2. Hair bundle morphology in OHCs and IHCs from Ush1g\(^{fl}\)/Myo15-cre\(^{−}\) P8 mice. Scanning electron microscopy analysis of OHCs and IHCs from the apex (Upper) and from the middle region of the cochlea (Lower) in Ush1g\(^{fl}\) and Ush1g\(^{fl}\)/Myo15-cre\(^{−}\) P8 mice. In the apical region, the hair bundles of Ush1g\(^{fl}\)/Myo15-cre\(^{−}\) IHCs and OHCs are cohesive. In the apical region, the hair bundles of Ush1g\(^{fl}\)/Myo15-cre\(^{−}\) IHCs and OHCs are cohesive, the tip-links are present, and prolate-shaped stereocilia tips are systematically observed. In the middle region, no differences were detected between Ush1g\(^{fl}\) and Ush1g\(^{fl}\)/Myo15-cre\(^{−}\) OHCs. In contrast, the presence of nonprolate-shaped stereocilia tips within the middle row of stereocilia was frequently detected in Ush1g\(^{fl}\)/Myo15-cre\(^{−}\) IHCs, and the number of tip-links that could be detected in these cells was reduced. At P9, additional hair bundle anomalies appeared in the Ush1g\(^{fl}\)/Myo15-cre\(^{−}\) IHCs from the mid to the apex of the cochlea; specifically, some stereocilia within the small and middle rows had reduced heights. No defects were found in OHCs from the cochlear apex at this stage, but in the middle region of the cochlea, we found the same anomalies as in IHCs. At P22, the reduction of the stereocilia length dramatically worsened, and some of the stereocilia from the small row had even disappeared in both IHCs and OHCs. Notably, the size of the stereocilia that compose the tall row was unchanged in Ush1g\(^{fl}\)/Myo15-cre\(^{−}\) mutants. (Scale bar: 1 μm.)

Fig. 3. Analysis of stereocilia length in IHCs and OHCs from P8 and P9 Ush1g\(^{fl}\)/Myo15-cre\(^{−}\) mice. Data corresponding to Ush1g\(^{fl}\) and Ush1g\(^{fl}\)/Myo15-cre\(^{−}\) mice are indicated in blue and in red, respectively. Five cells were analyzed in each group. The length of every measurable stereocilium from the middle and small rows was normalized to the mean length of stereocilia in the tall row (L\(_2\)/L\(_1\) and L\(_3\)/L\(_1\), respectively; mean ± SEM). The numbers (mean ± SEM) of tip-links detected in the apical (apex) and middle (mid) regions of the cochlea are indicated by histograms (Right panels). In Ush1g\(^{fl}\)/Myo15-cre\(^{−}\) IHCs (Upper panels), there is a progressive reduction of the stereocilia length in the middle and small rows and a parallel decrease of the number of tip-links detected, compared with Ush1g\(^{fl}\) IHCs (two-way ANOVA, P < 10\(^{−8}\) for both comparisons). In Ush1g\(^{fl}\)/Myo15-cre\(^{−}\) OHCs (Lower panels), a decrease of the stereocilia length and number of tip-links is also observed (two-way ANOVA, P < 10\(^{−8}\) for both comparisons). In Ush1g\(^{fl}\)/Myo15-cre\(^{−}\) P9 mice, note that some stereocilia have completely disappeared in both IHCs and OHCs (red dots on the x axis), specifically, 4% of stereocilia from the small row in IHCs of the mid cochlear region, 3% of stereocilia from the small row in OHCs of the apical region, and 15% and 32% of the stereocilia from the middle and small rows in OHCs of the mid region, respectively.
reactivity is specific to sans and persists in the mutant mice, indicating the non-specificity of these antibodies. We produced another polyclonal antibody directed against the SAM domain of sans (Materials and Methods). This antibody labeled the stereocilia in Ush1g−/− mice, but not in Ush1g+/+ mice, thereby proving the specificity of the stereocilia labeling (Fig. 4D). In the cochlea, sans was detected in the apical region of the hair bundles as early as embryonic day 16.5 (E16.5), and at P1 it was present at the hair bundle tips, as were the other four USH1 proteins (17). We conclude that sans belongs to the USH1 protein network that is required for the cohesion of hair bundles in cochlear hair cells during the early stages of their development. Thereafter, at P8, spots of sans labeling were observed in the apical region of all three stereocilia rows (Fig. 4C). Immunogold labeling confirmed the presence of sans at the tip of the small and middle stereocilia rows (Fig. 4D). Notably, the labeling of the small and medium rows was always located at the tips of stereocilia. In Ush1g−/−Myo15-cre+/− mice at P8, sans labeling was no longer detected in IHCs, but it was still present in OHCs, in accordance with the preservation of their MET currents at this stage (Fig. 4C).

In the absence of harmonin (Ush1c−/−) or myosin VIIa (Myo7afl/flfl4626SB/4626SB), sans was properly located at the apical end of the hair bundles in IHCs and OHCs at P1. In contrast, sans could not be detected in Cdhn23−/− and Pcdh15−/−/− mutant mice that lack cadherin-23 and protocadherin-15, respectively (Fig. 4B). We thus asked whether sans interacts with the cytoplasmic regions of these cadherins. By coexpressing sans with a chimeric cadherin composed of the extracellular region of E-cadherin and the cytoplasmic region of either cadherin-23 or protocadherin-15 (CD1, CD2, or CD3 isoforms; Materials and Methods) in transfected cells, between sans and the CD3 isoform of protocadherin-15 that is believed to form the lower part of the tip-link (8, 32), we conclude that sans belongs to the molecular complex present at the lower insertion point of the tip-link. However, we cannot exclude the possibility that sans also belongs to the molecular complex associated with the upper insertion point of the tip-link because the protein was also detected on the lateral side of some stereocilia in the taller row (Fig. 4) and was recruited to the plasma membrane when either cadherin-23 or protocadherin-15 (except the CD1 isoform) is present (Fig. S8). In the developing hair bundle, sans is thus likely to be present at both ends of the transient lateral links that are made of cadherin-23 and protocadherin-15. On the basis of the immunodetection of sans in the region of the tip-link lower insertion point in the stereocilia from the small and middle rows at later stages, as well as the interaction, in cotransfected cells, between sans and the CD3 isoform of protocadherin-15 that is present in OHCs, in accordance with the preservation of their MET currents at this stage (Fig. 4D) and was recruited to the plasma membrane by cadherin-23 in cotransfected cells (Fig. S8). Notably, the disappearance of the tip-links that we observed in IHCs from Ush1g−/−Myo15-cre+/− mice fully accounts for the decrease of the MET current amplitude in these cells. The less-than-1-d delay between the time when sans becomes undetectable and the time when tip-links disappear and the MET current decreases suggests that sans is involved in either the maintenance of the tip-link or its renewal.

Materials and Methods

Experiments on mice were carried out according to Institut National de la Santé et de la Recherche Médicale and Institut Pasteur welfare guidelines.

Whole-Mount Immunofluorescence. Rabbit antisera were raised against recombinant proteins encompassing the SAM domain (aa 369–455) of sans (GenBank accession no. NM_176847), and intracellular fragments of the protocadherin-15 CD1 (aa 1612–1726; GenBank accession no. Q99PJ1), CD2 (aa 1652–1790; GenBank accession no. Q02M28), or CD3 (aa 1522–1682; GenBank accession no. Q02M20) isoforms. Antibodies were affinity-purified against the corresponding antigens coupled to an NHS column (GE Healthcare). We used the N1 polyclonal antibody to detect cadherin-23 (3).
accession no. NP_001165405) or mouse protocadherin-15 (CD1, CD2, and CD3 isoforms; GenBank accession nos. NM_001142740, HQ420254, and HQ404375, respectively) were engineered for expression in COS-7 cells (2).

Scanning Electron Microscopy. Mouse inner ears were processed with osmium tetroxide/thiocarbohydrazide method as previously described (33). Samples were analyzed by field emission scanning electron microscopy operated at 5 kV (Jeol JSM6700F).

Immunogold Labeling. For transmission electron microscopy, gold-labeled tissues were washed, refixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 containing 1% tannic acid, washed in buffer, and postfixed in 1% osmium tetroxide as previously described (32).

In Vivo and in Vitro Electrophysiological Recordings and Data Analysis. ABRs, CM, and DPOAEs were recorded in anesthetized mice and analyzed as described previously (34, 35).

Electrophysiological cell recordings were performed on cochlear and utricular explants from mice aged between P1 and P8 (cochlear hair cells, n = 286; vestibular hair cells, n = 54), as reported (22). The probe used for mechanical stimulation of the hair bundles was secured to a stack-type piezoelectric actuator (PA8/12; Piezosystem Jenas) driven by a low-voltage power supply (30V300, Piezosystem Jena). As measured with offline displacement a millisecond monitor containing photodiodes, the first two milliseconds of the time course of probe motion were well described by an exponential rise with a time constant of 100 μs (22). Data were analyzed in Matlab, version 7.0 (MathWorks), P(0.05) curves were fitted with a three-state Boltzmann relation (22). For sensitivity measurements, the mean value of the three-state Boltzmann relation derivative was calculated for displacements corresponding to P0 values between 0.2 and 0.8.

Statistical Analysis. Statistical significance was tested by using either two-way analysis of variance (two-way ANOVA) coupled to the Bonferroni posttest or two-tailed unpaired t test with Welch’s correction using Prism software (GraphPad). Statistical significances are indicated on the figures (Figs. 1 and 3 and Figs. 52–57); the designations (n), (*), (**), and (***)) correspond to nonsignificant (P > 0.05), P < 0.05, P < 0.01, and P < 0.001, respectively.

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2. Boëda B, et al. (2002) Myosin VIIa, harmonin and cadherin 23, three Usher I gene isoforms; GenBank accession nos. NM_001142740, HQ420254, and HQ404375, respectively) were engineered for expression in COS-7 cells (2).


**Supporting Information**

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**Fig. S1.** Engineering of **Ush1g** knockout mice and **Myo15-cre** mice. (**A**) Schematic of the recombinant **Ush1g** alleles. A targeting vector was designed in which **loxP** sites were introduced upstream and downstream of **Ush1g** exon 2, and a neo cassette flanked with **FRT** sites as selectable marker was introduced downstream of exon 2. The targeting construct was electroporated into embryonic stem cells from the 129S1/SvlmJ mouse strain, and positive ES cells were selected by their resistance to G418. Stem cells carrying the targeted construct were injected into blastocysts from C57BL/6J mice to obtain chimeric mice. After germline transmission, mice were crossed with C57BL/6J mice producing Flp recombinase to remove the neo cassette. The **Ush1g** fl/fl mice (MGI:4361359) lack the neo cassette and behave like wild-type (+/+) mice. **Ush1g** fl/fl mice were crossed either with the PGK-Cre transgenic mouse strain carrying the cre recombinase gene driven by the early acting phosphoglycerate kinase-1 gene promoter or with **Myo15-cre** recombinant mice carrying the cre recombinase gene driven by the myosin-15 gene promoter which, in the inner ear, deletes only the flanked fragment in hair cells. Genotyping of **Ush1g** recombinant animals was carried out by means of two PCR amplifications, using either oligo-3 (5′-GTCAAAGGATCAGATCACTCGCAG-3′) and oligo-1 (5′-GGGAGTCGGCTTAACACCACATTG-3′) to detect the wild-type (323-bp amplicon) or flanked (423-bp amplicon) alleles or oligo-3 and oligo-4 (5′-CAGTTTCCCCATGTTGATCACCAAC-3′) to detect the presence of a deleted allele lacking **Ush1g** exon 2 (322-bp amplicon). All studies were performed on mixed C57BL/6–129/Sv genetic backgrounds. (**B**) Schematic of the **Myo15-cre** allele engineered for this study. The cre recombinase gene was placed under the control of the **Myo15** promoter: a targeting construct was designed containing a hygromycin resistance cassette flanked with **FRT** sites and introduced in C57BL/6J mice blastocysts, and after germline transmission, mice were crossed with C57BL/6J mice producing Flp recombinase to remove the hygromycin cassette (MGI:4361284). The heterozygote **Myo15-cre** mice behave like wild-type (+/−) mice. Genotyping of the animals was carried out by means of two PCR amplifications, using either oligo-2 (5′-GTCAAAGGATCAGATCACTCGCAG-3′) and oligo-1 (5′-GGGAGTCGGCTTAACACCACATTG-3′) to detect the wild-type (323-bp amplicon) or flanked (423-bp amplicon) alleles or oligo-3 and oligo-4 (5′-CAGTTTCCCCATGTTGATCACCAAC-3′) to detect the presence of a deleted allele lacking **Ush1g** exon 2 (322-bp amplicon). All studies were performed on mixed C57BL/6–129/Sv genetic backgrounds. (**C**) Schematic of the **Myo15-cre** allele engineered for this study. The cre recombinase gene was placed under the control of the **Myo15** promoter: a targeting construct was designed containing a hygromycin resistance cassette flanked with **FRT** sites and introduced in C57BL/6J mice blastocysts, and after germline transmission, mice were crossed with C57BL/6J mice producing Flp recombinase to remove the hygromycin cassette (MGI:4361284). The heterozygote **Myo15-cre** mice behave like wild-type (+/−) mice. Genotyping of the animals was carried out by means of two PCR amplifications, using either oligo-2 (5′-GTCAAAGGATCAGATCACTCGCAG-3′) and oligo-1 (5′-GGGAGTCGGCTTAACACCACATTG-3′) to detect the wild-type allele or cre-R (5′-TGTTGACGATCGAGGTTGGG-3′) to detect the **Myo15-cre** allele (450-bp amplicon). X-Gal staining on ROSA26−/−**Myo15-cre** mice. The temporal-spatial expression pattern of cre driven by the **Myo15** promoter in the inner ear was assessed by crossing **Myo15-cre** mice with ROSA26-lacZ reporter mice (1). The cre-driven lacZ expression was studied in the inner ear of ROSA26−/−**Myo15-cre** mice using X-Gal histochemistry. LacZ expression was first detected in vestibular hair cells at E19, and in hair cells from the cochlear base at P0. At P4, lacZ expression was detected in all cochlear hair cells.

Fig. S2. Analysis of the auditory function in Ush1g<sup>fl/fl</sup> P13, P15, and P21 mice and in Ush1g<sup>−/−</sup> P21 mice. (A, first graph) Auditory brainstem response (ABR) thresholds (mean ± SEM) in Ush1g<sup>fl/fl</sup> (black line) and Ush1g<sup>fl/fl</sup>/Myo15-cre<sup>+/−</sup> (blue line) P13 mice for 5- to 40-kHz tone bursts. Ush1g<sup>fl/fl</sup>/Myo15-cre<sup>+/−</sup> mutants show a total absence of evoked response (two-way ANOVA, P < 10<sup>−3</sup>). (second graph) Growth functions of cochlear microphonics (CM) (mean ± SEM) at increasing stimulus level from 75 to 105 dB for Ush1g<sup>fl/fl</sup> mice and from 90 to 100 dB for Ush1g<sup>fl/fl</sup>/Myo15-cre<sup>+/−</sup> mice. (third and fourth graphs) DPOAE amplitude (mean ± SEM) at frequency 2<sub>f<sub>1</sub></sub> −<sub>f<sub>2</sub></sub> for<br>f<sub>2</sub> = 10 kHz and f<sub>2</sub> = 15 kHz in Ush1g<sup>fl/fl</sup> and Ush1g<sup>fl/fl</sup>/Myo15-cre<sup>+/−</sup> P13 mice. The DPOAE amplitude was recorded in response to two equal-level primary tones, f<sub>1</sub> and f<sub>2</sub>, with f<sub>2</sub>/f<sub>1</sub> = 1.20. Cubic DPOAEs were elicited by a CubeDis system (Mimosa Acoustics, v2.43). Frequency f<sub>2</sub> was swept at one-tenth-octave steps from 4 to 20 kHz (levels increased stepwise from 30 to 70 dB SPL). For f<sub>2</sub> = 10 kHz, Ush1g<sup>fl/fl</sup>/Myo15-cre<sup>+/−</sup> mice show detectable DPOAEs for stimulus level from 60 to 70 dB, whereas no DPOAEs are detected for f<sub>2</sub> = 15 kHz. (B, first graph) ABR thresholds (mean ± SEM) in Ush1g<sup>fl/fl</sup> (black line) and Ush1g<sup>fl/fl</sup>/Myo15-cre<sup>+/−</sup> (blue line) P15 mice for 5- to 40-kHz tone bursts. Ush1g<sup>fl/fl</sup>/Myo15-cre<sup>+/−</sup> mutants show a total absence of evoked response (two-way ANOVA, P < 10<sup>−3</sup>). (second graph) Growth functions of CM (mean ± SEM) at an increasing stimulus level from 75 to 105 dB. (third and fourth graphs) DPOAE amplitude (mean ± SEM) at frequency 2<sub>f<sub>1</sub></sub> −<sub>f<sub>2</sub></sub> (f<sub>1</sub>/f<sub>2</sub> = 1.20) in Ush1g<sup>fl/fl</sup>, Ush1g<sup>−/−</sup>, and Ush1g<sup>fl/fl</sup>/Myo15-cre<sup>+/−</sup> P15 mice. Ush1g<sup>−/−</sup> and Ush1g<sup>fl/fl</sup>/Myo15-cre<sup>+/−</sup> mice have no detectable DPOAEs. Dashed line represents noise floor below 65 dB SPL and instrumental distortion above 65 dB SPL. (C, first graph) ABR thresholds (mean ± SEM) in Ush1g<sup>fl/fl</sup> (black lines), Ush1g<sup>−/−</sup> (pink line), and Ush1g<sup>fl/fl</sup>/Myo15-cre<sup>+/−</sup> (purple line) P21 mice for 5- to 40-kHz tone bursts. Ush1g<sup>−/−</sup> and Ush1g<sup>fl/fl</sup>/Myo15-cre<sup>+/−</sup> mutants show a total absence of evoked response (two-way ANOVA, P < 10<sup>−3</sup>). (second graph) Growth functions of CM (mean ± SEM) at an increasing stimulus level from 75 to 105 dB. (third and fourth graphs) DPOAE amplitude (mean ± SEM) at frequency 2<sub>f<sub>1</sub></sub> −<sub>f<sub>2</sub></sub> (f<sub>1</sub>/f<sub>2</sub> = 1.20) in Ush1g<sup>fl/fl</sup>, Ush1g<sup>−/−</sup>, and Ush1g<sup>fl/fl</sup>/Myo15-cre<sup>+/−</sup> P21 mice. Ush1g<sup>−/−</sup> and Ush1g<sup>fl/fl</sup>/Myo15-cre<sup>+/−</sup> mice have no detectable DPOAEs. Dashed line represents noise floor below 65 dB SPL and instrumental distortion above 65 dB SPL.
Fig. S3. Hair cell morphology and mechanoelectrical transduction current recordings in inner hair cells (IHCs) and outer hair cells (OHCs) from Ush1g<sup>−/−</sup> P5 mice. (A, Top) Scanning electron microscopy analysis of IHCs from Ush1g<sup>fl/fl</sup> and Ush1g<sup>−/−</sup> P5 mice. (Scale bar: 1 μm.) In Ush1g<sup>−/−</sup> IHCs, some stereocilia of the second row (about 10%) have a prolate end shape, which suggests that some tip-links are still present. Note the reduced length of most stereocilia from the small and medium rows. (Middle) Examples of transduction current recordings in an Ush1g<sup>fl/fl</sup> IHC (Left panel in dark-red) and an Ush1g<sup>−/−</sup> IHC from P5 mice while applying different displacement steps with a glass probe in the excitatory direction and a 150-nm step in the inhibitory direction (calibrated voltage command of the stimulator at the top). (Bottom) Mean maximum transduction currents and <i>P</i><sub:o</sub>(<i>X</i>) curves plotted for Ush1g<sup>fl/fl</sup> and Ush1g<sup>−/−</sup> P5 mice. The maximal amplitudes of the transduction current are 51 ± 9 pA and 471 ± 40 pA in Ush1g<sup>−/−</sup> and Ush1g<sup>fl/fl</sup> IHCs, respectively (unpaired <i>t</i> test, <i>P</i> < 10<sup>−4</sup>). The averaged sensitivity of the transduction currents to hair bundle displacement in the mutant IHCs is 0.99 ± 0.09 μm<sup>−1</sup> and 2.80 ± 0.15 μm<sup>−1</sup> in Ush1g<sup>−/−</sup> and Ush1g<sup>fl/fl</sup> IHCs, respectively (unpaired <i>t</i> test, <i>P</i> < 10<sup>−4</sup>).

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(B, Top) Scanning electron microscopy analysis of OHCs from Ush1g<sup>fl/fl</sup> and Ush1g<sup>−/−</sup> P5 mice. Note the fragmented aspect of the hair bundles and the reduced length of most stereocilia from the small and medium rows. However, one can recognize the stereocilia of the first row and some stereocilia of the second row. (Scale bar: 1 μm.) (Middle) Examples of transduction current recordings in a Ush1g<sup>fl/fl</sup> OHC and two Ush1g<sup>−/−</sup> OHCs from P5 mice. (Bottom) Mean maximum transduction currents and <i>P</i><sub:o</sub>(<i>X</i>) curves plotted for Ush1g<sup>fl/fl</sup> and Ush1g<sup>−/−</sup> P5 mice. The maximal amplitudes of the transduction current are 91 ± 24 pA and 696 ± 50 pA in Ush1g<sup>−/−</sup> and Ush1g<sup>fl/fl</sup> OHCs, respectively (unpaired <i>t</i> test, <i>P</i> < 10<sup>−3</sup>). The averaged sensitivity values are 1.51 ± 0.24 μm<sup>−1</sup> and 2.85 ± 0.26 μm<sup>−1</sup> in Ush1g<sup>−/−</sup> and Ush1g<sup>fl/fl</sup> OHCs, respectively (unpaired <i>t</i> test, <i>P</i> < 10<sup>−4</sup>). In addition, <i>X</i><sub>0.5</sub> is 745 ± 59 nm and 217 ± 20 nm in Ush1g<sup>−/−</sup> and Ush1g<sup>fl/fl</sup> IHCs, respectively (unpaired <i>t</i> test, <i>P</i> < 10<sup>−3</sup>).
Fig. S4. (A) Mechanoelectrical transduction current recordings in inner hair cells (IHCs) and outer hair cells (OHCs) from Cdh23<sup>fl/fl</sup> and Cdh23<sup>−/−</sup> P5 mice. Examples of transduction currents in IHCs (Upper) and OHCs (Lower) from Cdh23<sup>fl/fl</sup> and Cdh23<sup>−/−</sup> P5 mice in response to a 40-ms mechanical stimulation of the hair bundle. No current is recorded in the Cdh23<sup>−/−</sup> IHC (gray) and OHC (light purple). (B–D) Analysis of the auditory function in Cdh23<sup>fl/fl</sup>Myo15-cre<sup>+</>/−<sup> P16, P17, P20, and P22. (B) Auditory brainstem response (ABR) thresholds in Cdh23<sup>fl/fl</sup> (black lines) and Cdh23<sup>fl/fl</sup>Myo15-cre<sup>+</>/−<sup> (red lines) P16–P22 mice for 5- to 40-kHz tone bursts (mean ± SEM). Cdh23<sup>fl/fl</sup>Myo15-cre<sup>+</>/−<sup> mutants show progressive hearing loss from P16 to P22 and have profound hearing impairment at P22 (two-way ANOVA, P < 10<sup>−4</sup>). (C) Amplitude of the distortion-product otoacoustic emissions (DPOAE) (mean ± SEM) recorded at frequency 2<sup>f</sup>1 − <sup>f</sup>2 for a 60-dB SPL two-tone stimulus (<sup>f</sup>1/<sup>f</sup>2 = 1.20) in Cdh23<sup>fl/fl</sup> and Cdh23<sup>fl/fl</sup>Myo15-cre<sup>+</>/−<sup> P16, P17, P20, and P22 mice. Cdh23<sup>fl/fl</sup>Myo15-cre<sup>+</>/−<sup> mice have no detectable DPOAE at P22. (D) Growth functions of cochlear microphonics (CM) (mean ± SEM) at an increasing stimulus level from 75 to 105 dB in Cdh23<sup>fl/fl</sup> and Cdh23<sup>fl/fl</sup>Myo15-cre<sup>+</>/−<sup> P22 mice. (E) Hair bundle morphology in Cdh23<sup>fl/fl</sup>Myo15-cre<sup>+</>/−<sup> OHCs. Scanning electron microscopy analysis of OHCs in the mid region of the cochlea from Cdh23<sup>fl/fl</sup> (Upper) and Cdh23<sup>fl/fl</sup>Myo15-cre<sup>+</>/−<sup> (Lower) P16 and P22 mice. At P16, the hair bundles of Cdh23<sup>fl/fl</sup>Myo15-cre<sup>+</>/−<sup> OHCs are cohesive, but some stereocilia of the small row already have reduced heights. At P22, the reduction of the stereocilia length has dramatically worsened in the small and middle rows, and some of the stereocilia from the small row have even disappeared. Notably, the length of the stereocilia in the tall row is unchanged. (Scale bar: 1 μm.) (F) Analysis of stereocilia length in IHCs and OHCs from P16 and P22 Cdh23<sup>fl/fl</sup>Myo15-cre<sup>+</>/−<sup> mice. Data corresponding to Cdh23<sup>fl/fl</sup> and Cdh23<sup>fl/fl</sup>Myo15-cre<sup>+</>/−<sup> mice are indicated in blue and in red, respectively. Five cells were analyzed in each group. The length of every measurable stereocilium from the middle and small rows was normalized to the mean length of stereocilia in the tall row (L<sub>2</sub>/L<sub>1</sub> and L<sub>3</sub>/L<sub>1</sub>, respectively; mean ± SEM). The numbers (mean ± SEM) of tip-links detected are indicated by histograms (Right panels). In both Cdh23<sup>fl/fl</sup>Myo15-cre<sup>+</</sup> IHCs (Upper panels) and OHCs (Lower panels), there is a progressive reduction of the stereocilia length and a parallel decrease of the number of tip-links detected, compared with Cdh23<sup>fl/fl</sup> IHCs and OHCs (two-way ANOVA, P < 10<sup>−2</sup> for all comparisons). In Cdh23<sup>fl/fl</sup>Myo15-cre<sup>+</</sup> P22 mice, note that some stereocilia have completely disappeared in both IHCs and OHCs (two-way ANOVA, P < 10<sup>−2</sup> for all comparisons). In Cdh23<sup>fl/fl</sup>Myo15-cre<sup>+</</sup> P22 mice, note that some stereocilia have completely disappeared in both IHCs and OHCs (two-way ANOVA, P < 10<sup>−2</sup> for all comparisons).
Fig. S5. Analysis of mechanoelectrical transduction current adaptation in inner hair cells (IHCs) from Ush1g<sup>fl/fl</sup>/Myo15-cre<sup>−/+</sup> P7 mice. Mechanoelectrical transduction currents in cochlear hair cells from the region that is ∼40% of the total length of the cochlea from the apex. (A) Examples of transduction currents in midcochlear IHCs from Ush1g<sup>fl/fl</sup> (black) and Ush1g<sup>fl/fl</sup>/Myo15-cre<sup>−/+</sup> (blue) P7 mice. Mean maximum current amplitude is 616 ± 67 pA and 664 ± 71 pA for Ush1g<sup>fl/fl</sup> and Ush1g<sup>fl/fl</sup>/Myo15-cre<sup>−/+</sup> IHCs, respectively (unpaired t test, P = 0.63). The P<sub>0</sub>(X) curves can be superimposed with values of averaged sensitivity 2.03 ± 0.10 μm<sup>−1</sup> and 1.85 ± 0.14 μm<sup>−1</sup> for Ush1g<sup>fl/fl</sup> and Ush1g<sup>fl/fl</sup>/Myo15-cre<sup>−/+</sup>, respectively (unpaired t test, P = 0.35). In addition, no change in X<sub>0.5</sub> could be detected in the mutant IHCs with values 248 ± 23 nm and 273 ± 34 nm in Ush1g<sup>fl/fl</sup> and Ush1g<sup>fl/fl</sup>/Myo15-cre<sup>−/+</sup>, respectively (unpaired t test, P = 0.55). (B) We characterized the adaptation in Ush1g<sup>fl/fl</sup> and Ush1g<sup>fl/fl</sup>/Myo15-cre<sup>−/+</sup> IHCs in terms of its extent and kinetics. The adaptive decline of the transduction current I as a function of time t was fitted by the double exponential relation 
\[
I(t) = A_F \{ \exp\left(-\frac{(t-t_0)}{\tau_F}\right)\} + A_S \{ \exp\left(-\frac{(t-t_0)}{\tau_S}\right)\} + A_{SS}
\]
From the fit, we deduced the fast and slow adaptation rates, 1/τ<sub>F</sub> and 1/τ<sub>S</sub>, and the proportions, A<sub>F</sub>/(A<sub>F</sub>+A<sub>S</sub>) and A<sub>S</sub>/(A<sub>F</sub>+A<sub>S</sub>) respectively, as well as the extent of adaptation 1 - A<sub>SS</sub>/(A<sub>F</sub>+A<sub>S</sub>+A<sub>SS</sub>) (1). Statistical significance was tested by using either two-way analysis of variance coupled to the Bonferroni posttest (two-way ANOVA) or two-tailed unpaired t test with Welch’s correction using the Prism software (GraphPad). We compared the extent of adaptation, the fast adaptation proportion, and the fast and slow adaptation rates in Ush1g<sup>fl/fl</sup> and Ush1g<sup>fl/fl</sup>/Myo15-cre<sup>−/+</sup> mice, and no change could be detected in the mutant IHCs (two-way ANOVA, P = 0.34, P = 0.60, P = 0.42, and P = 0.17, respectively).

Fig. S6. Analysis of mechanoelectrical transduction current adaptation in Ush1g^{fl/fl}Myo15-cre^{+/−} inner hair cells (IHCs) from the apex. We characterized the adaptation in Ush1g^{fl/fl} and Ush1g^{fl/fl}Myo15-cre^{+/−} IHCs from the cochlear apex in terms of its extent and kinetics. The adaptive decline of the transduction current $I$ as a function of time $t$ was fitted by the double exponential relation

$$I(t) = A_F \{\exp\left(-\frac{(t-t_0)}{\tau_F}\right)\} + A_S \{\exp\left(-\frac{(t-t_0)}{\tau_S}\right)\} + A_{SS}.$$  

In this equation, $t_0$ is the time at which the stimulus was applied; the fast and slow components of adaptation are characterized by their magnitudes, $A_F$ and $A_S$, and time constants, $\tau_F$ and $\tau_S$, respectively; and $A_{SS}$ describes the transduction current at steady state. From the fit, we deduced the fast and slow adaptation rates, $1/\tau_F$ and $1/\tau_S$, and the proportions, $A_F/(A_F+A_S)$ and $A_S/(A_F+A_S)$, respectively, as well as the extent of adaptation $1 - A_{SS}/(A_F+A_S+A_{SS})$ (1). Statistical significance was tested by using either two-way analysis of variance coupled to the Bonferroni posttest (two-way ANOVA) or two-tailed unpaired $t$ test with Welch’s correction using the Prism software (GraphPad). We compared the extent of adaptation, the fast adaptation proportion, and the fast and slow adaptation rates in Ush1g^{fl/fl} and Ush1g^{fl/fl}Myo15-cre^{+/−} mice, and no change could be detected in the mutant IHCs (two-way ANOVA, $P = 0.78$, $P = 0.44$, $P = 0.08$, and $P = 0.16$, respectively).

Mechanoelectrical transduction current recordings in outer hair cells (OHCs) from Ush1g\(^{fl/fl}\)Myo15-cre\(^{-/-}\) P8 mice. To test if hair cell function was affected despite the absence of morphological changes, we analyzed mechanoelectrical transduction currents at P8 in hair cells from the cochlear apical region (A and B: ~35% the total length of the cochlea from the apex) and the middle region (C and D: ~55% the total length) in Ush1g\(^{fl/fl}\) and Ush1g\(^{fl/fl}\)Myo15-cre\(^{-/-}\) mice. (A) Examples of transduction currents in apical OHCs from Ush1g\(^{fl/fl}\) (black) and Ush1g\(^{fl/fl}\)Myo15-cre\(^{-/-}\) (blue) P8 mice. Mean maximum current amplitude is 1,014 ± 82 pA and 866 ± 67 pA for Ush1g\(^{fl/fl}\) and Ush1g\(^{fl/fl}\)Myo15-cre\(^{-/-}\) inner hair cells (IHCs), respectively (unpaired t test, \(P = 0.19\)). The \(P_o(\chi)\) curves can be superimposed, with values of averaged sensitivity of 3.89 ± 0.38 μm\(^{-1}\) and 3.41 ± 0.18 μm\(^{-1}\) for Ush1g\(^{fl/fl}\) and Ush1g\(^{fl/fl}\)Myo15-cre\(^{-/-}\), respectively (unpaired t test, \(P = 0.28\)). In addition, no change in \(X_{0.5}\) could be detected in the mutant OHCs, with values of 188 ± 23 nm and 196 ± 12 nm in Ush1g\(^{fl/fl}\) and Ush1g\(^{fl/fl}\)Myo15-cre\(^{-/-}\), respectively (unpaired t test, \(P = 0.9\)). (B) We characterized the adaptation in Ush1g\(^{fl/fl}\) and Ush1g\(^{fl/fl}\)Myo15-cre\(^{-/-}\) OHCs from the cochlear apex in terms of its extent and kinetics. We compared the extent of adaptation, the fast adaptation proportion, and the fast and slow adaptation rates in Ush1g\(^{fl/fl}\) and Ush1g\(^{fl/fl}\)Myo15-cre\(^{-/-}\) mice, and no change could be detected in the mutant OHCs (two-way ANOVA, \(P = 0.51, P = 0.05, P = 0.73, P = 0.96\), respectively). (C) Examples of transduction currents in an Ush1g\(^{fl/fl}\) OHC (black) and an Ush1g\(^{fl/fl}\)Myo15-cre\(^{-/-}\) OHC (pink) from the cochlear middle at P8. Mean maximum current amplitude is 963 ± 91 pA and 1,009 ± 60 pA for Ush1g\(^{fl/fl}\) and Ush1g\(^{fl/fl}\)Myo15-cre\(^{-/-}\) OHCs, respectively (unpaired t test, \(P = 0.69\)). The \(P_o(\chi)\) curves can be superimposed with values of sensitivity 3.73 ± 0.27 μm\(^{-1}\) and 3.19 ± 0.70 μm\(^{-1}\) for Ush1g\(^{fl/fl}\) and Ush1g\(^{fl/fl}\)Myo15-cre\(^{-/-}\), respectively (unpaired t test, \(P = 0.48\)). In addition, no change in \(X_{0.5}\) could be detected in the mutant IHCs, with values of 179 ± 19 nm and 213 ± 29 nm in Ush1g\(^{fl/fl}\) and Ush1g\(^{fl/fl}\)Myo15-cre\(^{-/-}\) IHCs, respectively (unpaired t test, \(P = 0.34\)). (D) We characterized the adaptation in Ush1g\(^{fl/fl}\) and Ush1g\(^{fl/fl}\)Myo15-cre\(^{-/-}\) OHCs from the cochlear middle in terms of its extent and kinetics. We compared the extent of adaptation, the fast adaptation proportion and the fast and slow adaptation rates in Ush1g\(^{fl/fl}\) and Ush1g\(^{fl/fl}\)Myo15-cre\(^{-/-}\) mice, and no change could be detected in the mutant OHCs (two-way ANOVA, \(P = 0.5, P = 0.05, P = 0.91, P = 0.96\), respectively).
Fig. S8. Protocadherin-15 CD2 and CD3 and cadherin-23 colocalize with sans at the plasma membrane in cotransfected COS-7 cells. (A) COS-7 cells were transiently transfected to produce one of the protocadherin-15 (pcdh15) CD1, CD2, or CD3 isoforms and/or sans. In the single-transfected cells, protocadherin-15 CD1, CD2, and CD3, but not sans, are targeted to the plasma membrane. In cotransfected cells, sans is recruited at the plasma membrane only in cells that also produce protocadherin-15 CD2 or CD3, but not CD1. (B) COS-7 cells were transiently transfected to produce cadherin-23 (cdh23) and/or sans. In cotransfected cells producing sans and cadherin-23, sans is recruited at the plasma membrane, where it colocalizes with cadherin-23. (Scale bars: 5 μm.)