SUPPLEMENTARY MATERIALS AND METHODS

Generation of *PTXa* mice

To produce the *PTXa* line described here, a *mycPTXa* PCR fragment was cloned into *pBigT* (Addgene #21270) using EcoRI/Nhe(blunt) and SacI (Srinivas et al., 2001). The *BigT-mycPTX* fragment was then inserted into *pRosa26PA* (Addgene #21271) using PacI and AsclI. The linearized plasmid was used as a vector for homologous recombination to insert a *SA-(loxP-PGKneo-tpA-loxP)-mycPTXa-bpA* cassette of 4kb at the *Rosa26* locus using CRISPR/Cas9 and fertilized oocyte injection, as depicted in Fig. S2A.

Antibodies and Imaging

Primary antibody used were rabbit anti-mInsc (raised against long isoform-specific MRRPPGDGDSTGEG peptide; 1:1000), rabbit anti-LGN (gifts from F. Matsuzaki, RIKEN, and Q. Du, Georgia Regents University, and Sigma HPA007327 lot A41537; both 1:1000), rabbit anti-Gαi (Sigma G4040 lot 89H0210; 1:600), rabbit anti-Egfp (ThermoFisher A11122 lot 1691382; 1:1000), mouse anti-myc (9e10, SCBT sc-40 lot F1710; 1:500), goat anti-Cdh23 (SCBT sc-26338 lot B2604; 1:100), chicken anti-PDZD7 (N-ter; gift from Jun Yang, U. of Utah; 1:500) and mouse anti-Eps8 (BD Biosciences 610143 lot 4330935; 1:200). F-actin (F-act) was labeled with AF488 or rhodamine-conjugated phalloidin (ThermoFisher A12379, R415). Images were captured using a Zeiss LSM710 confocal or a Leica DM6000B epifluorescence microscope and processed using Adobe Photoshop CS6.

Quantitative analysis

To quantify the fraction of apical LGN protein respectively present at the bare zone and stereocilia tips (Fig. 4C), Image J was used to determine the Raw Integrated Density (sum of pixel values) in each compartment, correcting for background signal on the same image. For each cell, the ratio of tip and bare zone intensity was calculated as the fraction of total intensity. Ratios for 9 OHCs representing all rows and 3 IHCs were averaged for each newborn, and n=4 controls ($LGN^{DEL/+}$) and n=3 mutants ($LGN^{DEL/DEL}$) were plotted for OHCs, and n=3 controls and n=4 mutants were plotted for IHCs.

To quantify stereocilia height, cochlear samples were tilted during mounting and imaging to limit parallax issues. Scanning electron microscopy images were analyzed using ImageJ's measurement tool and cell counter plugin. While the tilt applied ranged between 50-90°, care was taken to have similar tilts among directly compared littermate samples. Stereocilia diameter was measured in the distal portion. Stereocilia were considered to be a row within the bundle if they were regularly spaced and spanned at least 50% of the width of the bundle. Graphics and statistics were produced using Graphpad Prism.

REFERENCES

Srinivas, S., Watanabe, T., Lin, C. S., William, C. M., Tanabe, Y., Jessell, T. M. and Costantini, F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC developmental biology* **1**, 4.

SUPPLEMENTARY FIGURES

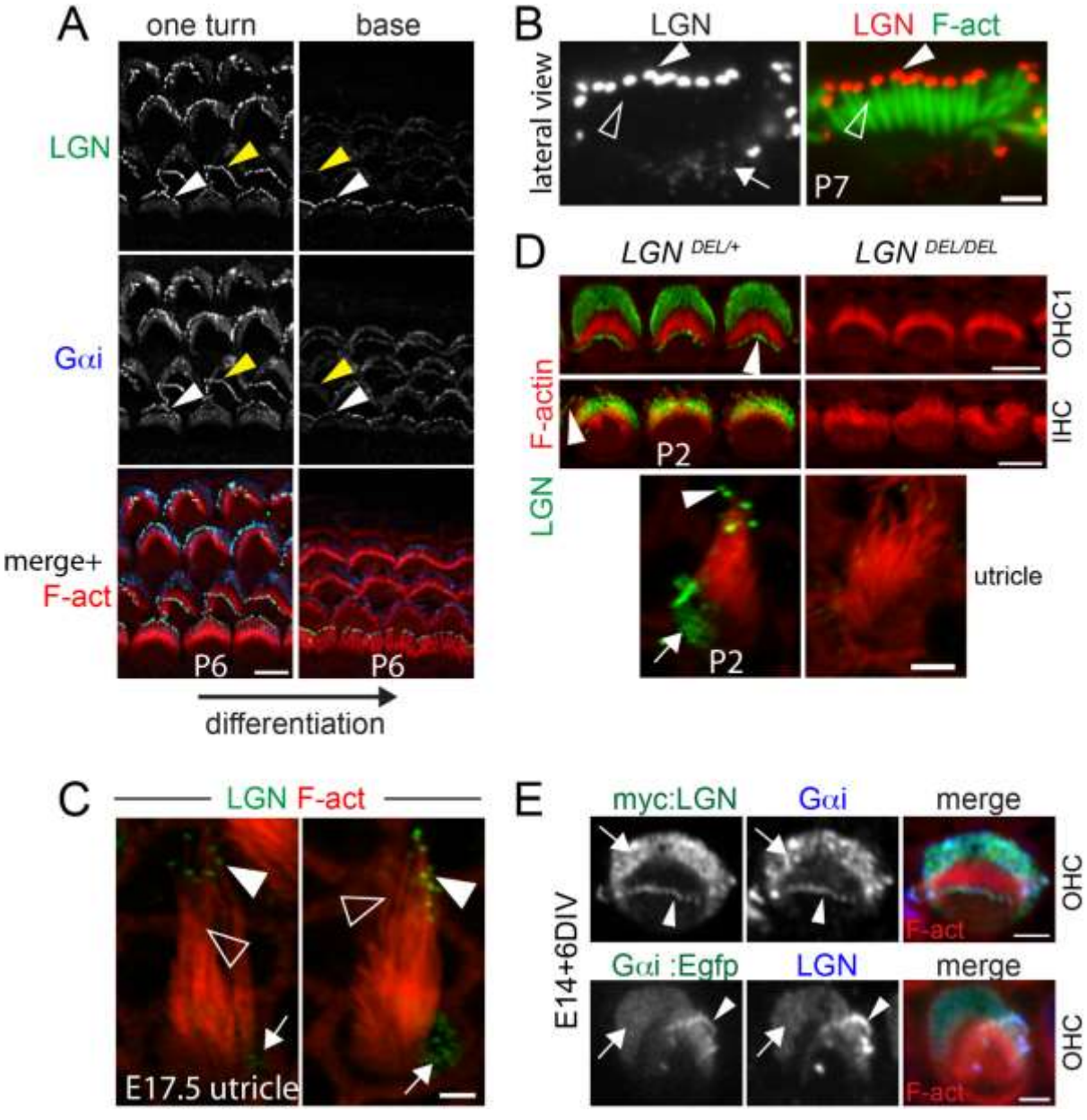

Figure S1. LGN and Gαi localize at the distal tip of stereocilia in the tallest row. (A) LGN and Gαi co-immunostaining at P6 in a less differentiated (one turn) and more differentiated (base) region of the cochlea. Note how protein accumulation at stereocilia tips decreases in OHCs (yellow arrowhead) compared to IHCs (white arrowhead). **(B)** Lateral side view of an IHC immunostained for LGN at P6. In this view, the bare zone staining (arrow) is not superimposed with the bundle, and no LGN signal is visible at tips in the second row (hollow arrowhead) in spite of overexposure and saturated signal in the first row (solid arrowhead). **(C)** LGN immunostaining in utricular HCs at E17.5. As in the cochlea, LGN is detected specifically at the tip of the tallest stereocilia (arrowheads) in addition to the bare zone (arrow). **(D)** LGN immunostaining in $LGN^{DEL/+}$ and $LGN^{DEL/DEL}$ cochlear and utricular samples. Antibody detection of LGN at stereocilia tips (arrowheads) is specific, like at the bare zone. **(E)** Myc-tagged LGN recapitulates endogenous LGN distribution at the bare zone (arrow) and stereocilia tips (arrowheads), and colocalizes with endogenous Gαi in OHCs. Reciprocally, Egfp-tagged Gαi colocalizes with endogenous LGN. In spite of excess fusion protein, a single line of dots (arrowheads) denotes specific enrichment in the tallest row and absence from the shorter rows. Cochleas were electroporated at E14.5 and cultured for 6 days. Solid arrowheads: stereocilia tips in the first row; hollow arrowheads: stereocilia tips in the second row; arrow: bare zone. All images are en-face views of the apical surface of cochlear or utricular sensory epithelium. Scale bars: 5μm (A; D, top), 2μm (B-C; D, bottom; E).

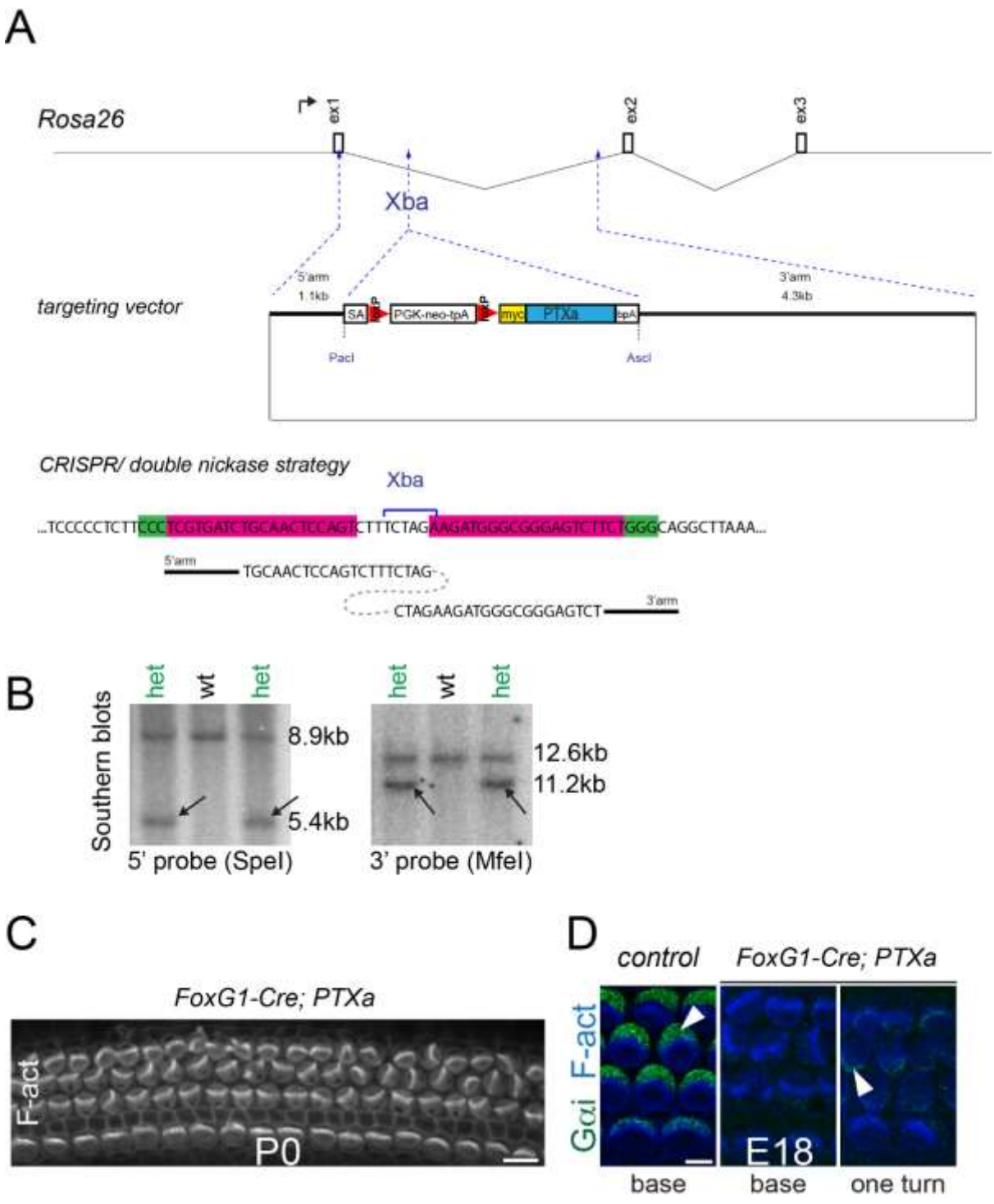

Figure S2. Design and characterization of the *Rosa26-(loxP-stop-loxP)-mycPTXa* mouse model. (A) Scheme of the CRISPR/Cas9 strategy used to insert a 4kb *SA-(loxP-stop-loxP)-mycPTXa* cassette at the Xba site of the *Rosa26* locus (Soriano, 1999). The guide RNA and PAM sequences are indicated in pink and green, respectively. See Methods for details. **(B)** Southern blots used to validate founder animals and their progeny for proper targeted insertion at the *Rosa26* locus. Probes flanking the homology arms on each side were used with the indicated restriction digests. **(C)** When *mycPTXa* is activated in otic progenitors using the *FoxG1-Cre* driver, organ of Corti defects appear restricted to apical HC differentiation, with notably severe bundle misorientation at P0. **(D)** PTXa severely disrupts Gαi accumulation at the HC apical membrane, although leftover protein can be detected in less differentiated HCs (right-most panel). Scale bar: 10μm (C), 5μm (D).

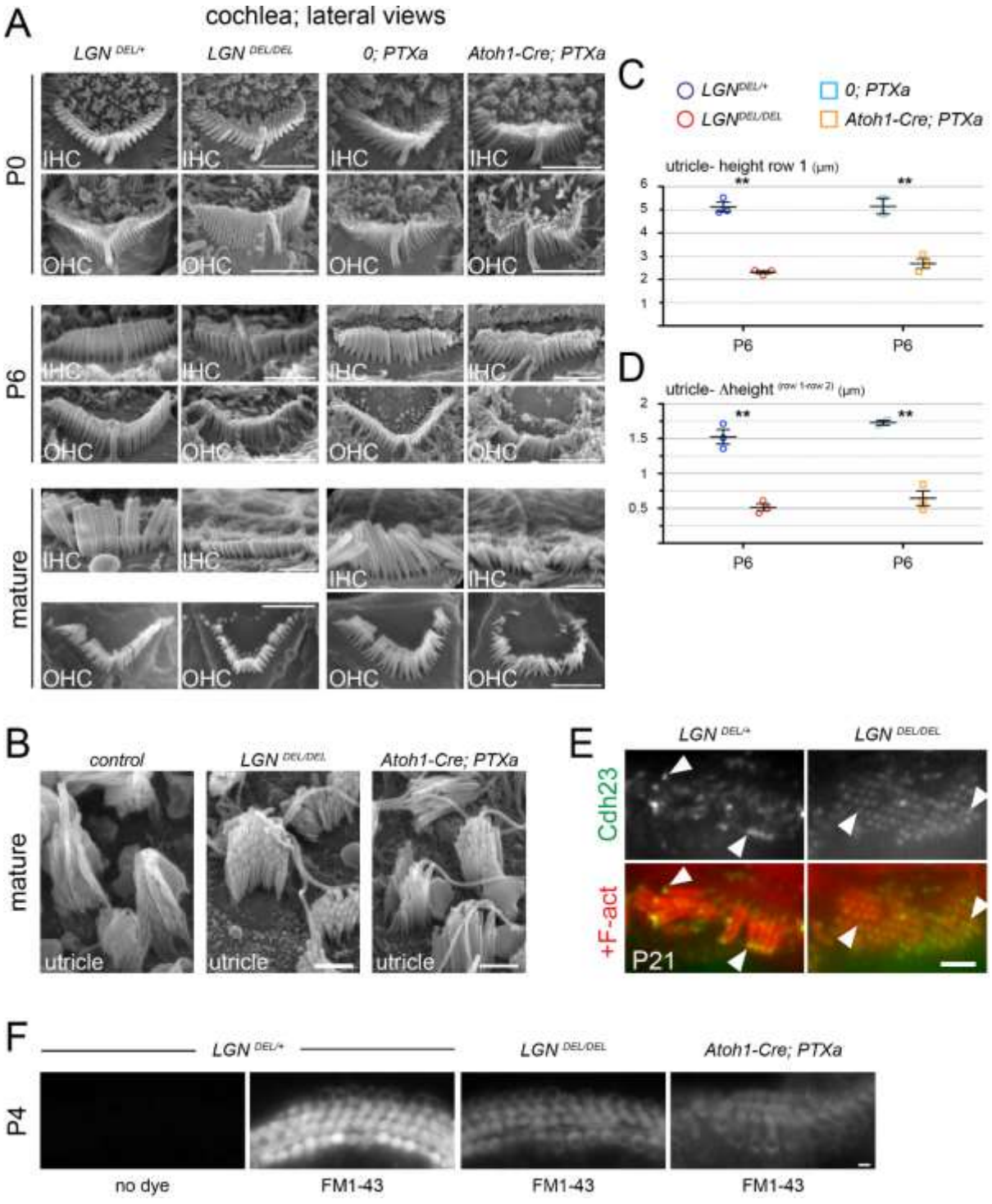

Figure S3. Hair bundle defects in absence of LGN and Gαi function. (A) Close-up *lateral* views of representative single IHCs (top) and OHCs (bottom) at P0, P6 and mature stages imaged with scanning electron microscopy. Mature *LGN* and *PTXa* samples are P14 and P22, respectively. **(B)** Hair bundle defects in striolar HCs in the utricle at mature stages. **(C-D)** Utricular HC quantification of stereocilia height in the first row (C) and height differential between the first and second row (D) in the striolar region at P6. Average per animal ± sem is plotted for n=3 animals. Welch's t-test (ns: $P>0.05$; * $P\leq0.05$; ** $P\leq0.01$). Quantifications are detailed in Table S2. **(E)** Cdh23 immunostaining at P21 in IHCs. Signal near stereocilia tips (arrowheads) is retained in *LGN* mutants, suggesting tip-links are present. **(F)** FM1-43 dye uptake experiment at P4 for the indicated genotypes. Both *LGN* and *PTXa* mutant HCs are still able to incorporate the dye, suggesting mechanosensory channels are functional. Scale bar: 2μm (A, B, E), 10μm (F).

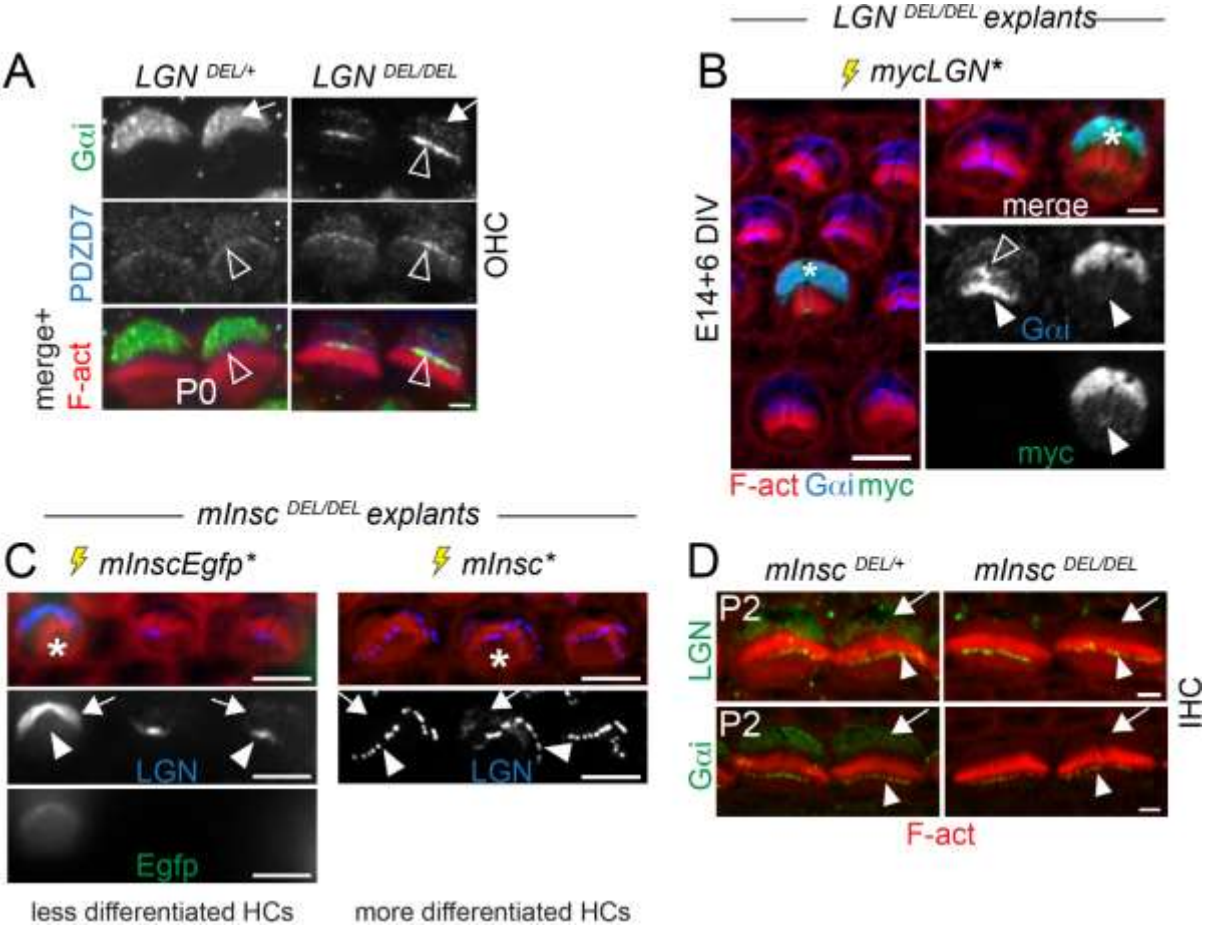

Figure S4. Balanced LGN-Gαi distribution between the bare zone and stereocilia tips. (A) Gαi immunostaining at P0. Ectopic Gαi accumulation at stereocilia base (hollow arrowheads) is seen in *LGN* mutants, as shown by colocalization with the USHER2 protein PDZD7 (blue). **(B-C)** Single HC protein rescue in mutant explants restores proper balance of partner proteins between the bare zone and stereocilia tips. **(B)** $LGN^{DEL/DEL}$ explants electroporated at E14.5 with mycLGN construct and cultured for 6 days. Transfected hair cells (asterisks) have a restored balance of Gαi between the bare zone (arrow) and stereocilia tips (arrowhead), with lower amount at tips and higher amount at the bare zone. **(C)** $mInsc^{DEL/DEL}$ explants electroporated at E14.5 with mInscEgfp construct, and cultured for 6 days. *Left panels:* transfected hair cells (asterisk) have a restored balance of LGN between the bare zone (arrow) and stereocilia tips (arrowhead), with lower amounts at tips and higher amount at the bare zone. *Right panels:* in more differentiated HCs, the mInsc construct rescues LGN enrichement at the bare zone (arrow), but does not affect normal LGN enrichment at tips (arrowheads). mInsc was electroporated without reporter in this experiment. **(D)** Normal distribution of LGN (top) and Gαi (bottom) at stereocilia tips at P2 in $mInsc^{DEL/DEL}$. Note both proteins are still strongly downregulated at the bare zone (arrows). Scale bar: 2μm (A; B, right; D), 5μm (B, left; C).

REFERENCES

Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* **21**, 70-71.

Movie 1. Free swimming behavior of control and mutant *LGN* and *PTXa* mice. Mice were introduced to an open pool and their behavior recorded. Twenty-second clips are shown for each genotype: $LGN^{DEL/+}$, $LGN^{DEL/DEL}$, *0; PTXa*, and *Atoh1-Cre; PTXa*. Mutant animals show less organization of their swimming movements and escape search compared to controls.

Table S1

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Table S2

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