

Expanded View Figures

Figure EV1. OHC electromotility remains unaffected in *Lrba* mutants.

Prestin-driven electromotility remains functional in Lrba-KO OHCs (p15-16).

- A Motor protein levels in the OHC membrane leaflet were determined electrophysiologically by analyzing the nonlinear capacitance (NLC) of WT and *Lrba*-KO OHCs in response to a stair-step protocol.
- B, C Representative current traces to quantify; (B) NLC voltage dependence; and (C) peak capacitance. In these latter experiments, no statistically significant differences between WT and *Lrba* mutants could be detected (WT n = 10, *Lrba*-KO n = 9; P = 0.063; Student's t-test). Data are presented as means \pm s.e.m.



Figure EV2. Normal maturation, synapse count, and synaptic function in *Lrba* mutant IHCs.

- A, A' Spotlike BK channel (green) staining at the IHC neck region indicates normal IHC maturation in *Lrba* mutant mice; IHCs have been counterstained for otoferlin (magenta). Scale bar: 5 µm.
- B, B' Synapse count in apical turn IHCs from p14–16 WT and *Lrba*-KO mice, as assessed by presynaptic CtBP2 (magenta) and postsynaptic GluA2/3 (green) co-staining remains unaltered, as quantified in (B'). Data are presented as means ± s.e.m. The dashed line in (B) outlines a single IHC, and asterisks indicate nuclei. Scale bar: 10 µm.
- C-C" Electrophysiologically recorded whole-cell Ca²⁺ current-voltage relationship from p14-17 IHCs show similar voltage dependence and current amplitudes in both genotypes. (C') Exocytic Δ Cm in response to step depolarizations to the respective maximum Ca²⁺ current potential for either 20 ms (to deplete readily releasable vesicles) or 200 ms (to probe sustained exocytosis) revealed no statistically significant difference in synaptic release between WT (n = 18; N = 16) and *Lrba*-KO IHCs (n = 10; N = 7) (P = 0.24 and P = 0.51, respectively; Student's *t*-test). (C") Similarly, release efficiencies for both depolarization durations (i.e., $\Delta Cm \text{ per } Q_{Ca2+}$) appear unchanged in Lrba mutants. Data are presented as means \pm s.e.m.



Figure EV3. LRBA partially colocalizes with the intracellular transport vesicle-associated protein Rab11 at the kinociliar pore of cochlear hair cells.

- A p7 wild-type organs of Corti were co-stained with specific antibodies against Rab11 (green) and LRBA (magenta). Fluorophore-conjugated phalloidin (blue) was used to visualize actinrich stereocilia. Representative single optical section of an organ of Corti overview at the height of the cuticular plates. Scale bars: 5 um.
- B, C
 Representative magnified views of a single (B)
 OHC and (C) IHC, respectively. Scale bars:
 5 μm.
- D, D' Detailed incremental z-stacks of the dashed boxes in (B and C), illustrating the overlapping signal between Rab11 and LRBA (as indicated by "white pixels") in ascending optical planes (step size 200 nm) from the hair cell neck (N) through the cuticular plate (CP) of a representative (D) OHC and (D') IHC. Scale bars: 1 μm.
- E-F' Pixel-based intensity analysis performed on the representative example images shown in panels (B and C) for LRBA and Rab11 or (E'-F') LRBA and phalloidin as an internal control. Pearson's correlation coefficients (P_r; marked in red in the respective panels) were determined by fitting the data sets with linear regressions and illustrate the positive correlation for LRBA and Rab11. In contrast, LRBA and phalloidin signals show a negative correlation and hence do not colocalize (please also refer to Fig 4F-F" in this context). Scale bars: 1 μ m.



Figure EV4. Unaltered targeting of whirlin to stereociliar tips and normal planar cell polarity in Lrba mutant hair cells.

- A, A' Whirlin (magenta) is found at stereociliar tips of p8 OHCs and IHCs of both, (A) wild-type and (A') *Lrba* mutant animals (blue and white arrows in the insets indicate first- and second-row stereocilia, respectively, for representative OHCs at adjusted brightness for clarity). Stereocilia have been counterstained using fluorophore-coupled phalloidin (green). Scale bars: 5 μm.
- B, B' Lrba mutant hair cells exhibit normal planar cell polarity, as assessed by calculating the degrees of deviation (°) from an axis perpendicular to the apico-basal axis of the organ of Corti that runs through the center of each individual hair bundle (illustrated in B for an OHC; dashed blue lines indicate the perpendicular axes, dashed white line and white arrow illustrate the degrees of deviation). Scale bars: 5 μm.
- C-C" Quantification of the data from (B) (in C: P = 0.33, in C': P = 0.56; Student's t-test). Numbers indicate the number of hair cells analyzed. (C") Simplified graphical representation of the approximate kinocilia insertion points for OHCs and IHCs in wild-type and Lrba-KOs. Data are presented as means \pm s.e.m.
- D–D" Basal body and kinocilium localization are unaffected by stereociliar loss in *Lrba*-KO hair cells. Regardless of the extent of stereociliar loss in *Lrba* mutants (D'), stereociliar pivot points can still be observed (D"). Samples were stained with specific antibodies against pericentrin (green; basal body) and acetylated tubulin (magenta; kinocilia) as well as fluorophore-conjugated phalloidin (blue, actin-rich stereocilia). Scale bars: 5 µm.



Figure EV5. No major loss of cochlear hair cells can be observed in 29-week-old *Lrba* mutants and the afferent innervation pattern is maintained; moreover, vestibular hair cells do not show a prominent hair bundle phenotype.

- A, A' Representative confocal maximum projections of 29-week-old (A) wild-type and (A') *Lrba*-KO organs of Corti stained with a specific antibody against the exocytic protein otoferlin (magenta). Actin-rich structures were counterstained with fluorophore-conjugated phalloidin. Scale bars: 10 μm.
- B, B' IHC afferent innervation patterns in (B) wild-type and (B') *Lrba* mutants are maintained after 29 weeks. Specimen were stained with specific antibodies against NF200 (green; SGN afferent fibers) and the IHC-specific marker Vglut3 (magenta). Scale bars: 10 μm.
- C-C" Utricular hair cells also express LRBA at the fonticulus, but do not show any obvious hair bundle deficits at an age where cochlear hair bundles are degenerated. (C) Detail of a single utricular hair bundle illustrating LRBA localization at the fonticulus. Overviews of (C') wild-type and (C") Lrba-KO hair bundles from p22 utricles. Scale bars: 5 μm in (C); 10 μm in (C' and C").