The BEACH protein LRBA is Required for Hair Bundle Maintenance in Cochlear Hair Cells and for Hearing

Short title: **LRBA is Required for Hair Bundle Maintenance in Cochlear Hair Cells**

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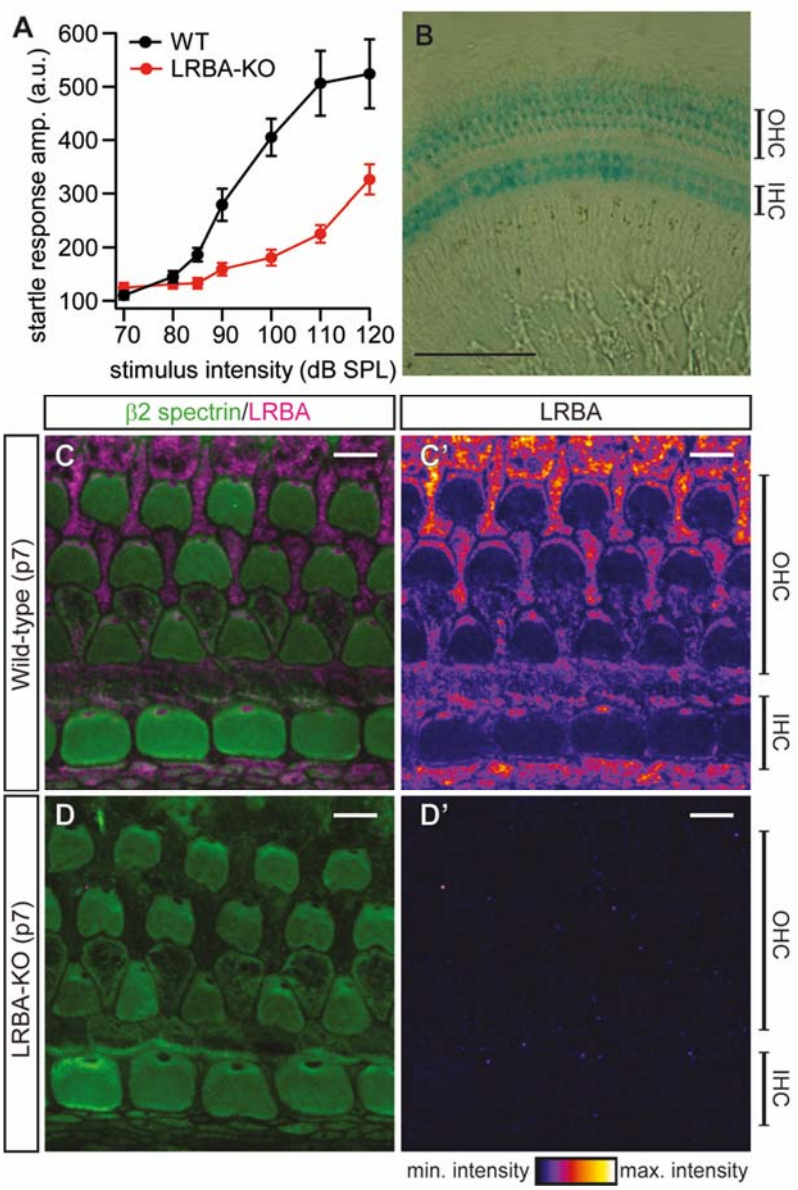
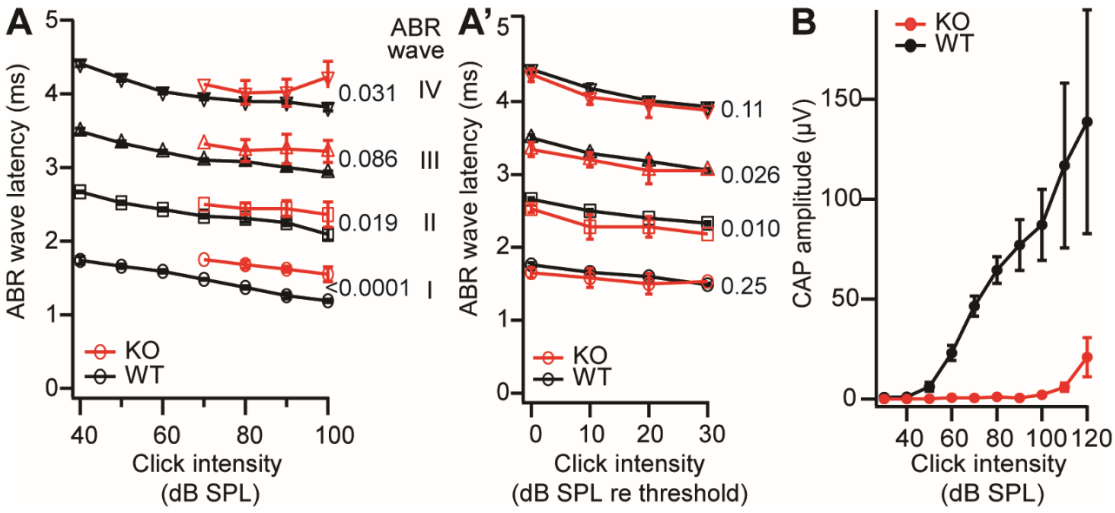


Appendix Figure S1 - LRBA is specifically expressed in IHC, OHC and supporting cells, and reduced acoustic startle responses in Lrba-KO mice.

(**A**) Acoustic startle response amplitudes were measured using a commercially available system (Med Associates Inc., VT, USA with "Advanced Startle" software) based on the protocol used in IMPRESS from the International Mouse Phenotyping Consortium (IMPC, see www.mousephenotype.org/impress). Background noise was 65 dB. Each session was initiated with a 5-min-acclimation period followed by five presentations of leader startle pulses (110 dB) that were excluded from statistical analysis. Startle responses to 40 msec white noise bursts of varying intensity were significantly reduced in *Lrba*-KO compared to WT animals (n=10 each, mean ± SEM; $p<0.0001$, 2-way ANOVA).

(**B**) Representative LacZ staining in the apical coil of an organ of Corti (p24) of a LRBA reporter mouse demonstrates the expression of LRBA in IHC, OHC and possibly in supporting cells. Scale bar: 100 µm.

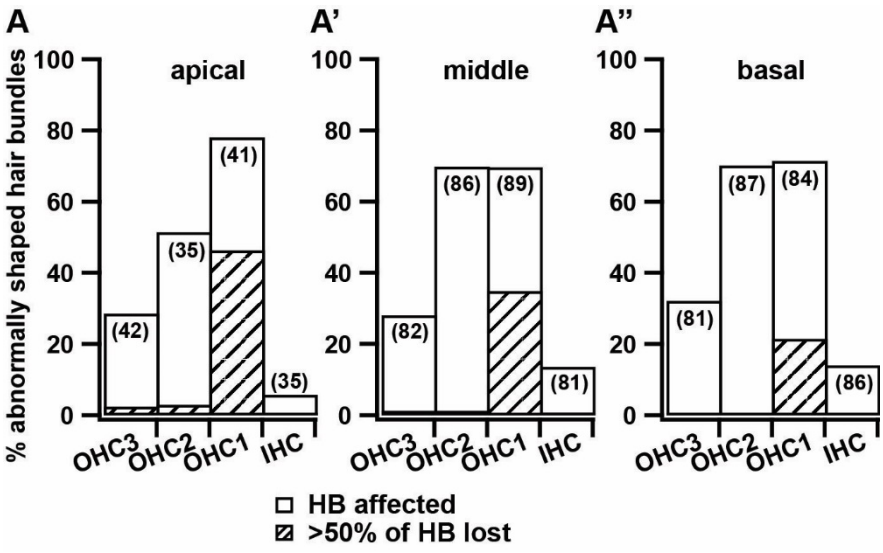
(**C,D**) Cochlear whole mount preparations from wild-type (**C**) and *Lrba*-KO littermate mice (**D**) processed and imaged in parallel with identical settings show the absence of LRBA immunofluorescence in the KO in IHC, OHC and supporting cells. C', D' show LRBA immunofluorescence signal illustrated with an intensity coded look-up table for improved visualization of the fluorescence intensity distribution. Scale bar: 5 µm.



Appendix Figure S2 - ABR latency and electrocochleography analysis.

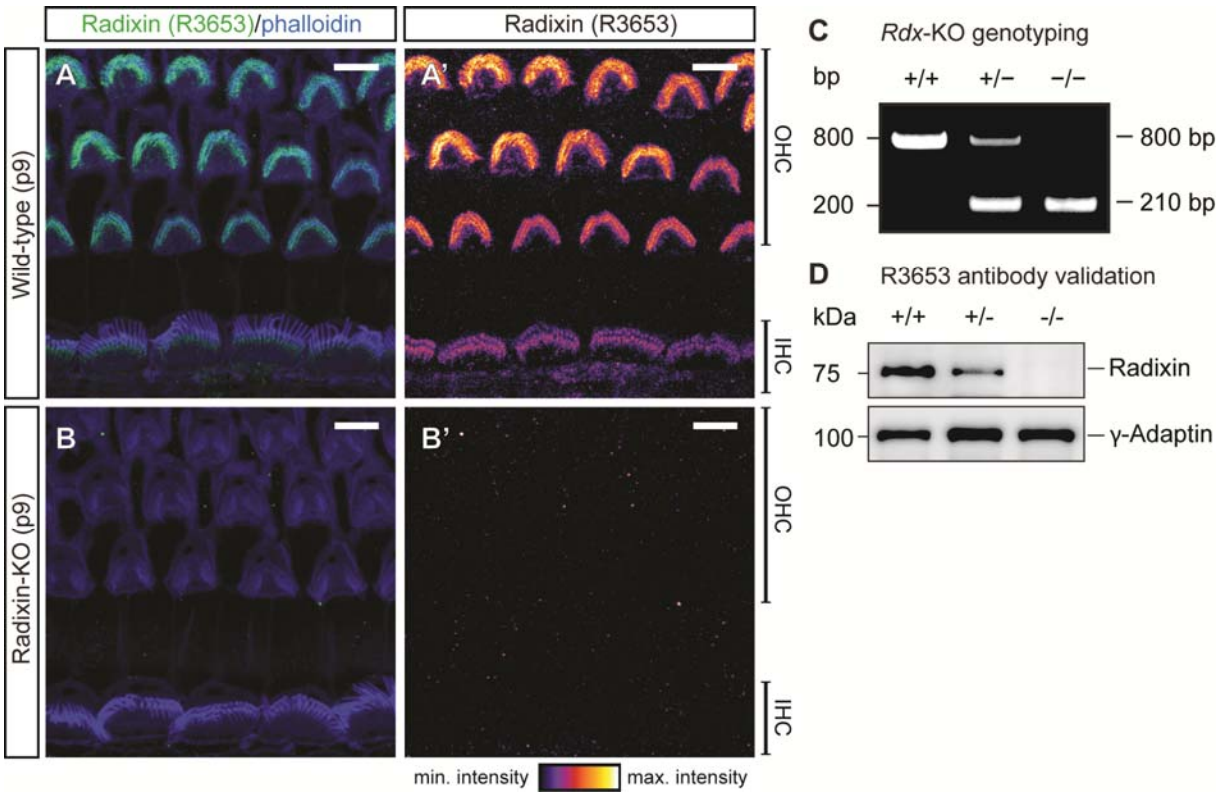
(**A**) ABR latencies in *Lrba*-KO mice (n=8) are increased compared to WT (mean ± SEM, n=9; age group 4-5 weeks) when comparing for same stimulus intensities but not after correcting for the loss of cochlear sensitivity (**A'**). Numbers indicate p values of genotype factor from 2-way ANOVA, 70-100dB (A) or 0-30dB (A'). The slight reduction in corrected latency is likely due to the shallower amplitude growth function.

(**B**) Amplitudes (means ± SEM, n=8 each, age 4-5 weeks) of the SGN compound action potential, assessed by electrocochleography, show similar changes as ABR wave I (Amplitude difference between genotypes $p<0.0001$, 2-way ANOVA).



Appendix Figure S3 - ***The severity of the Lrba-KO associated hair bundle phenotype is dependent on the tonotopic position.***

(**A-A'''**) Frequency (in %) of abnormally shaped hair bundles in *Lrba*-KO hair cells from SEM images of p15 *Lrba*-KO and wild-type littermates of (**A**) apical, (**A'**) medium and (**A''**) basal turns, respectively. Hair bundles from the outermost OHC row (OHC3; i.e. furthest from the IHCs) seem to be the least affected (~30%), while the first and second OHC rows show more abundant structural deficits (~70%), which appear to be conserved throughout the cochlea; however, the severity of these deformities in OHCs (i.e. complete loss of one entire half of the hair bundle; as indicated by the hatched bars) appears to follow a tonotopic gradient, where basal OHCs are less affected than apical OHCs. In contrast, deterioration of IHC hair bundles could generally be observed less frequently (<15%) and appeared to be comparable independent of tonotopic position.



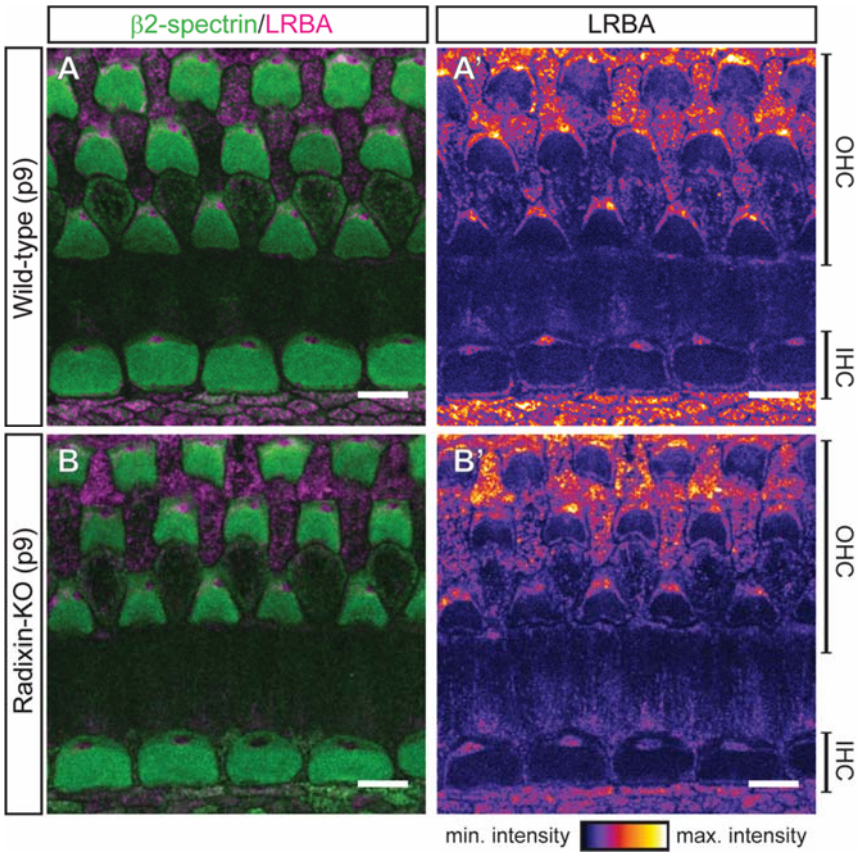
Appendix Figure S4 - *Radixin antibody specificity validation in the organ of Corti.*

To ensure target protein specificity of the commercially available anti-radixin antibody used in this study (R3653; Sigma Aldrich), we performed immunolabeling experiments of organs of Corti from age-matched wild-type control and *Rdx*-KO [1,2] animals at p9.

(**A-B'**) While R3653 prominently labelled OHC hair bundles – and, to a much lesser degree IHC stereocilia – in wild-type controls (**A**), hair bundles from (**B**) *Rdx*-KO hair cells did not show any significant and/or specific labelling. (**A'-B'**) RDX immunofluorescence signal illustrated with an intensity coded look-up table for improved visualization of the fluorescence intensity distribution, where warmer colors indicate higher intensity. All immunolabellings where performed in parallel under identical conditions to enable direct comparison of *Rdx*-KO and wildtype tissues and confirm target specificity under the given conditions. A'-B'.

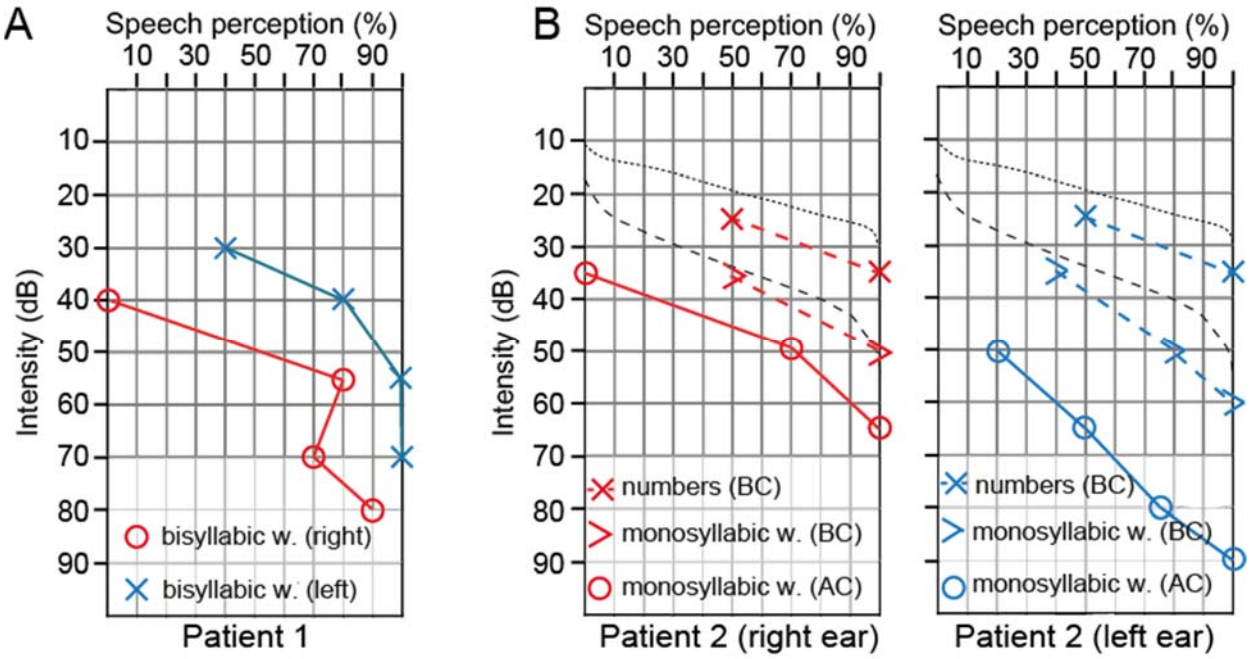
(**C-D**) *Rdx*-KO genotyping clearly identifies KO mutants (age: 17 weeks). (**D**) R3656 antibody validation on cochlear extracts of WT (+/+), heterozygous (+/-) and *Rdx*-KO (-/-) animals by Western blotting.

1. Kikuchi S, Hata M, Fukumoto K, Yamane Y, Matsui T, Tamura A, Yonemura S, Yamagishi H, Keppler D, Tsukita S, et al. (2002) Radixin deficiency causes conjugated hyperbilirubinemia with loss of Mrp2 from bile canalicular membranes. *Nat Genet* **31**: 320–325.
2. Kitajiri S, Fukumoto K, Hata M, Sasaki H, Katsuno T, Nakagawa T, Ito J, Tsukita S, Tsukita S (2004) Radixin deficiency causes deafness associated with progressive degeneration of cochlear stereocilia. *J Cell Biol* **166**: 559–570.



Appendix Figure S5 - LRBA expression appears unaltered in RDX-KO mice.

(**A-B'**) Cochlear whole mount preparations from p9 (**A-A'**) wild-type and (**B-B'**) *RDX*-KO mice processed and imaged in parallel with identical settings show a comparable LRBA distribution pattern in both genotypes. A' and B' show LRBA immunofluorescence signal illustrated with an intensity coded look-up table for improved visualization of the fluorescence intensity distribution. Scale bar: 5 µm.



Appendix Figure S6 - Speech audiometry in Lrba patients.

(**A**) Speech audiometry in patient 1 (in French, bisyllabic Fournier test, monaural presentation): elevated thresholds but close to normal discrimination at high intensities. In the Lafon test (free field presentation), speech discrimination was moderately impaired in quiet (25/30 correct) but severely deteriorated in background noise (11/30). (**B**) Speech audiometry in patient 2 (in German, Freiburger test, monaural presentation): mildly elevated speech perception thresholds for four-syllable numbers (x) and monosyllabic words (>) when presented by bone conduction (normal values: dotted/dashed black lines). Using air conduction headphones (o), perception of monosyllabic words is mildly impaired on the left and moderately impaired on the right side. Speech discrimination at high intensities was not impaired.