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

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 15 December 2016

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge the potential interest of the findings. However, they also raise a number of - sometimes overlapping - issues that would need to be addressed before publication. Importantly, referee 1 and 2 both point out that the data describing and validating the LRBA knockout is not provided in the manuscript. As the proof that the studied mutant allele is indeed a null allele is essential for the presented findings, some data on the design and functionality of the knockout has to be included in the manuscript, even if a more detailed description will be published elsewhere. Moreover, all experiments involving human subjects have to be approved by an Ethics committee. Please confirm that this was also the case for patient 2 and please provide evidence confirming that informed consent to publish the data was obtained from all subjects. Please also see our Author Checklist concerning required information and documentation for studies involving human subjects (available for download on our homepage/Author Guidelines). Moreover, I suggest removing the country-assignment of the patient.

Given the overall constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome

of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

REFEREE REPORTS

Referee #1:

The manuscript from Vogl and co-authors (The BEACH protein LRBA is required for hair bundle maintenance in cochlear hair cells and for hearing) provides convincing data that in mouse a recessive mutant allele of Lrba is associated with hearing loss and several lines of evidence indicate hair cell stereocilia bundle degeneration after normal appearing development of these cells. In large part, the manuscript is nicely written. However, there are many issues (many of them, but not all, are minor and easily corrected) listed below that need attention and with revision would significantly improve the manuscript.

1. Abstract, last sentence, Comment about two patients seems tacked on to the end of the abstract. The statement lacks details such as to the mutant alleles of LRBA found in the two subjects.

2. Was study of human subjects approved by an IRB/Ethics Committee/Helsinki Committee? Did the subjects/parent give written informed consent to participate in the study prior to undertaking the study? In the last sentence, under study approval, the authors only state that "Patient 1 and parents of patient 2 gave informed consent to publish the data."

3. Introduction, 2nd paragraph, line 7, change "carries" to "has"

4. Discussion, 2nd page, 2nd to last line, provide a reference for RDX-interacting protein Nherf2.

5. Results, The ko of LRBA is not described in this manuscript and is said to be a deletion of exon 3 that shifts the reading frame when presumably exon 2 is spliced to exon 4. A description of the wild type LRBA gene needs to be added to the text and/or as a figure and some form of convincing data must be provided that this mutant allele is a knockout of protein expression or at least what is expressed is nonfunctional. Western data is not provided. Expression of LRBA or a lack of it is not provided. The authors indicate that these data are to be provided elsewhere. Unacceptable -- not good science.

6. Results, the LRBA mouse is reported to show a "reduced startle response". How was the startle response quantified as authors claim a reduction.

7. Do your results regarding prominent immunolocalization signals of LRBA in inner and outer hair cells correspond to what has been reported for LRBA in the Harvard SHIELD and U of Maryland gEAR databases?

8. For human mutations, give the accession number for the DNA sequence and protein c.5047C>T, c.1420G>A, and c.2834_2837delTCTT need a GenBank or RefSeq accession number.

9. Discussion, first paragraph, "...hair bundle defect induces significant hearing loss, but..." "induces" is not the right word.

10. Discussion, The authors mention that "...the large size of the LRBA protein impede further biochemical studies to..." Plenty of even larger proteins have been studies biochemically after purification. Western blots also possible.

11. Discussion, something is wrong with the phrase "despite of unchanged numbers..." delete "of"?

12. Figure 1, legend, There is no way of knowing if LRBA is highly expressed in hair cells from your immunolocalization images unless somehow you quantify expression. LRBA may be easily detectable but that's the best you can say.

13. Figure 1 supplement, panel B, how was startle response amplitudes quantified?

14. In the immunolocalizations figure 4, is your antiserum detecting boththe long and short isoforms of LRBA? In figure 4, it would be helpful in the interpretation of the data to have a cartoon of the LRBA gene, LRBA protein and the location of the epitope for the antisera.

15. In humans LRBA is located on chromosome 4q. Is LRBA in or outside the DFNB26 deafness interval on 4q31.3? The gene underlying DFNB26 has not been reported.

16. Figure 7, "Hearing impairment in LRBA-deficient patients" How do you know they are deficient for LRBA, if by deficient you mean "null". If not, maybe better terminology than "deficient" could be used here.

Referee #2:

Vogl and colleagues show that the protein LRBA is required for maintenance of cochlear hair bundles, particularly those of OHCs. The data largely support their hypotheses, although the manuscript could probably stand to be shortened. There is a considerable amount of data described in detail that is essentially negative; while I appreciate it was a lot of work to collect, it is distracting for the reader who is trying to understand the consequences of loss of LRBA. In particular, the authors present a large amount of data on cochlear and auditory nerve physiology that could be distilled considerably, given that they were able to narrow in on hair cell function, especially bundle mediated transduction.

The authors are technically correct in saying that the KO leads to impaired cochlear amplification and reduced synaptic transmission, but their data suggests that these effects are entirely due to disrupted bundles and altered transduction. The dye labeling experiments they used are inadequate to measure transduction (see below). Of course, it would be nice to look at transduction in mice that are old enough to display the profound phenotype, but measurement of transduction in older mouse hair cells is difficult if not impossible at the moment. Nevertheless, the authors need to be precise about primary vs. secondary defects.

Finally, it is true that there are some similarities between the Rdx and Lrba knockouts; perhaps this could be investigated more. Mechanistically, give the state of knowledge about function of RDX and LRBA, do you have a hypothesis for a functional connection between the two proteins?

One last major comment. Remember that in mouse, gene symbols are first letter capitalized, all italics; protein symbols are all caps, not italicized. There is some inconsistency in formatting of symbols.

Comments:

1. Always use page numbers and it is better to use line numbers as well. It is very annoying to not have the page numbers; I have added them to my copy of the manuscript, with page #1 being the title page.

2, Page 4, second line. A phenotype cannot cause hearing loss-hearing loss IS a phenotype.

3. Page 5, first Results paragraph. If the immunoblot data are important to this manuscript (and I think they are), then you must show them here-not "to be described elsewhere."

4. Page 5, second Results paragraph. Do we really believe the immunolabeling results fully? The KO control is good but isn't fully consistent with the beta-gal labeling data-there just OHC and IHC labeling. Is there really any evidence for supporting cells, Reissner's membrane, and the stria? Higher magnification of the appropriate regions of control and KO would be valuable.

5. Page 5, last paragraph. Thresholds are displayed in Fig. 2A (not 2B-C); 2B-2C shows amplitudes. Correct the text.

6. Page 6, first paragraph. "...involves a defect of cochlear amplification..." is misleading. That

suggests a specific defect in the amplification mechanism, but all the evidence points to disturbed hair bundles and hence aberrant mechanoelectrical transduction instead.

7. Page 6. I am confused; the text indicates that SGN firing rates were distributed towards high SR fibers in the KOs (I see Fig. 3A), but the SGN firing rates shown in Fig. 3C don't show this at all. Is the spontaneous firing of those units not shown in Fig. 3C? The text also later refers to "...the reduction in spontaneous SGN rates...", which is directly contradictory to the first statement on firing rate distribution.

8. Page 7. The whole section on OHC electromotility could be condensed down to one sentence: "There was no effect of LRBA loss on prestin function."

9. Page 10, top. The FM1-43FX data do not necessarily implicate transduction. A 45 sec incubation is too low to restrict dye entry to transduction channels (people usually use 5-10 sec). There are transduction channel-independent pathways for dye entry specifically into hair cells (>>supporting cells). To show that this dye application tests transduction channels you must use a control with a channel blocker.

10. Page 11, top. Quantitative immunocytochemistry is difficult to do accurately. Are the antibodies validated against knockouts?

11. Figure 4. In A-D, it is very difficult to understand the distribution of the channels. Probably drop the phalloidin and just show two-color with spectrin and LRBA.

12. Figure 4. The heat map lookup table is terrible for displaying the LRBA signal. First, you need a key showing the relationship between color and level; heat maps are notorious for allowing a distorted view. Much better yet would be to just show the images in grayscale, which allows a good representation of level.

Referee #3:

In this very nice manuscript, Vogl et al. have studied the function of the BEACH protein LRBA in the inner ear. Nothing is known about the function of BEACH proteins in the inner ear. The authors demonstrate that mutation of LRBA in mice leads to progressive hearing loss. The manuscript is very comprehensive and studies protein expression and localization, evaluates hair cell and neuronal function physiologically combined with immunolocalization studies to provides insights into the mechanism by which LRBA affects the peripheral auditory sense organ. The data demonstrate a profound degeneration of hair bundles that starts from the center of the bundle, a rare phenotype that has so far only been observed in hair cell lacking radixin. The authors provide evidence that LRBA and radixin might act in a common pathway thus linking two genes associated with disease into a common molecular pathway. This is a very beautiful contribution and the findings are novel and interesting. I have a few suggestions for improvement:

1.They should refer in the text to Fig. 1B', which shows that the immunolocalization signals observed in wild-type mice are specific.

2.Figure 4 could be described a bit better. What is the yellow signal in Fig. A'-D' and A'-D'. They should describe better "intensity coded lock-up". I am not sure what that means. It would be useful to show costaining with a kinociliary marker. Finally, is the peculiar BEACH expression pattern maintained even after degeneration of the kinocilium?

3. Describe in Figure 5 the magenta and yellow signal.

4. The authors state in results that hair bundles were abnormal after the onset of hearing but their data in Figure 4 reveal abnormalities already before the onset of hearing.

1st Revision - authors' response 15 May 2017

We would like to thank the reviewers for their appreciation of our work and constructive criticism that helped us improve our manuscript. We have addressed all points of the reviewers and marked the changes in red font.

Referee #1:

The manuscript from Vogl and co-authors (The BEACH protein LRBA is required for hair bundle maintenance in cochlear hair cells and for hearing) provides convincing data that in mouse a recessive mutant allele of Lrba is associated with hearing loss and several lines of evidence indicate hair cell stereocilia bundle degeneration after normal appearing development of these cells. In large part, the manuscript is nicely written. However, there are many issues (many of them, but not all, are minor and easily corrected) listed below that need attention and with revision would significantly improve the manuscript.

We thank the reviewer for her/his appreciation of our work and helpful comments on how to further improve our manuscript.

1. Abstract, last sentence, Comment about two patients seems tacked on to the end of the abstract. The statement lacks details such as to the mutant alleles of LRBA found in the two subjects.

We now changed the abstract and added the mutations: "Clinical data obtained from two patients with LRBA gene mutations (homozygous c.5047C>T and c.1420G>A/c.2834_2837delTCTT) suggest that LRBA-deficiency may likewise cause syndromic sensorineural hearing impairment in humans, albeit less severe than in our mouse model."

2. Was study of human subjects approved by an IRB/Ethics Committee/Helsinki Committee? Did the subjects/parent give written informed consent to participate in the study prior to undertaking the study? In the last sentence, under study approval, the authors only state that "Patient 1 and parents of patient 2 gave informed consent to publish the data."

Yes, we obtained ethics committee approval and informed consent before undertaking the study and now added the specific information to the text. It now reads: "Patient 1 gave informed consent to publish his audiological data specifically acquired for the purpose of this study, which was obtained after approval from the ethics committee of the University of Liège (Nr. B707201420571, 2014/74). The family of patient 2 gave written consent to share clinical and laboratory data for scientific publications (University Freiburg Ethic Committee protocol no. 282/11)."

3. Introduction, 2nd paragraph, line 7, change "carries" to "has"

We thank the reviewer for pointing this out and changed the text to: *"Each IHC synapse is characterized by a synaptic ribbon,…"*

4. Discussion, 2nd page, 2nd to last line, provide a reference for RDX-interacting protein Nherf2.

We have now included (Yun et al, 1997) at the respective position in the text as it provides the initial description of Nherf2/E3KARP/SLC9A3R2

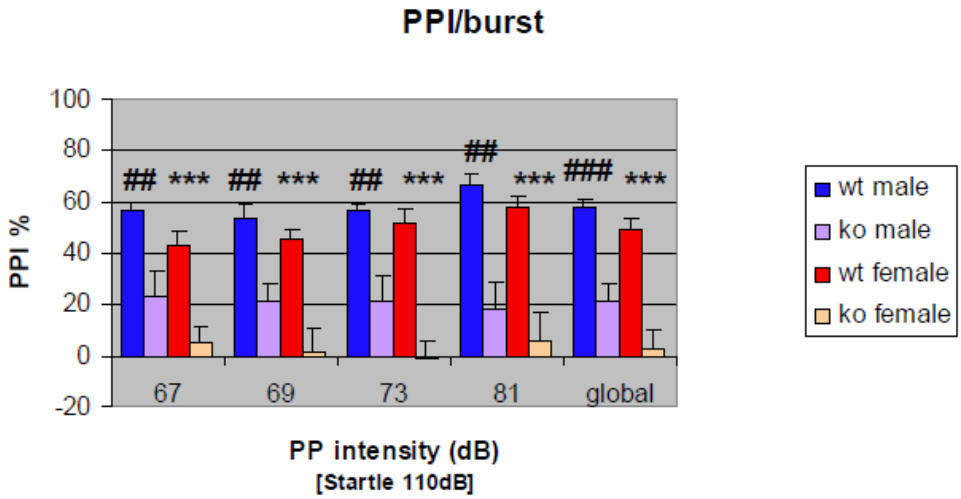
5. Results, The ko of LRBA is not described in this manuscript and is said to be a deletion of exon 3 that shifts the reading frame when presumably exon 2 is spliced to exon 4. A description of the wild type LRBA gene needs to be added to the text and/or as a figure and some form of convincing data must be provided that this mutant allele is a knockout of protein expression or at least what is expressed is nonfunctional. Western data is not provided. Expression of LRBA or a lack of it is not provided. The authors indicate that these data are to be provided elsewhere. Unacceptable -- not good science.

Unfortunately, the acceptance for publication of a separate manuscript (Kurtenbach et al.) which describes in detail the generation of this KO mouse is still pending. We attach the manuscript to this revision letter for reviewers to judge the data. After consultation with the editor, we were kindly

offered that - in case of acceptance of our MS - publication of our work would be withheld until the Kurtenbach et al. paper is accepted for publication at a different journal. We agreed with the editor and the Kilimann group to accept this kind offer and added the Kurtenbach et al. reference at the respective positions in the text. We have now added a higher magnification example of LRBA immunostainings on p7 organs of Corti that were performed in parallel between WT and KO, which also show the absence of LRBA staining in the KO to Appendix Figure S1.

6. Results, the LRBA mouse is reported to show a "reduced startle response". How was the startle response quantified as authors claim a reduction.

The acoustic startle response in the *Lrba*-KO mice was measured using the protocol described below, a shortened version of which can now also be found in the supplementary data (legend of Appendix Figure S1). The acoustic startle response is a defensive reaction to an intense auditory stimulus that induces a so-called startle reflex. This reflex can be influenced by the hearing ability of the mice, the state of their neuromuscular recruitment ability and the emotional condition of the animal. In the case of the *Lrba*-KO mice, the clearly decreased acoustic startle reactivity in the mutant mice likely reflects the sensorineural hearing loss in these mice. The graph showing the results of this analysis can also be found in the supplementary data (Appendix Figure S1). We measured the acoustic startle response to several acoustic startle sound stimuli from 70 to 120 dB. In addition, we also measured the ability of a milder acoustic stimulus (a prepulse) to inhibit the acoustic startle and found a significant reduction of prepulse inhibition in LRBA KO mice (graph below), suggesting impaired sound perception. However, we did not include that data into the MS, as we felt that the big difference in acoustic startle reactivity between *Lrba*-KO and WT, to which prepulse inhibition experiments are normalized, made the result difficult to interpret.



Prepulse inhibition (PPI) of the acoustic startle response protocol:

Animals were separated based on sex, but not genotype. PPI was assessed using a startle apparatus setup (Med Associates Inc., VT, USA) including four identical sound-attenuating cubicles. The protocols were written using the Med Associates “Advanced Startle” software. Experiments were carried out between 08:30h and 17:00h. Background noise was 65 dB, and startle pulses were bursts of white noise (40 msec). A 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. Trial types for the PPI included four different prepulse intensities (67, 69, 73, 81 dB); each prepulse preceded the startle pulse (110 dB) by a 50 msec inter-stimulus interval. Each trial type was presented 10 times in random order, organized in 10 blocks, each trial type occurring once per block. Inter-trial intervals varied from 20-30 sec. This protocol is based on the protocol used in IMPRESS from the International Mouse Phenotyping Consortium (IMPC, see www.mousephenotype.org/impress), adapted to the specifications of our startle equipment.

7. Do your results regarding prominent immunolocalization signals of LRBA in inner and outer hair

cells correspond to what has been reported for LRBA in the Harvard SHIELD and U of Maryland gEAR databases?

Yes, we thank the reviewer for this suggestion. The SHIELD database reports the expression of LRBA in hair cells and gEAR refers to this. Using RNA-Seq based expression analysis, LRBA expression was found in cochlear and utricular hair cells as well as supporting cells. We have now included the appropriate references (Shen *et al*, 2015; Scheffer *et al*, 2015) at the respective position in the text.

8. For human mutations, give the accession number for the DNA sequence and protein c.5047C>T, c.1420G>A, and c.2834_2837delTCTT need a GenBank or RefSeq accession number.

We now added the accession numbers (NM_006726.4, NP_001186211.2, ENST00000510413) as provided in the original publications in the Abstract and main text and also include the information that lack of LRBA expression was shown by Western Blot in both patients. Abstract, last sentence: Clinical data obtained from two patients with LRBA gene mutations (homozygous c.5047C>T and c.1420G>A/c.2834_2837delTCTT) suggest that LRBA-deficiency may likewise cause syndromic sensorineural hearing impairment in humans, albeit less severe than in our mouse model.

Results (page 11/12, middle and last paragraph): …a 31 year-old man who carried a homozygous c.5047C>T mutation in the *LRBA* gene (NM_006726.4; patient P3 in Lopez-Herrera *et al*, 2012) leading to the lack of LRBA expression as confirmed by Western blot… 11 year old girl with confirmed loss of LRBA expression due to a compound heterozygote c.1420G>A stop codon and c.2834_2837delTCTT frame shift mutation in the *LRBA* gene, again leading to complete loss of LRBA protein as confirmed by Western blot (NP_001186211.2 / ENST00000510413 patient 105-1 in Gámez-Díaz *et al*, 2016).

9. Discussion, first paragraph, "...hair bundle defect induces significant hearing loss, but..." "induces" is not the right word.

This has now been changed to *"...hair bundle defect leads to significant hearing loss, but..."*

10. Discussion, The authors mention that "...the large size of the LRBA protein impede further biochemical studies to..." Plenty of even larger proteins have been studies biochemically after purification. Western blots also possible.

We agree with the reviewer's comment and concede that our phrasing may have been misleading here. In discussions with several specialists on biochemistry we had come to the conclusion that without a good candidate protein and given the restricted availability of the LRBA antibody, the cochlea was not a suitable system to answer such questions – given the small amounts of tissue that can be obtained from the cochlea. Hence, we rather meant to directly refer to biochemical studies that might reveal the direct interaction partners of LRBA. Moreover, we feel that a more detailed biochemical study beyond the auditory system is beyond the scope of our current study. Therefore, we have now removed this sentence.

11. Discussion, something is wrong with the phrase "despite of unchanged numbers..." delete "of"?

done

12. Figure 1, legend, There is no way of knowing if LRBA is highly expressed in hair cells from your immunolocalization images unless somehow you quantify expression. LRBA may be easily detectable but that's the best you can say.

We agree with the argument of the reviewer and have changed our statement to:
"LRBA immunoreactivity could be detected in hair cells,…"

13. Figure 1 supplement, panel B, how was startle response amplitudes quantified?

For a detailed answer to this point, please refer to our answer to question 6 here.

14. In the immunolocalizations figure 4, is your antiserum detecting both the long and short isoforms of LRBA? In figure 4, it would be helpful in the interpretation of the data to have a cartoon of the LRBA gene, LRBA protein and the location of the epitope for the antisera.

According to UniProt, there is currently three described mouse LRBA splice variants (referred to as LRBA alpha/beta/gamma) that differ exclusively in the extent of the outermost C-terminal sequence (i.e. between aa 2569 and aa 2856). Therefore, our antibody should recognize all currently reported LRBA isoforms as it targets aa 942-1296. As suggested by the reviewer, we have now included a cartoon of the LRBA protein in Figure 4A, in which the binding site of the antibody is schematically illustrated. Like the mouse line, the antibody will be described in the Kurtenbach et al. MS, which we attach to this submission for your confidential information. We have now extended this information in the methods section by further referring to the Kurtenbach et al. paper: "…was raised against recombinant mouse LRBA aa 942-1296 (Fig. 4A; a sequence with no similarity to Neurobeachin, targeting coding exon 22 and the initial 24 codons of exon 23 of mouse LRBA; Kurtenbach et al.), and…"

15. In humans LRBA is located on chromosome 4q. Is LRBA in or outside the DFNB26 deafness interval on 4q31.3? The gene underlying DFNB26 has not been reported.

Indeed, the LRBA gene is coded in the human DFNB26 deafness locus. We had mentioned this in an earlier version of the MS but had decided to remove it to avoid confusion. The locus includes other candidate genes, the genetics of DFNB26 is quite complicated (involving a genetic modifier) and in an informal personal communication a human geneticist had stated that he/she had preliminary evidence for a different gene being responsible. Also, the phenotype of our mice and LRBA-deficient humans appeared to be different from the DFNB9 patients: DFNB9 has been described to cause severe deafness and no other symptoms, while LRBA deficiency is mostly associated with a severe immunodeficiency syndrome and, in our cases, mild to moderate hearing impairment.

We now reinserted a statement in the discussion: Interestingly, human non-syndromic autosomal recessive deafness DFNB26 has been described to be associated with a gene locus (4q31) in the chromosomal region where the BEACH protein lipopolysaccharide-responsive beige-like anchor protein (LRBA) is encoded (4q31.3, Riazuddin *et al*, 2000). However, the genetics of DFNB26 is complex (involving a genetic modifier) and it remains questionable whether LRBA mutations are involved. The clinical phenotype is clearly different, as DFNB26 patients suffer from severe deafness, which has not been described in LRBA-deficient humans so far. Instead, their clinical phenotype is dominated by a severe immunodeficiency syndrome (Lopez-Herrera *et al*, 2012).

16. Figure 7, "Hearing impairment in LRBA-deficient patients" How do you know they are deficient for LRBA, if by deficient you mean "null". If not, maybe better terminology than "deficient" could be used here.

All three mutations have been associated with stop codons (page 993 (P3, our patient 1) in Lopez-Herrera et al., 2012, Table II (patient 1, our patient 2) in Gamez-Diaz et al, 2016.
In both patients, an initial western blot screen in EBV cell extracts had indicated the absence of LRBA expression. We now specify this in at the respective positions in the results section (please also refer to our response to comment 8).

Referee #2:

Vogl and colleagues show that the protein LRBA is required for maintenance of cochlear hair bundles, particularly those of OHCs. The data largely support their hypotheses, although the manuscript could probably stand to be shortened. There is a considerable amount of data described in detail that is essentially negative; while I appreciate it was a lot of work to collect, it is distracting for the reader who is trying to understand the consequences of loss of LRBA. In particular, the authors present a large amount of data on cochlear and auditory nerve physiology that could be distilled considerably, given that they were able to narrow in on hair cell function, especially bundle mediated transduction.

We thank the reviewer for her/his appreciation of our work and the helpful comments and suggestions on our manuscript. We somewhat shortened the MS by partly moving the IHC and OHC cell physiology to the expanded view/supplement sections. We refrained from shortening the *in vivo* recording parts, because it is, to our knowledge, the detailed description (including single auditory nerve fiber recordings) of a mutant with aberrant but not fully abolished mechanoelectrical transduction in OHC and probably also IHC. We think that for specialists interested in the heterogeneity of IHC synaptic function, the finding of a shifted spontaneous rate distribution might be interesting. Yet we did not want to stress this point too much, as it partly remains speculative and is rather complicated for non-specialists.

The authors are technically correct in saying that the KO leads to impaired cochlear amplification and reduced synaptic transmission, but their data suggests that these effects are entirely due to disrupted bundles and altered transduction. The dye labeling experiments they used are inadequate to measure transduction (see below). Of course, it would be nice to look at transduction in mice that are old enough to display the profound phenotype, but measurement of transduction in older mouse hair cells is difficult if not impossible at the moment. Nevertheless, the authors need to be precise about primary vs. secondary defects.

We now replaced the FM dye labeling experiment (Fig. 5) by a new dataset with a shorter incubation period as suggested by the reviewer. Moreover, we changed the text slightly to explain better that – for us – the main outcome was that FM uptake was homogenous, not "mosaic", as observed in many Usher mutants, where the function of mechanotransduction channels is compromised (for a current example please refer to e.g. Pan et al. 2017 Nat. Biotechnol. Mar;35(3):264-272. doi: 10.1038/nbt.3801). Hence, we can speculate that mechanotransduction appears unaltered in the mutants prior to stereociliar loss. Unfortunately, as the reviewer admits, direct measurements of transduction in adult animals are difficult or impossible. To further address this point, we attempted to quantify the FM uptake of wildtype and mutant hair cells in direct comparison by imaging both preparations side-by-side simultaneously in real time, while infusing the dye through the bath solution, but did not feel confident about the results due to a range of technical challenges. Given the obvious absence of a part of the OHC hair bundles (and thus reduction in the number of available MET channels) and disruption of the IHC hair bundle coherence, it seems quite plausible to assume reduced transduction currents in the mutant. In addition, ECochG summating potentials, thought to mostly arise from IHC transduction, are strikingly reduced, also at high intensities where active amplification should play a minor role.

Finally, it is true that there are some similarities between the Rdx and Lrba knockouts; perhaps this could be investigated more. Mechanistically, give the state of knowledge about function of RDX and LRBA, do you have a hypothesis for a functional connection between the two proteins?

We added Western Blot data which confirms our immunohistochemical analysis which showed a reduction in Rdx expression in *Lrba*-KO hair cells and we believe that the hearing phenotype of the *Lrba*-KO mice can mostly be ascribed to this deficiency. Indeed, the morphological phenotypes and the developmental time courses are comparable and the differences may be due to the residual Rdx expression. However, the reduction in Nherf2 and other mechanisms may contribute. Regarding the functional connection between LRBA and Rdx, we can only vaguely speculate. The subcellular distributions are quite different, arguing against a direct interaction. As BEACH proteins have been implicated in intracellular trafficking events and given the clustering of LRBA in the important intracellular transport pathways around the cuticular plate, we tend to believe that LRBA plays a role in directing Rdx and maybe other proteins to the apical compartments of hair cells. Interestingly, in our newly added WB analysis, we observed a progressive reduction of RDX and Nherf2, which could not be detected in p7 cochlear extracts. Hence, we hypothesize that is finding is due to a specific reduction of stereociliar RDX and Nherf2 purely in developing hair bundles and may disappear in the 'background' of global RDX/Nherf2 expression in the other cell types of the cochlea. However, after 7 weeks, a specific reduction of RDX and Nherf2 levels can be observed in *Lrba*-KO samples, further implicating an intimate – although likely not direct/physical – link between LRBA and radixin levels (Fig. 6J-K). We have now included the Western blot analysis of RDX and Nherf2 in Figure 6J-L and extended the respective

Results section: "Since RDX and Nherf2 levels were most strongly affected in these experiments, we next employed protein biochemistry to independently verify our findings with a distinct

experimental approach. Here, Western analysis of cochlear extracts revealed a progressive reduction of RDX and Nherf2 expression in *Lrba*-KO samples, thereby further implicating an intimate – although, due to the distinct subcellular expression patterns seemingly indirect – link between LRBA and RDX/Nherf2 (Fig. 6J-L). Intriguingly, in these latter experiments, we failed to detect a reduction in whole cochlear RDX or Nherf2 levels from early postnatal samples (p7; Fig 6J, K, L). Given the strongly reduced stereociliar IF signal at this developmental stage, these findings may appear rather counter-intuitive. To explain this conundrum, we propose a scenario where compartmental RDX and Nherf2 targeting to the hair bundle may be specifically compromised without affecting overall cochlear protein levels during early postnatal development; however, with advancing adolescence, the disruption of this pathway may lead to increased RDX and Nherf2 mistargeting and degradation, thereby contributing to the progressive nature of the observed phenotype. In this context, it also has to be taken into consideration that both, RDX and Nherf2 are expressed in a range of other cochlear supporting cell types (Scheffer et al. 2015; Shen et al. 2015), where they might exhibit distinct temporal expression patterns and initially mask the specific reduction observed in hair bundle signals. Finally, our findings that LRBA expression and subcellular distribution appears to be unaffected by loss of RDX again implicates RDX as a downstream target from LRBA (Appendix Figure S5)."

One last major comment. Remember that in mouse, gene symbols are first letter capitalized, all italics; protein symbols are all caps, not italicized. There is some inconsistency in formatting of symbols.

We apologize for our mistake, which has been corrected now, and hope we did not miss any instances.

Comments:

1. Always use page numbers and it is better to use line numbers as well. It is very annoying to not have the page numbers; I have added them to my copy of the manuscript, with page #1 being the title page.

We apologize for this inconvenience and have changed this for the revised version of the manuscript.

2, Page 4, second line. A phenotype cannot cause hearing loss-hearing loss IS a phenotype.

To rectify this issue, we have now changed the respective text to: *"Here, we describe a similar phenotype in another mouse mutant…"*

3. Page 5, first Results paragraph. If the immunoblot data are important to this manuscript (and I think they are), then you must show them here, not "to be described elsewhere."

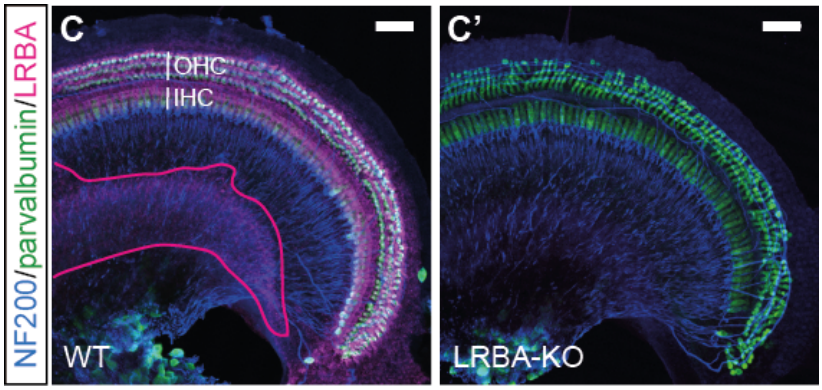
Unfortunately, the acceptance for publication of a separate manuscript (Kurtenbach et al.) which describes in detail the generation of this KO mouse is still pending. We attach the manuscript to this revision letter for reviewers to judge the data. The editor already looked at it and kindly offered that, in case of acceptance of our MS, she could withhold publication of our work until the Kurtenbach et al. paper is accepted at another peer-reviewed journal. We agreed with her and the Kilimann group to accept this kind offer.

4. Page 5, second Results paragraph. Do we really believe the immunolabeling results fully? The KO control is good but isn't fully consistent with the beta-gal labeling data-there just OHC and IHC labeling. Is there really any evidence for supporting cells, Reissner's membrane, and the stria? Higher magnification of the appropriate regions of control and KO would be valuable.

We agree with the reviewer that the X-Gal-staining quite clearly shows labeling of inner and outer hair cells, but we find it difficult to judge for supporting cells because it mostly overlap with the hair cells. We now include a different image with slightly better magnification and resolution in which a faint blue staining "on top of" the IHC row can be observed (Appendix Figure S1), seemingly labeling supporting cells. Unfortunately, we do not have better images and the reporter mouse line is not available to us anymore. In our immunohistochemistry experience, the labelling of supporting

cells tended to be much stronger in neonatal than in older animals. Thus, in our opinion, the X-Gal results (done in animals aged 24 days) are consistent with the immunolabeling imaging. Moreover, the RNAseq data provided in the SHIELD Harvard Inner Ear Laboratory database supports our IF findings. To add proof regarding the specificity of staining, we now added images of cochlear whole mount preparations stained for LRBA from WT and KO littermates as Appendix Figures S1C-D which clearly show positive staining in WT supporting cells and complete absence in the KO and also refer to this in the Figure legend of Figure 1. In fact, during the course of the entire project, it is worth mentioning that we have never seen any sort of labelling with this antibody in *Lrba*-KO tissue of any kind.

Regarding Reissner's membrane, the immunostaining signal was relatively weak but consistently observed, we therefore wanted to mention it. In the whole mount preparation shown in Figure 1, we see LRBA labeling in the remnant of Reissner's membrane. During preparation, we tried to remove this structure (edge outlined in pink) which partly overlaps with the spiral lamina (outlined in pink in the image below, modified from Figure 1C). Such a staining pattern has never been observed in the KO controls. Functionally, it would be difficult to ascribe a clear significance to LRBA expression in Reissner's membrane.



In the case of the stria vascularis – again – there was a reproducible immunostaining signal located exactly where we would expect the marginal cells of the stria vascularis. We excluded a strial dysfunction as a cause of the amplification deficit in a pilot experiment by our colleague Anna Gehrt, which showed a preserved endocochlear potential of 113 mV in a single tested knockout animal. Since this number is normal in comparison to the data in the literature (for reference values please refer to, e.g. Fig. EV1J in Buniello et al. EMBO Mol Med. 2016 Mar 1;8(3):191-207. doi: 10.15252/emmm.201505523) and did not seem to explain the ABR threshold shift. Therefore, we did not pursue this question further, as these experiments are technically highly demanding. We did not report the data in the MS because it was only a single pilot experiment without appropriate WT control. We now rephrased our description as follows: "Immunolocalization analysis in cochleae of p15 wild-type mice revealed prominent LRBA expression in IHCs and OHCs. Weaker immunostaining signals were observed in several types of supporting cells, the lining of the stria vascularis and Reissner's membrane, but not in spiral ganglion neurons (SGNs) (Figure 1A-B). Specific LRBA expression in IHC, OHC and supporting cells was confirmed by positive LacZ staining of organs of Corti from gene-trap mice expressing β-galactosidase under the control of the LRBA gene promoter (Appendix Figure S1B) and by the complete absence of staining in LRBA-KO mice (Figure 1B', Appendix Figure S1C-D). "

5. Page 5, last paragraph. Thresholds are displayed in Fig. 2A (not 2B-C); 2B-2C shows amplitudes. Correct the text.

done

6. Page 6, first paragraph. "...involves a defect of cochlear amplification..." is misleading. That suggests a specific defect in the amplification mechanism, but all the evidence points to disturbed hair bundles and hence aberrant mechanoelectrical transduction instead.

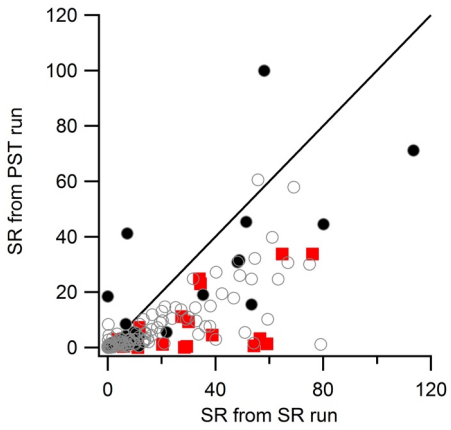
At this point of the MS we only have systems physiology data indicating a defect of cochlear amplification which could be caused by any deficit in general cochlear function (e.g. cochlear micromechanics, EP generation, transduction), or active amplification. We will later show that this is indeed caused by aberrant OHC mechano-electrical transduction. To avoid confusion, we now modified the statement to: "…and involves a primary or secondary defect of cochlear amplification, the mechanism of which remained to be determined. Next, we set out to narrow down the site of lesion and further characterized the sound encoding deficit in the absence of LRBA by recording spontaneous and sound-evoked action potential firing of postsynaptic SGNs in vivo."

7. Page 6. I am confused; the text indicates that SGN firing rates were distributed towards high SR fibers in the KOs (I see Fig. 3A), but the SGN firing rates shown in Fig. 3C don't show this at all. Is the spontaneous firing of those units not shown in Fig. 3C? The text also later refers to "...the reduction in spontaneous SGN rates...", which is directly contradictory to the first statement on firing rate distribution.

We apologize for this big mistake of having written "…the reduction in spontaneous SGN rates…" which is clearly wrong. Spontaneous rates are indeed higher in the mutants (mean of 36.6 Hz in mutants vs 20.3 in wildtypes) and this has now been corrected. One difficulty in the analysis of spontaneous rate distributions is that due to their skewness, relatively large sample sizes are required. The SR dataset in Figure 3A is based on a larger sample than the other datasets which are always acquired later in time when the signal to noise ratio often has deteriorated or units have been lost. We therefore have more confidence in SR analyses from the SR runs than from PST or rate level function runs. Like in the SR analysis in Figure 3A, the spontaneous rates are also higher in the mutants in the rate level functions shown in Figure 3C. In this smaller dataset, the average lowest (subthreshold) rate is 29.7 in mutants and 21.1 in wildtypes. We thus think, that the data in Figures 3A and 3C are quite comparable.

However, the end of the poststimulus time histogram in Figure 3D indeed looks different, and we find this difficult to explain. We initially suspected a systematic spike detection problem, but this error was easily ruled out when we double-checked the original recording traces. In the dataset used, mutants did not have bigger stimulus artifacts due to higher stimulus intensities in the mutants which might have made it necessary to raise the trigger levels for spike detection. The signal to noise ratio was very satisfactory in the presented dataset.

In the PST runs, the spontaneous rate averaged from the end of the non-stimulated part of the PSTH run was 13 in mutants and 10 in wildtypes. In contrast, the spontaneous rates from the 10-30 second long spontaneous rate recordings from the same units are 29.3 Hz in mutants vs 13.7 in mutants, respectively. Thus it seems like in both genotypes, spontaneous spiking following maximal stimulation does not recover completely until 150 ms after the end of the stimulus. We show this in the following graph, where we plot the two different spike rate measurements from the spontaneous rate run (x: measurement for 10 seconds, additional 10 or 20 seconds when SR is low) against the spontaneous rate determined from the PST run (y: 50-140 ms after stimulus end for 5 Hz stimulation). It is obvious that (with few exceptions) in mutants (red), wildtypes (black) and a larger wildtype dataset from our database (grey) the spontaneous rate determined from the SR run is systematically lower than the one from the PST run.



Our interpretation of this graph is that even in wildtypes, maximal stimulation probably leads to some depletion of the RRP at the IHC ribbon synapse which reduces spontaneous spiking for a period of at least about 150ms. In the absence of a genotype-dependent effect in our forward masking experiments (Figure 3E), we do not have a good explanation why this effect may be slightly more pronounced in LRBA mutants. Since we tend to believe that it is a problem of sampling, we refrain from discussing it in the manuscript and rather slightly shortened the time axis of the PST graph (Figure 3D) to avoid confusion of the reader.

8. Page 7. The whole section on OHC electromotility could be condensed down to one sentence: "There was no effect of LRBA loss on prestin function."

We appreciate the suggestion of the reviewer and – to streamline the manuscript – have now dropped the OHC electromotility part from the main text. However, since LRBA has previously been implicated in intracellular trafficking events, we believe that it is important to show that the phenotype we describe is due to a specific effect on the hair bundles, without affecting general hair cell physiology. Moreover, since NLC measurements do not only provide insights into prestin availability, but also OHC functional maturation, we believe that this data is essential to show normal development of LRBA-KO hair cells. Hence, we have decided to include this data set in the Expanded View and Appendix sections.

9. Page 10, top. The FM1-43FX data do not necessarily implicate transduction. A 45 sec incubation is too low to restrict dye entry to transduction channels (people usually use 5-10 sec). There are transduction channel-independent pathways for dye entry specifically into hair cells (>>supporting cells). To show that this dye application tests transduction channels you must use a control with a channel blocker.

This is a very important point raised by the reviewer and we have now repeated these experiments with the commonly used application protocol of 10 sec to avoid the utilization of endocytotic uptake pathways (Fig. 5C). We did not aim to provide a quantitative comparison of FM dye uptake between WT and KO hair cells and rather aimed to focus on the fact that FM uptake is homogenous in the KO and not mosaic, as seen in some Usher mutants. Similar to the latter, we would expect to see "patchy" uptake in *Lrba*-KO animals as the mechanotransduction deficits will not affect all hair cells to the same degree and differential effects could hence be expected; however, according to our data, this does not seem to be the case. We have now changed the text at this position in the results section to point this out to the reader more clearly: "Next, we set out to probe hair cell mechanotransduction channel function of early postnatal IHCs and OHCs of both genotypes by transient exposure to the lipophilic styryl dye FM1-43FX (Gale et al. 2001). In these experiments, we chose p5 animals (i.e. a developmental age group unaffected by hair bundle degeneration), as hair cells from these preparations would reveal functional mechanotransduction deficits occurring alongside – but independent from – stereociliar loss. Here, we would expect to observe mosaic FM1-43FX uptake in case that functional mechanotransduction is compromised. However, the homogenous styryl dye uptake pattern found in these experiments suggests seemingly normal mechanotransduction channel function in *Lrba*-KO hair cells (Fig. 5C). Thus, our findings are consistent with…"

10. Page 11, top. Quantitative immunocytochemistry is difficult to do accurately. Are the antibodies validated against knockouts?

We agree with the reviewer that this is an essential point. The chosen anti-Nherf1 antibody (Abcam) has previously been used and validated in a study investigating the auditory phenotype of Nherf1-KO animals (Supplementary Figure S3A in Kamiya et al. 2014). Similarly, the same study also validated the anti-Nherf2 antibody (Sigma Aldrich) in a Nherf2-KO mouse line (Supplementary Figure S9B in Kamiya et al. 2014). Due to the characteristic expression patterns of all investigated proteins in the hair bundles and microvilli of surrounding supporting cells that are reminiscent of the findings of Kamiya and colleagues in hair bundles of mouse OHCs at comparable age (i.e. p5-p15), we are confident that our data do not show an artifact, but indeed reflect specific labelling of the target proteins. The used anti-radixin antibody (Sigma Aldrich) has previously been employed by Khan et al. 2007 to visualize radixin expression in the cochlea. Based on the conserved sequence used for immunization and identical expression pattern as shown in Kitajiri et al. 2004 – who used a

radixin-KO mouse model to validate their own custom anti-radixin antibody – it seemed likely to faithfully report radixin expression in the organ of Corti. Nonetheless, we now performed control experiments with our Sigma Aldrich antibody, which clearly show the absence of radixin staining from radixin-KO hair bundles and further confirmed the antibody specificity by Western blot analysis (Appendix Figure S4). In addition, to further validate our IF results, we have now performed Western blot analysis on cochlear extracts, which confirms the reduction of radixin expression in *Lrba*-KO organs of Corti (Fig. 6J-L).

11. Figure 4. In A-D, it is very difficult to understand the distribution of the channels. Probably drop the phalloidin and just show two-color with spectrin and LRBA.

We agree with the reviewer and for the sake of clarity have now dropped the phalloidin staining form panels A-D, as suggested.

12. Figure 4. The heat map lookup table is terrible for displaying the LRBA signal. First, you need a key showing the relationship between color and level; heat maps are notorious for allowing a distorted view. Much better yet would be to just show the images in grayscale, which allows a good representation of level.

We thank the reviewer for this comment and have now included the missing color key(s) reflecting the relationship between color and fluorescence intensity level in the respective Figure(s). We respectfully disagree about the improvement by monochrome images, as we believe that heat-maps generally provide a better idea of intensity distribution and hence give a clearer understanding of the spatial distribution pattern of a given protein than a two-dimensional look-up table. Moreover, this is a purely illustrative – and commonly used – tool and does not have any impact on quantitative analysis. However, in case the reviewer, as well as the editor insist on this point, we are willing to replace the figures by grayscale ones.

Referee #3:

In this very nice manuscript, Vogl et al. have studied the function of the BEACH protein LRBA in the inner ear. Nothing is known about the function of BEACH proteins in the inner ear. The authors demonstrate that mutation of LRBA in mice leads to progressive hearing loss. The manuscript is very comprehensive and studies protein expression and localization, evaluates hair cell and neuronal function physiologically combined with immunolocalization studies to provides insights into the mechanism by which LRBA affects the peripheral auditory sense organ. The data demonstrate a profound degeneration of hair bundles that starts from the center of the bundle, a rare phenotype that has so far only been observed in hair cell lacking radixin. The authors provide evidence that LRBA and radixin might act in a common pathway thus linking two genes associated with disease into a common molecular pathway. This is a very beautiful contribution and the findings are novel and interesting. I have a few suggestions for improvement:

We thank the reviewer for her/his appreciation of our work and the helpful suggestions.

1.They should refer in the text to Fig. 1B', which shows that the immunolocalization signals observed in wild-type mice are specific.

We now wrote: "Immunolocalization analysis in cochleae of p15 wild-type mice revealed prominent LRBA expression in IHCs and OHCs. Weaker immunostaining signals were observed in several types of supporting cells, the lining of the stria vascularis and Reissner's membrane, but not in spiral ganglion neurons (SGNs) (Figure 1A-B). Specific LRBA expression in IHC, OHC and supporting cells was confirmed by positive LacZ staining of organs of Corti from gene-trap mice expressing β-galactosidase under the control of the LRBA gene promoter (Appendix Figure S1B) by the absence of staining in LRBA-KO mice (Figure 1B', Appendix Figure S1C-D)." We also added a second example of WT and KO immunolocalization studies performed in parallel to Appendix Figure S1 to clearly show the specificity of the labelling with higher magnification.

2.Figure 4 could be described a bit better. What is the yellow signal in Fig. A'-D' and A'-D'. They

should describe better "intensity coded lock-up". I am not sure what that means. It would be useful to show costaining with a kinociliary marker.

We apologize for having forgotten to add a color key in the Figure so that the intensity-based look-up table is more understandable. We now include it in the revised figure. Moreover, we have now updated our statement in the text to make this more clear: "…LRBA clustered at a plane beneath the cuticular plate and was highly enriched – i.e. showed the highest fluorescence intensity signal (i.e. yellow to white pixels in Fig. 4A'-D'') – in the areas void of cuticular-plate actin, especially where the kinocilium passes through the cuticular plate (the so-called fonticulus/kinociliar pore; Fig 4A'-D''). Here, LRBA appeared to line the pore (Figure 4E-E'') and its distribution pattern overlapped with Rab11,…" Additionally, we have now added a double labelling of LRBA and the kinociliar marker 'acetylated tubulin' to clearly show LRBA localization at the base of the kinocilium (Fig 4E"). Unfortunately, a triple labelling of the cuticular plate, LRBA and the kinocilium within the same preparation was not possible due to antibody species conflicts. However, we are confident that this current version of the Figure better visualizes the LRBA expression pattern at the kinocoiliar pore.

Finally, is the peculiar BEACH expression pattern maintained even after degeneration of the kinocilium?

In this mouse strain, kinociliar degeneration appears to be completed around p9/p10, hence Figure 4D reflects the representative LRBA distribution in the absence of the kinocilium. Moreover, we have also looked at the expression pattern of LRBA in after the onset of hearing (i.e. p15) and found the exact same localization and distribution. We now refer to these findings in the respective text: "This subcellular distribution pattern at the fonticulus and pericuticular necklace (Hasson et al, 1997) was maintained even after the degeneration of the kinocilium – which occurs by p10 in this mouse strain – and even past the onset of hearing (data not shown)".

3. Describe in Figure 5 the magenta and yellow signal.

As in Figure 4, we now include a color key for the intensity-based look-up table and added at all respective positions in the Figure legends: "…images are shown with an intensity coded look-up table for improved visualization of the fluorescence intensity distribution."

4. The authors state in results that hair bundles were abnormal after the onset of hearing but their data in Figure 4 reveal abnormalities already before the onset of hearing.

We thank the reviewer for pointing out this issue and have now altered the text to clarify this point: "…develop normally and then degenerate prior to the onset of hearing: (i)…"

2nd Editorial Decision 08 June 2017

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As I already indicated in my previous mail, referee 2 and 3 are now very positive about the study but referee 1 raises concerns regarding the LRBA mutation in patient 1/patient 3 (c5047C>T). This referee is concerned that the mutation might represent a polymorphism since no data on 100 matched control individuals is presented. As you already outlined in your response to this mail you relied on the earlier publication by Lopez-Herrera et al (Am J Hum Genet 2012) for describing the mutation of this patient. This study identified the mutation by sequencing the patient and two hundred alleles from European healthy controls. Please provide a more detailed explanation and clear reference to this earlier dataset in your manuscript and address the remaining concerns of referee 1.

Browsing through the manuscript myself, there are several things that we need from the editorial side before we can proceed with the official acceptance of your study:

- All materials and methods should be part of the main manuscript. Therefore, please move the

supplementary methods to the main text.

- Statistics: please indicate the number of analysed samples/animals in the legends of Fig 2C, EV2C and EV3E-F as appropriate.

- Since all corresponding authors are required to supply an ORCID ID for their name to ensure unambiguous name assignment, I kindly ask you to provide an ORCID for the co-corresponding author Christian Vogl. Please find the instructions on how to link your ORCID to your profile in our online Authors Guidelines.

- We routinely perform a quality check of all images prior to publication. This scan triggered the appearance of vertical lines in Fig. EV2A' and S1D. These might represent an artefact that occurred during image compression. However, to rule out any ambiguity we routinely ask for the source data in such cases. Could you please provide the original data/images for these two panels?

I am looking forward to seeing a final version of your manuscript as soon as it is ready.

REFEREE REPORTS

Referee #1:

The revised manuscript from Christian Vogl and co-authors is much improved. Please find below a short list of issues and typos that can be quickly corrected. However, one problem is more serious. On page 12, line 19, patient 1 is said to have a c.5047C>T variant in the LRBA gene (NM_006726.4). This accession number is for the protein and genomic sequence and not the cDNA so that this reviewer can't figure out exactly where c.5047C>T is located and the change it makes to the protein sequence. In the genomic sequence provided in NM_006726.4, there is no C nucleotide at nucleotide 5047. Revise the manuscript to include the following: (1) Provide an accession number for the LRBA cDNA sequence. (2) Indicate the exon of LRBA in which the c.5047C>T variant occurs. (3) State the change at the protein level that results from this variant. (4) Provide data from at least 100 ethnically matched control individuals (200 chromosomes) that the variant is not a polymorphism. (5) Indicate the allele frequency in the public data bases for the c.5047C>T variant. (6) If c.5047C>T changes the amino acid sequence of LRBA, state the predicted consequences to the function of LRBA protein of this variant. Add some of this information to the abstract so that a reader understands the consequences of the C>T transition variant.

Other issues:

- Abstract, line 3, "which have been attributed a multitude..." is awkward English.
- Abstract, line 8, change wording to "LRBA-deficiency is associated with a reduced abundance of ..."
- Abstract, line 10, "central parts of the stereociliary bundle." is vague and does not make much sense. Do you mean the F-actin core of a stereocilium?
- Page 5, Kitajiri et al., did report that stereocilia degenerated in his Rdx knockout mouse but only in the organ of Corti and not stereocilia of vestibular hair cells, similar to your Lrba knockout, that you are publishing elsewhere.
- Page 5, line 12, "are involved in human disease..." provide some specific information about this disease and add the MIM number for the disorder.
- Page 5, line 19, add a clause defining "prepulse inhibition"
- Page 6, line 10, correct the typo "obvious" not "obvFious"
- Page 8, line 26, change "..appeared to line.." to "...appeared to surround..." There is no evidence for a liner.
- Page 13, line 22, delete "first" Surely this study is not the "first" detailed and multidisciplinary description, and surely not the "first" showing partial bundle defects that leads to a significant but not complete hearing loss. Avoid hyperbole.

Referee #2:

I am satisfied with the authors' responses to my original review.

Referee #3:

The authors have responded well to the reviewers comments. In my opinion they have clarified outstanding issues and the manuscript is suitable for publication. I think it will be of great interested to the research community.

2nd Revision - authors' response 27 July 2017

Referee #1

The revised manuscript from Christian Vogl and co-authors is much improved. Please find below a short list of issues and typos that can be quickly corrected. However, one problem is more serious. On page 12, line 19, patient 1 is said to have a c.5047C>T variant in the LRBA gene (NM_006726.4). This accession number is for the protein and genomic sequence and not the cDNA so that this reviewer can't figure out exactly where c.5047C>T is located and the change it makes to the protein sequence. In the genomic sequence provided in NM_006726.4, there is no C nucleotide at nucleotide 5047. Revise the manuscript to include the following: (1) Provide an accession number for the LRBA cDNA sequence. (2) Indicate the exon of LRBA in which the c.5047C>T variant occurs. (3) State the change at the protein level that results from this variant. (4) Provide data from at least 100 ethnically matched control individuals (200 chromosomes) that the variant is not a polymorphism. (5) Indicate the allele frequency in the public data bases for the c.5047C>T variant. (6) If c.5047C>T changes the amino acid sequence of LRBA, state the predicted consequences to the function of LRBA protein of this variant. Add some of this information to the abstract so that a reader understands the consequences of the C>T transition variant.

We completely relied on the earlier publication by Lopez-Herrera et al (Am J Hum Genet 2012) for describing the mutation and immunological phenotype in our patient 1 / their patient 3, and we now cite that reference more clearly.

That MS states:
- That sequencing was based on RefSeq NM_006726.2 (and discusses in more detail possible sequence variants, page 988)
- The amino acid substitution (p.Arg1683) , affected exon (30) and accession number (ss479152581) in dbSNP (table 2)
- "Which introduced a stop codon" (page 993)
- "No LRBA was detected in EBV cell extracts of the affected individual (Figure 2B)" (page 993)
- "Two hundred alleles from European healthy controls were sequenced for exon 30, and no mutation was found." (page 993)

Regarding the cDNA sequence, the current RefSeq NM_006726.4 indeed is for mRNA but that should correspond to cDNA except for the T/U exchange. For the mutation, if one checks from the first A of ATG (244 in this case), one can find the C in position 5047+244=5291 which belongs to a CGA codon which codes for Arginin and a mutation to T would make a TGA which is a stop codon. To make the references to the clinical genetics papers more visible and include more information, we now write in the results section:

“Patient 1 a 31-year-old male (described as patient P3 in [28]) carries a homozygous nonsense mutation in the LRBA gene (c.5047C>T, p.Arg1683*, reference sequence NM_006726.2), which truncates the protein after 59% of its length and leads to loss of detectable LRBA expression in EBV-transformed B cells from the patient [28].”

AND Patient 2, an 11-year old girl (described as patient#1, code 105-1 in [29]) is compound heterozygous for two nonsense/frameshift mutations (c.1420G>A/p.Q473* and c.2834_2837delTCTT/pE945Efs, reference sequences ENST00000510413 and NP_001186211.2) which truncate the protein after 17% and 33%, respectively, of its length and abolish LRBA expression [29].”

Abstract, line 3, ".. which have been attributed a multitude..." is awkward English.

Changed to “which have been attributed various cellular functions”.

Abstract, line 8, change wording to "LRBA-deficiency is associated with a reduced abundance of ..."

Done.

Abstract, line 10, "central parts of the stereociliary bundle." is vague and does not make much sense. Do you mean the F-actin core of a stereocilium?

We meant the central parts of the hair bundle, not of the individual stereocilium. We changed the wording to “loss of structural integrity of the central parts of the hair bundle”.

Page 5, Kitajiri et al., did report that stereocilia degenerated in his Rdx knockout mouse but only in the organ of Corti and not stereocilia of vestibular hair cells, similar to your Lrba knockout, that you are publishing elsewhere.

We unfortunately do not understand this comment. We stated in the MS “Radixin-deficiency in mice leads to early postnatal hair bundle degeneration [12]. Here, we describe a similar phenotype in another mouse mutant…” , which does not contradict your statement. In addition, we write in the discussion: “Indeed, the hair bundle phenotype of Lrba mutants closely resembles the one observed in RDX-KO animals, in which the hair bundle of cochlear hair cells also develops normally until at least p6 but loses its central aspect by p14, while vestibular hair cells are unaffected [12]. “ We believe that the differences in severity and time course might be due to residual radixin expression in the LRBA mutants.

Page 5, line 12, "are involved in human disease..." provide some specific information about this disease and add the MIM number for the disorder.

We would like to leave the original text (“several of the nine identified human BEACH proteins, including LRBA, are involved in human diseases [reviewed in 14].”) because a discussion would go beyond the scope of this MS. Indeed several of the 9 human BEACH domain proteins have been associated with diseases as diverse as immune disorders, cancer, glioma, gray platelet syndrome, systemic lupus erythematosus, mental retardation etc. We prefer to discuss the phenotype of LRBA deficiency and for the rest, we included the reference to a recent review by Cullinane et al.

Page 5, line 19, add a clause defining "prepulse inhibition"

We removed “prepulse inhibition” because we are not showing the data. Since auditory startle responses were severely impaired in the mutants, we cannot be certain that the PPI data is valid, even if it is normalized to the reduced amplitude.

Page 6, line 10, correct the typo "obvious" not "obvFious"

Done.

Page 8, line 26, change "..appeared to line.." to "...appeared to surround..." There is no evidence for a liner.

We see a fine layer of LRBA signal at the border of the fonticulus (the channel through the dense cuticular plate) which surrounds the kinocilium as long as it is present. “to line” is the best term we know for this kind of localization (less commonly used alternatives: “to edge”, to seam”). We would prefer it over “to surround” because that would suggest a localization outside the fonticulus, thus within the cuticular plate, which is not what we see. We now changed to “appeared to outline” which hopefully is a bit clearer.

Page 13, line 22, delete "first." Surely this study is not the "first" detailed and multidisciplinary

description, and surely not the "first" showing partial bundle defects that leads to a significant but not complete hearing loss. Avoid hyperbole.

Done. (I would not know any that has all the morphology and IHC and OHC electrophysiology and auditory nerve fiber recordings).

Referee #2:

I am satisfied with the authors' responses to my original review.

Thank you!

Referee #3:

The authors have responded well to the reviewers comments. In my opinion they have clarified outstanding issues and the manuscript is suitable for publication. I think it will be of great interested to the research community.

Thank you!

3rd Editorial Decision 07 August 2017

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Dr. med. Nicola Strenzke
Journal Submitted to: EMBO reports
Manuscript Number: EMBOR-2016-43689V2

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number[s] of the manuscript draft or figure legend[s] where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com	Antibodypedia
http://1degreebio.org	1DegreeBio
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo	ARRIVE Guidelines
http://grants.nih.gov/grants/olaw/olaw.htm	NIH Guidelines in animal use
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm	MRC Guidelines on animal use
http://ClinicalTrials.gov	Clinical Trial registration
http://www.consort-statement.org	CONSORT Flow Diagram
http://www.consort-statement.org/checklists/view/32-consort/66-title	CONSORT Check List
http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun	REMARK Reporting Guidelines (marker prognostic studies)
http://datadryad.org	Dryad
http://figshare.com	Figshare
http://www.ncbi.nlm.nih.gov/gap	dbGAP
http://www.ebi.ac.uk/ega	EGA
http://biomodels.net/	Biomodels Database
http://biomodels.net/miriam/	MIRIAM Guidelines
http://jjj.biochem.sun.ac.za	JWS Online
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was chosen for the various experiments according to typical numbers of observations in the respective fields (e.g. immunohistochemistry, cellular or systems electrophysiology, electron microscopy).
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For all experiments, the number of animals/cells is indicated in respective sections in the manuscript. For in vivo studies, the numbers of animals needed was estimated before the study based on appropriate power statistics using freeware GPower 3.1 program depending on the type of statistical test which we expected to use.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No animals were excluded from analysis. For other experiments, exclusion criteria were pre-established; e.g. any experiment in which technical problems occured that affected the data itself or when the immunofluorescence intensity was too low were excluded. In cellular patch clamp experiments, experiments were excluded if the cell was unhealthy, which becomes obvious when the Ca2+ current is small. For single unit studies, data in which the signal to noise ratio was not sufficient to separate action potentials from noise was excluded based on pre-established criteria before further analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	There was no treatment of animals. Animals across all experiments were used in littermate pairs whenever possible and genotypes were (re-) confirmed after the respective experiment.
For animal studies, include a statement about randomization even if no randomization was used.	Randomization was not executed, but mutants were compared/processed in parallel to littermate controls whenever possible.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Data analysis was automatized as much as possible (e.g. immunofluorescence analyses, single unit recording spike detection) and for subjective judgements (e.g. signal to noise ratio in single unit recordings) the analyzing person was blinded. Re-genotyping was performed in all instances.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was performed. Mutants were always processed in parallel with littermate controls. For morphological and systems physiology studies, data was typically double-checked by another observer.
5. For every figure, are statistical tests justified as appropriate?	In all instances, data were tested for normality and equality of variance. Based on this, the appropriate statistical tests were used to test for statistical significance, indicated in the figure legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	For samples with n =7 or more, we used the Jarque-Bera test to test for normal ditribution. For sampels with n<7 we used the Komogorov-Smirnov-Test.
Is there an estimate of variation within each group of data?	We tested for equal variation with the F-test.
Is the variance similar between the groups that are being statistically compared?	Most data are provided as means +- standard error of the mean. For non-normal distributions, box plots are used, as specified in the respective section in the figure legends. For small sample sizes, the individual values are presented in addition.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Most antibodies we used are commercially available and we indicate the catalog numbers in the methods section. For the LRBA antibody, we write: "A rabbit antiserum was raised against recombinant mouse LRBA aa 942-1296 (a sequence with no similarity to Neuroboeachin), and affinity-purified on the same fusion protein." Further details will be published in a separate MS by Kurtenbach et al. prior to publication of this article.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	no cell lines were used

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	The generation of the LRBA knockout mice will be published in a separate MS by Kurtenbach et al. prior to publication of this article. Mice of both sex were used at the ages indicated in the figure legends.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal handling was in accordance with national animal care guidelines and all experiments were reviewed and approved by the animal welfare committees of the University of Göttingen and the State of Lower Saxony.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We confirm compliance with the ARRIVE guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	see methods section
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	see methods section
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A

14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	N/A
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Raw and analyzed data are stored on servers of the InnerEarLab and the GWDG (Göttingen) according to institutional guidelines. They are available on demand.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	Raw and analyzed data are stored on servers of the InnerEarLab and the GWDG (Göttingen) according to institutional guidelines. They are available on demand.
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	We confirm compliance with referencing and citation guidelines.
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No, we do not think that there is any aspect in our study falling under dual use research restrictions.