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Tryptophan-rich basic protein (WRB) mediates insertion of the tail-anchored protein otoferlin and is required for hair cell exocytosis and hearing

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Additional Correspondence:	06 January 2016
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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.)

Additional Correspondence 06 January 2016

Thank you for submitting your manuscript to The EMBO Journal. Three good experts in the fields of hearing/IHC and TA protein insertion pathway have now reviewed your study.

As you can see below, your study received a bit of a mixed response. Referees #1 and 2 are generally supportive, while Referee #3 finds the advance and insight provided into protein trafficking not sufficient to consider publication here. I have discussed the comments of referee #3 further with referee #2. We are both in agreement that if you can conclusive show that the loss of Otoferlin is the key cause for the phenotypes seen in the WRB KO mice then the manuscript would become suitable for publication here. Such additional insight would show that despite many TA proteins that at least in this context that only one or a very few TA proteins might be crucial for the phenotype - it would imply a robustness in this pathway. However, this would require Otoferlin rescue experiments as indicated by referee #3. As it is not clear if such an experiment can be done plus the outcome is also unclear I have decided to do a pre-consultation with you before I take the decision on the manuscript. I think this is the most productive way forward - it is clear what we need and the question is if such experiments are technical feasible.

I would therefore like to ask you to send me a response to the referees' comments and let me know

what can be done about the Otoferlin rescue experiment. If you have any further questions please don't hesitate to contact me.

REFEREE REPORTS

Referee #1

This report focuses on a group of proteins, the TA proteins, which are post-translationally inserted into the endoplasmic reticulum via the TRC40 pathway. The focus was via otoferlin, a TA protein expressed in hair cells.

The results first demonstrated that the TRC40 pathway is essential for hearing in zebrafish. An in vitro analysis further demonstrated that the TRC40 pathway is the key mediator of otoferlin insertion. Wrb, when reduced specifically in hair cells and some neurons using a Vglut3-Cre, led to hearing impairment. Furthermore, Wrb disruption led to reduced otoferlin levels, Sound encoding was further found to be compromised. The combination of experiments led the authors to hypothesize that hearing impairment caused by Wrb disruption was caused by a reduced vesicle replenishment, leading to a smaller readily releasable pool at the hair cell synapse. As a result, the TRC40 pathway is essential for TA protein processing in the sensory epithelium of the inner ear. An elegant combination of experiments using zebrafish and mice, as well as in vitro assays, electrophysiology and immunohistochemistry, was used to demonstrate the above premise.

The results demonstrate the first detailed analysis of TA proteins associated with deafness and provide a detailed mechanism of this pathway with respect to hearing and the inner ear.

Referee #2

This manuscript contains a substantial amount of work using multiple experimental systems. Overall it provides lucid evidence that a lack of WRB is linked to deafness via its role in the biosynthesis of the tail-anchored membrane protein otoferlin, and provides a credible molecular basis for this outcome.

This reviewer was specifically tasked with commenting on the studies of otoferlin membrane insertion that is contained in the manuscript.

Major points:

1. In Fig. 1. Shows that a loss of the pinball wizard/ WRB gene in zebrafish results in a substantial reduction in Otoferlin expression. This can be rescued by expression of WRB-GFP, as can a defect in the acoustic startle reaction of zebrafish larva that is also detected in the WRB mutant. These data are solid and well controlled.

2. Fig. 2. Uses an established in vitro assay to investigate the requirement for the TRC40/WRB pathway in order for otoferlin to insert into the ER membrane. The N-glycosylation, and hence membrane insertion, of a shortened form of Otoferlin requires functional TRC40 and the process is inhibited by recombinant fragments of both WRB and CAML. Upon comparison to the previous study by (Favaloro et al 2010), the overall efficiency of otoferlin N-glycosylation appears substantially lower than that achieved with RAMP4 using the same approach (Favaloro et al 2010). It is also noteworthy that the difference between otoferlin membrane insertion with and without ATP is modest in comparison to the effect of ATP on RAMP4 insertion (Favaloro et al 2010). These points should be commented on in the text and/or authors' response. These data support the principal conclusion in relation to loss of otoferlin function upon perturbation of WRB.

3. Fig. 3. Shows a clear correlation between an absence of WRB in Inner Hair Cells and impaired hearing.

4. Fig. 4. Convincingly shows that otoferlin levels are reduced in IHCs from mice that lack WRB expression in this tissue.

5. The remaining three figures of the main text address the molecular basis for the effects of a reduction in otofrelin levels in IHCs.

Minor comments:

1. Page 5: replace "ER standing" with "ER resident"

2. Page 5: "we found spot-like Wrb-GFP signal co-localized with a recombinant fluorescent ER marker". Figure 1B is small, but is seems to show there is a partial co-localization between the WRB and ER labels?

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(TA) proteins. Some intermediate factors are absent.

4. Page 5. .....startle reflex..... should be (Figure 1I) not (Figure 1J).

5. The authors should specify what form of otoferlin was used for the in vitro studies shown in Figure 2 and why a shortened form of the protein was needed for these experiments. They should also define the TRC40 mutant that is used in Figure 2.

6. Page 10. The section starting "In addition, we found that flat subplasmalemmal cisternae....." and concluding "might reflect an up-regulation of perisynaptic rough ER, aiming to compensate for impaired TA protein insertion" is rather opaque and could be more clearly expressed.

Referee #3

As stated in the first paragraph of the discussion, this study reports "the requirement of the TRC40 pathway for efficient [insertion] of the TA protein otoferlin in sensory HCs" and "reveals a critical requirement of the TRC40 pathway for TA protein processing in the sensory epithelium of the ear." These conclusions are supported by the data in this paper, along with a fairly extensive characterization of the HC function in tissue-specific knockouts for WRB.

While there is certainly value in determining the importance of basic cellular pathways in different cell types, tissues, and organisms, these findings do not conceptually change our understanding of this TA protein insertion pathway. It is expected that disruption in HCs of a widely used and conserved trafficking pathway results in HC-related phenotype(s) consistent with impairment of clients of this pathway. As noted by the authors, disruption of this pathway is anticipated to have pleiotropic consequences based on studies in vitro and in yeast, and demonstrated in WRB mutant zebrafish in the accompanying Lin et al. manuscript. Thus, the major advance in the current study is the suggestion that the cause of the WRB phenotype in HCs is due primarily to impaired otoferlin insertion. However, identifying otoferlin as another client of this pathway is to be expected from its topology, and the proposition that this particular client is the primary cause of the phenotype is not strongly supported (see below). Thus, from the standpoint of protein trafficking, the study as currently presented is more suited to a specialized journal because the broader conceptual advance would seem to be relatively limited. Whether the advances related to auditory biology are more widely interesting is for experts in that area to judge.

Specific suggestion:

The authors show that disruption of WRB results in reduced otoferlin, and approximately phenocopies otoferlin knockouts. While this provides circumstantial evidence that the phenotype of WRB disruption in HCs is via otoferlin, this is not a very strong conclusion. The reason is that many other TA proteins (of which ~133 are expressed in HCs according to Lin et al.) are likely to be partially or severely disrupted, and these may well also contribute to the phenotype. If the authors wish to point the finger at otoferlin, they would need to show that a WRB knockout can be rescued by a version of otoferlin that bypasses the TRC/GET pathway for insertion. If substantial rescue is observed, one can then conclude that although the TRC/GET pathway handles many clients, the key

one in HCs is otoferlin. The title of the study is therefore not really appropriate unless such a rescue is demonstrated.

There are two ways to attempt rescue. The first is to simply over-express otoferlin to compensate for loss of WRB. As shown in Schuldiner et al. (2008) for yeast, individual phenotypes associated with a disrupted GET pathway can be bypassed by overexpressing the relevant TA protein associated with that phenotype. The second, perhaps more elegant, strategy is to extend the C-terminus of otoferlin (with GFP, for example). This would convert otoferlin into a co-translational substrate (see Stefanovic and Hegde, 2007, for an example) that is no longer dependent on WRB. Because the functional parts of otoferlin are cytosolic and in the membrane, a GFP on the other side of the membrane is not likely to affect its function. If such a construct rescued the phenotype, this would convincingly show otoferlin as the key TRC/GET pathway client responsible for HC function.

1st Editorial Decision 08 January 2016

Thank you for sending me the point-by-point response. I have now had a chance to take a look at it and I appreciate the described approach to carry out the Otoferlin rescue analysis. Should you be able to include such data then I would like to invite your to submit a suitably revised manuscript for our consideration.

You can use the link below to upload the revised manuscript.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://emboj.embopress.org/about#Transparent_Process

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

1st Revision - authors' response 29 May 2016

Response to the reviewers:

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

In order to further corroborate our hypothesis that impaired otoferlin insertion is a major mechanism in the auditory synaptopathy observed in young Wrb-deficient mice, we have i) performed otoferlin overexpression experiments in *pwi* zebrafish and ii) considered an alternative approach that targets ER-insertion of otoferlin without general disruption of the TRC40-pathway.
i) In order to test whether the impaired auditory function of *pwi* fish relates to otoferlin deficiency, we sought to override the disrupted ER-targeting by overexpression (Schuldiner *et al*, 2008) of otoferlin. Indeed, we could partially restore the startle response by otoferlin overexpression in a dose-dependent manner, suggesting that disrupted ER-targeting of otoferlin contributes to the impairment of auditory function in *pwi* fish (revised Figure 1).
ii) In order to take a complementary perspective on the otoferlin-insertion hypothesis of impaired auditory function in *Wrb* mutants, we searched for otoferlin missense mutations affecting the transmembrane domain that might interfere with its ER-insertion, as shown for the TA protein emerin in Emery-Dreifuss muscular dystrophy (Pfaff *et al*, 2016). Here we report on a newly identified mutation that affects the transmembrane domain of otoferlin and impairs hearing likely via disrupting the ER- insertion of otoferlin. The isoleucine deletion causes an auditory synaptopathy in a child that presents with a mild increase of pure tone thresholds but a lack of auditory brainstem responses and a dramatic reduction of the compound action potential of the auditory nerve, despite of normal cochlear amplification. Using our *in vitro* ER insertion assay we found that this mutation impaired otoferlin insertion. We feel that this new, otoferlin-centered

perspective supports our hypothesis that the function of the TRC40 pathway in inner hair cells is required for hearing primarily via efficient membrane insertion of otoferlin.

We would also like to mention that – in a parallel study of a knock-in mouse that carries an otoferlin mutation, which causes temperature-sensitive auditory synaptopathy in humans – we also found a vesicle replenishment phenotype (Strenzke et al., under review at EMBO J). Amongst the insights into the disease mechanism of otoferlin missense mutations provided by this study, is the demonstration that the rate of vesicle replenishment at the IHC ribbon synapse scales with the abundance of otoferlin. We take the phenotypic similarity - otoferlin reduction and impaired vesicle replenishment – as additional support for our hypothesis that the sound encoding deficit upon *Wrb*-disruption is primarily caused by reduced otoferlin abundance due to impaired membrane insertion.

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substantially lower than that achieved with RAMP4 using the same approach (Favaloro et al 2010). It is also noteworthy that the difference between otoferlin membrane insertion with and without ATP is modest in comparison to the effect of ATP on RAMP4 insertion (Favaloro et al 2010). These points should be commented on in the text and/or authors' response. These data support the principal conclusion in relation to loss of otoferlin function upon perturbation of WRB.

We fully agree with the reviewer and have included the requested discussion in the revised manuscript (both results: page 6 last paragraph; discussion: middle section of page 16). As stated above we have now included work on a mutation in OTOF that deletes an isoleucine in otoferlin's transmembrane domain, causes less efficient transport into the ER by the TRC40 pathway (tested in vitro) and leads to a human auditory synaptopathy, most likely via reducing the abundance of membrane-inserted otoferlin in inner hair cells. Moreover, we have studied the effect of the alternative insertion pathway using Hsc70/Hsp40 chaperones on insertion of otoferlin into microsome and did not detect an effect of Hsc70-immunodepletion prior to running the assay (Appendix Figure S1). Together this data supports our notion that efficient ER-insertion of otoferlin via the TRC40 pathway is critical for hearing.

Minor comments:

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done

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Co-localization is indicated by white color reflecting merge of Wrb-GFP and ER-tdTomato in this maximum projection of confocal sections. While there is substantial non-overlapping ER-tdTomato signal, most of the Wrb-GFP fluorescence seems to co-localize with ER-tdTomato fluorescence. In response to the reviewer's comment we have weakened the statement: "we found spot-like Wrb-GFP signal mostly co-localized with a recombinant fluorescent ER marker"

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done

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done

5. The authors should specify what form of otoferlin was used for the in vitro studies shown in Figure 2 and why a shortened form of the protein was needed for these experiments. They should also define the TRC40 mutant that is used in Figure 2.

done:
"The shortened otoferlin was used for better solubility of protein purified from E.coli. The TRC40gr mutant was described in (Favaloro et al., 2010)." (page 22; paragraph 'Protein purification and *in vitro* post-translational membrane insertion')

Moreover, further methods now included into Appendix Supplementary Methods (Appendix page 6f):
"For pQET328-10hisZZ-OTOFop, the coding sequence of Otoferlin (amino acids 1733-1997) was amplified from pEGFP-Otoferlin using the primers
TATACAGGTACCGAGCTGCGGGTCATCGTGTGGAACACAGACGAG and
TAGTATAAGCT

TTTAGCCCGTCTTGTTGGAGAAAGGCACGTAGAAGTTTGGGCCGGCCCCTAGGAGCTTC
TT containing KpnI and HinDIII restriction sites respectively. This PCR reaction introduces a C-terminal opsin tag containing a N-glycosylation site. The fragment was cloned into pQET328-10hisZZtev (Favaloro et al., 2010).
A NheI/AvrII fragment generated from pQET328-10hisZZ-OTOFop was cloned into pQE80-MBP-TRC40wt andpQE80-MBP-TRC40gr (Favaloro et al., 2010) to generate the constructs pT5L_T7-MBP-TRC40wt_hisZZ-OTOFop and pT5L_T7-MBP-TRC40gr_hisZZ-OTOFop respectively for bacterial expression of TRC40/OTOF complexes."

6. Page 10. The section starting "In addition, we found that flat subplasmalemmal cisternae....." and concluding "might reflect an up-regulation of perisynaptic rough ER, aiming to compensate for impaired TA protein insertion" is rather opaque and could be more clearly expressed.

done (page 10; bottom):
"In addition, we found that subplasmalemmal cisternae near synaptic sites were often decorated with electron-dense particles, most likely representing ribosomes (Figure 5B, B', C). Quantification of random ultrathin sections revealed that ~36% $Wrb^{fl/fl}$:Cre^{A} synapses showed such structures within a distance of 200 nm, or less, from the presynaptic density (n = 41 sections; see also tomographic reconstruction in Appendix Figure S3D-D'), whereas this was observed at only ~4% of $Wrb^{+/+}$:Cre^{A} synapses (n = 25 sections). This might reflect a compensatory up-regulation of perisynaptic ER, triggered by impaired TA protein insertion."

Referee #3

As stated in the first paragraph of the discussion, this study reports "the requirement of the TRC40 pathway for efficient [insertion] of the TA protein otoferlin in sensory HCs" and "reveals a critical requirement of the TRC40 pathway for TA protein processing in the sensory epithelium of the ear." These conclusions are supported by the data in this paper, along with a fairly extensive characterization of the HC function in tissue-specific knockouts for WRB.

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strongly supported (see below). Thus, from the standpoint of protein trafficking, the study as currently presented is more suited to a specialized journal because the broader conceptual advance would seem to be relatively limited. Whether the advances related to auditory biology are more widely interesting is for experts in that area to judge.*

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

Specific suggestion:

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one in HCs is otoferlin. The title of the study is therefore not really appropriate unless such a rescue is demonstrated.

There are two ways to attempt rescue. The first is to simply over-express otoferlin to compensate for loss of WRB. As shown in Schuldiner et al. (2008) for yeast, individual phenotypes associated with a disrupted GET pathway can be bypassed by overexpressing the relevant TA protein associated with that phenotype. The second, perhaps more elegant, strategy is to extend the C-terminus of otoferlin (with GFP, for example). This would convert otoferlin into a co-translational substrate (see Stefanovic and Hegde, 2007, for an example) that is no longer dependent on WRB. Because the functional parts of otoferlin are cytosolic and in the membrane, a GFP on the other side of the membrane is not likely to affect its function. If such a construct rescued the phenotype, this would convincingly show otoferlin as the key TRC/GET pathway client responsible for HC function.

We thank the reviewer for proposing experiments to further test our hypothesis that the hearing impairment in wrb-fish/mice right after onset of hearing is primarily caused by defective ER-insertion of otoferlin. We note that we clearly identified this as a hypothesis and discussed alternative possibilities in the manuscript. We realize that the title indeed went beyond this and changed it to:

"Tryptophan-rich basic protein (WRB) mediates insertion of the tail-anchored protein otoferlin and is required for hair cell exocytosis and hearing".

Regarding the conceptual advance resulting from this study and general relevance of the work we would like to stress that this is, to our knowledge, the first study revealing a critical function of the TRC40 pathway in mammalian neuronal tissue. It demonstrates a synaptic deficit due to impaired synaptic vesicle replenishment as the key functional deficit upon WRB-disruption with strong physiological and morphological evidence. The reduction of otoferlin and the similarity of the auditory phenotype to that of missense-mutants for otoferlin led us to formulate the otoferlin hypothesis of hearing impairment in wrb-fish/Wrb-mouse mutants.

The reviewer follows this line of argument, but concludes that all the findings were more or less expected. We respectfully disagree with this conclusion and stress that unlike the expected pleiotropic consequences of WRB-disruption we found a rather specific deficit of hair cell biology at least up to 3 weeks of age. Indeed, the normal formation, morphology and function of hair cell stereocilia (implying intact polarized trafficking and normal planar and apicobasal cell polarity) as well as the absence of synapse loss support the view of an initially synapse-specific hearing deficit likely primarily due to reduced otoferlin levels rather than a global failure of the hair cell due to defective TA-protein insertion. Moreover, the fact that the amount of membrane-proximal synaptic vesicles at active zones (EM data), Ca^{2+} channel activity and the size of the RRP *in vitro* remain unaltered (electrophysiological data), further suggests a specific synaptic deficit, namely in vesicular replenishment. The synaptic phenotype bears similarity to that observed in the otoferlin mutant *pachanga* (Pangrsic et al. 2010), but was less severe allowing much more detailed systems physiological analysis and which lends more confidence.

Following the reviewers advise we have performed the otoferlin overexpression experiment trying to override the insertion deficit. As neither we nor others have achieved transgenic expression of otoferlin in mouse IHCs (the size of otoferlin cDNA exceeds capacity of adeno-associated virus (AAV) that works best for viral-gene transfer into IHCs), we have turned to otoferlin overexpression in *pwi* fish. Towards this end we have injected otoferlin mRNA (based on mouse otoferlin cDNA) into zebrafish eggs in two different doses (1x and 2x mRNA). We observed a dose-dependent partial rescue of auditory function as tested by the startle response. While it is not yet clear which pathway(s) contribute to TRC40-independent insertion of otoferlin in hair cells, the results supports our hypothesis that disrupted ER-targeting of otoferlin contributes to the impairment of auditory function in *Wrb* mutants.

Unfortunately, the second proposal of a C-terminal fusion of a GFP to convert otoferlin into a co-translationally inserted protein is problematic. The lab of Christine Petit (Pasteur Institute) has recently generated a knock-in mouse with a C-terminally GFP-tagged otoferlin, which results in an IHC exocytosis phenotype similar to that found in our hair cell specific Wrb-KO mice (unpublished

data presented at meetings). At this point it is not clear whether this indicates i) an impairment of ER-insertion due to the C-terminal tag and/or ii) a disrupted function of the inserted protein.

In order to further test our otoferlin-hypothesis of the hearing impairment in *Wrb* mutants, we have considered an alternative approach that targets ER-insertion of otoferlin without general disruption of the TRC40-pathway. Specifically, we searched for otoferlin missense mutations affecting the transmembrane domain that might interfere with its ER-insertion as shown for the TA-protein emerin in Emery-Dreifuss muscular dystrophy (Pfaff *et al*, 2016). Here we report on a newly identified mutation that affects the transmembrane domain of otoferlin and impairs hearing likely via disrupting the ER- insertion of otoferlin (Results, page 13ff; Discussion, page 15 (bottom) and page 19 (middle section). The isoleucine deletion causes an auditory synaptopathy in a child that presents with a mild increase of pure tone thresholds but a lack of auditory brainstem responses and a dramatic reduction of the compound action potential of the auditory nerve despite normal cochlear amplification. Using our *in vitro* ER insertion assay we found that the mutation impaired otoferlin insertion.

We would also like to mention that – in a parallel study of a knock-in mouse that carries an otoferlin mutation, which causes temperature-sensitive auditory synaptopathy in humans – we also found a vesicle replenishment phenotype (Strenzke et al., under review at EMBO J). Amongst the insights into the disease mechanism of otoferlin missense mutations provided by this study, is the demonstration that the rate of vesicle replenishment at the IHC ribbon synapse scales with the abundance of otoferlin. We take the phenotypic similarity - otoferlin reduction and impaired vesicle replenishment – as additional support for our hypothesis that the sound encoding deficit upon *Wrb*-disruption is primarily caused by reduced otoferlin abundance due to impaired membrane insertion.

Acceptance 10 June 2016

Thank you for submitting your revised manuscript. Your study has now been re-reviewed by referee #2. As you can see below, the referee appreciates the introduced changes and support publication here.

I am therefore very pleased to accept the manuscript for publication here.

REFEREE REPORT

Referee #2:

The authors have added important additional data that convincingly addresses the issues I flagged with their original submission by providing more data and or revising the text. I find their identification and characterisation of a relevant human otoferlin mutant and the inclusion of partial rescue by overexpression in their fish model also enhance the paper and I believe this is an important and physiologically relevant study.

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: Tobias Moser
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2015-93565

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).

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 observation in the respective fields (e.g. biochemisty, 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.
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. Criteria for analyses were pre-established for all types of experiments.
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.	Animals across all experiments were used in littermate pairs and genotypes (re-) confirmed independently 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 always compared/processed in parallel to littermate controls. However, data of experimenters were typically double-checked by another observer and re-genotyping was performed in all instances.
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.	Although experimental blinding was performed whenever possible, the observed strong auditory, but also cellular phenotypes of mutant animals/hair cells revealed the genotypes during the respective experiment. However, for single unit recordings, the experiments were performed blindly and the genotype was made known to the experimenter only after the data collection and online analysis was complete.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Except for single unit recording studies, no blinding was performed. Mutants were always processed in parallel with littermate controls, data of experimenters were 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, subsequently, the appropriate statistical tests were used to test for statistical significance. (page 26)
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Data normality and variance were tested in all instances using Jarque-Bera and Kolmogorov-Smirnov tests, prior to the adequate statistical test (page 26).
Is there an estimate of variation within each group of data?	All data are provided with standard error of the mean, as specified in the respective section in the text.
Is the variance similar between the groups that are being statistically compared?	Equality of variance was preassessed with Jarque-Bera and Kolmogorov-Smirnov tests and the adequate statistical tests chosen based on the results, i.e. non-parametric tests in case of non-normal distribution or unequal variances, etc. (page 26).

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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).	The details of all antibodies are listed within the Material and Methods section (page 22-23)
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 in this study. All measurements from single cells were from acutely dissected tissue samples.

* 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.	Generation of Wrb fl/fl mice is described in Appendix (pages 1-2) in a C57BL/6 background. Transgenic Vglut3-Cre mice (CreA) are already published in (Jung et al, 2015). The Vglut3-ires-Cre knock-in mice (CreB, used by the Boston group) were generated by L. Vong and B. Lowel (strategy and characterization unpublished) and first used in (Lou et al, 2013).The generation of both reporter mouse lines has been described previously (Madisen et al, 2010; Nakamura et al, 2006). The generation of wrb (pwi) zebrafish has been described (Gross et al, 2005) (also see page 20 and Appendix pages 1-3).
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. All animal procedures performed at Harvard Medical School were approved by Harvard Medical School IACUC. (page 20)
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.	Approval was obtained from the Ethical Committee of Hospital Universitario Ramón y Cajal
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.	Approval was obtained from the Ethical Committee of Hospital Universitario Ramón y Cajal and in accordance with the 1964 Declaration of Helsinki. Written informed consent was obtained from all participants in the study (parents for themselves and for their son, who was minor) (page20)
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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.	NA
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.	NA

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	NA
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 participating institutions or 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).	
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	All relevant references ae included in the Material & Methods section (page 27ff) as well as the Appendix (page 17).
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.	NA

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.	NA