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Hair cell synaptic dysfunction, auditory fatigue and thermal sensitivity in otoferlin Ile515Thr mutants

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(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 09 June 2016

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by two referees and their comments are provided below.

As you can see, the referees find the analysis interesting and support publication here. Referee #1 raises a number of constructive comments that I would like to ask you to resolve in a revised version. I should add that it is EMBO Journal policy to allow only a single round of major revision and that it is therefore important to resolve the concerns raised at this stage.

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.

REFEREE REPORTS

Referee #1:

The manuscript by Strenzke et al. explores the roles of otoferlin in inner hair cells and tests the mechanisms of action of a human temperature sensitive otoferlin mutation using a novel mouse model (OtofI515T). Through a very extensive, comprehensive and impressive set of experiments involving many levels of analysis, the authors reach the conclusion that otoferlin plays a key role in synaptic vesicle reformation and the replenishment of the readily releasable vesicle pool.
The paper reports that mice with the OtofI515T mutation display phenotypes partially resembling those observed in patients, specifically the progressive hearing loss, but not temperature sensitivity. They also find that OtofI515T mutation causes reduction in the expression level and plasma membrane level of otoferlin protein, impairment of synaptic vesicle replenishment and sound encoding leading to defects in gap detection and auditory fatigue in vivo. Through additional experiments, the authors report that increase in body or bulla temperature further reduces membrane level of otoferlin, and they postulate that thermal sensitivity of human patients may be due to a combinatory effect of temperature and the presence of unique RXR motif in human version of otoferlin. The study is technically sound, detail-oriented and important for understanding the roles of oteferlin in genetic deafness.

Major concern:
The authors propose that the mutant mouse fails to exhibit temperature sensitivity due to absence of RXR motif (which is present in humans) in mouse otoferlin. However, as far I understand, one of the mouse otoferlin isoforms does have this motif as authors show in Figure EV4 (mouse var2). Secondly, some of the human otoferlin isoforms also lack this RXR motif, such as isoform b (NP_004793.2) and d (NP_919304.1). Thus, to support this conclusion, it will be important to show which otoferlin isoforms are expressed in mouse and human hair cells respectively. This can be done by isoform-specific RT-PCR or qPCR and should help address whether RXR motif is indeed the contributing factor.

Other comments:

The claims of quantification of otoferlin levels at the plasma membrane by immunostaining are not convincing as the images do not prove the localization. The quality and interpretability of the immuno-gold TEM images are also questionable. Since this is a key point of the study, the authors need to clarify and solidify these claims.

If I am not mistaken, the behavioral tests the authors used for testing the effects of the otof mutation are also commonly used to test for tinnitus. The authors need to discuss if they distinguish between hearing loss and the possibility that the otof mutation results in tinnitus.

Given the many different types of statistical analysis used in the different experiments, it is necessary that the authors list the specific tests used in the figure legend for each panel. There are several points in the paper in which statistical information is missing.

Page 5, Appendix Fig S1C and S1D were cited erroneously.

Page 10, "...the elevated cochlear temperature did not fully abolish sound encoding in OtofI515T/I515T mouse mutants, as it seems to be the case in human OtofI515T/R1116X patients. Thus, heat-induced cell physiological or ultrastructural effect of fever found in our mouse model might be even stronger in human patients." These two sentences are confusing and seemingly contradictory, the authors should elaborate.

Figure 6H-J, the apical and basal localization of Otoferlin was difficult to observe, particularly for the mutant, the authors should have additional panels with enhanced contrast.

Figure EV1, it would be helpful to include the DPOAE thresholds.

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Referee #2:

Mutations in the OTOF gene were discovered in 1999. Subsequent detailed work led to the finding that the protein encoded by this gene is required for exocytosis in inner hair cells and is associated with activity at the ribbon active zones of the hair cells. Most compelling, a number of missense mutations lead to a temperature sensitive auditory synaptopathy. The authors set out to create another mouse model for OTOF deafness. The novelty to this mouse is that it has an intermediate hearing defect, allowing the authors to answer an open ended question about synaptic sound encoding and the relationship of otoferlin function. While the mouse was quite similar to several human patients, they did not share the temperature sensitivity. This provided a hypothesis regarding the mechanism for the temperature sensitivity in humans.

A targeted knock-in strategy was employed to create the Ile515Thr mutation in mice and determined that they harbor a reduction in otoferlin levels. Patch clamp recordings were conducted to evaluate presynaptic inner hair cell function. While synaptic vesicle fusion was normal, there was impaired replenishment of vesicles. Extracellular recordings from individual SGNs demonstrated a reduction of sound encoding in synapses of the mutant mice. Prepulse inhibition of the acoustic startle response was used to detect a further impairment in the mice. The next major issue was to examine the reaction of the mutant mice to increased heat. There did not seem to be the same exacerbation of hearing loss as in humans. As a result, the differences between the human and mouse otoferlin were studied. A 20 amino acid region appears to be responsible for reducing plasma membrane localization, as seen by inserting into the mouse otoferlin cDNA and otoferlin null inner hair cells. Levels of otoferlin was further examined in hair cells exposed to heat, showing that increased heat reduces exocytosis and otoferlin membrane levels. Finally, an increase in the size of otoferlin-labelled endosomal vesicles and synaptic vesicles, as evaluated by electron microscopy and tomography.

Overall, this is a comprehensive report describing the mechanisms associated with the Ile515Thr mutation in mice, which serves as a model for hearing impairment due to OTOF mutations and the phenomenom of auditory fatigue. As heat exposure and increased hearing loss is quite unusual, it is fascinating that the reason behind this finding is now known. This work also highlights a crucial difference between humans and mice, with a plausible explanation for this difference on a molecular level.

Only change recommended:
Change I515T to Ile515Thr when describing the mutation, to adhere to nomenclature rules for mutations. When referring to the name of the mouse, can be kept at I515T for brevity - OtofI515T/I515T

1st Revision - authors' response 24 August 2016

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The paper reports that mice with the OtofI515T mutation display phenotypes partially resembling those observed in patients, specifically the progressive hearing loss, but not temperature sensitivity. They also find that OtofI515T mutation causes reduction in the expression level and plasma membrane level of otoferlin protein, impairment of synaptic vesicle replenishment and sound encoding leading to defects in gap detection and auditory fatigue in vivo. Through additional experiments, the authors report that increase in body or bulla temperature further reduces membrane level of otoferlin, and they postulate that thermal sensitivity of human patients may be due to a combinatory effect of temperature and the presence of unique RXR motif in human version of otoferlin. The study is technically sound, detail-oriented and important for understanding the roles of otoferlin in genetic deafness.

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

Major concern:
The authors propose that the mutant mouse fails to exhibit temperature sensitivity due to absence of RXR motif (which is present in humans) in mouse otoferlin. However, as far I understand, one of the mouse otoferlin isoforms does have this motif as authors show in Figure EV4 (mouse var2). Secondly, some of the human otoferlin isoforms also lack this RXR motif, such as isoform b (NP_004793.2) and d (NP_919304.1). Thus, to support this conclusion, it will be important to show which otoferlin isoforms are expressed in mouse and human hair cells respectively. This can be done by isoform-specific RT-PCR or qPCR and should help address whether RXR motif is indeed the contributing factor.

We agree with the reviewer that this is an important point that needs to be clarified. In case of the mouse, we performed a PCR that demonstrated that the majority of cochlear otoferlin lacks the RXR motif (Figure EV4). The sources for mature human inner ear mRNA, however, turned out to be very limited. We tried several approaches to solve this issue:

- We are grateful to Nahid Robertson and Cynthia Morton for providing us an unsubtracted cDNA library from human fetal inner ears (Robertson *et al*, 1994). This cDNA library was derived from 173 labyrinths of 16-22 week old human fetuses, which is just before the onset of hearing. Note that otoferlin is expressed in rodent inner hair cells already before the onset of hearing. However, fragments in this library are on average 500bp and the reverse transcription was primed with oligo-dT primers, such that the amplification of the RXR site which is around 3 kb upstream of the poly-A-tail was at the edge. As a control, we isolated mRNA from human brain tissue and transcribed this into cDNA (Thanks to Sabine Pfeifenbring and Wolfgang Brück, Dept. for Neuropathology, University Medical Center Göttingen, for providing the tissue). Otoferlin (with RXR motif) is supposed to be expressed in brain, however on a very low level. Using 40 PCR cycles and/or nested PCR, we could not amplify fragments from human otoferlin from both of these cDNA templates applying a series of different primer combinations.
- Our preferred attempt was and is to obtain fresh vestibular hair cells from acoustic neuroma surgery. We have not been lucky yet (a planned surgery was canceled on short notice because of cardiac problems), but we continue to make requests to more researchers and ENT surgeons and plan to do the experiment in any case, ideally to be included in the present MS at the stage of proof reading (one expanded view figure would needed to be changed). However, translabyrinthine surgery is only rarely performed and we do not yet know how long it would take to finish these experiments.
- In addition, we consulted experts on the field generating hair cell-like cells from human embryonic stem cells or iPS cells. Unfortunately, they told us that they think these hair cells are not mature hair cells; only very few of them express otoferlin at all (e.g., Ronaghi et al., 2014).
- Furthermore, trying to solve the issue *in silico* was also not successful (no sequences from human inner ear tissue in databases, no pathogenic nonsense mutations in the RXR motif).

We revised the manuscript now to make clear that there is no experimental prove which splice variant of otoferlin is indeed expressed in human auditory hair cells. We included the sequence of human isoform b in the multiple sequence alignment of Fig EV4. We rephrased the last two sentences in this paragraph (page 11), stating now:
Since the abundance of otoferlin at the plasma membrane seems to be most relevant for sound encoding in vivo, this might explain the more pronounced heat sensitivity in human patients. When comparing to the hearing phenotype of our mouse models, hearing at normal and elevated temperature in human Ile515Thr patients would best be explained by a mixture of the splice variant with RXR and the splice variant without being expressed in human IHCs.

In addition, we revised the discussion accordingly (page 17):
Direct comparison of mouse ABRs at febrile temperature with psychoacoustic testing of human patients is challenging. However, it seems that our mice are less susceptible to heat than human $OTOF^{I515T/R1116*}$ *subjects who exhibit a threshold elevation by ≥60dB and loss of speech perception at 38.1°C body temperature. Our data indicate that this may be due to the RXR motif sequence presumably present in human otoferlin which reduces the plasma membrane abundance of Ile515Thr-otoferlin beyond what we found in* $Otof^{I515T/I515T}$ *mice. The expression of this RXR motif*

depends on whether the first or the second splice acceptor site in exon 30 is used, which is currently unknown. Together, the lower cellular otoferlin protein levels due to the Ile515Thr mutation, the presumed presence of the RXR splice isoform which causes a weaker potency of the Ile515Thr-otoferlin for plasma-membrane localization, and a likely heat-induced protein unfolding provide a candidate mechanism for temperature-sensitive hearing loss in humans.

Other comments:

The claims of quantification of otoferlin levels at the plasma membrane by immunostaining are not convincing as the images do not prove the localization. The quality and interpretability of the immuno-gold TEM images are also questionable. Since this is a key point of the study, the authors need to clarify and solidify these claims.

We apologize that this essential point was so far not clear enough. We now added more experimental data supporting the finding that plasma membrane abundance correlates with hearing, and in addition we revised the respective figures for better visualization of this effect.
First, we performed a second immunofluorescence analysis on $Otof^{+/+}$, $Otof^{I515T/I515T}$ and $Otof^{Pga/Pga}$ IHCs with a different antibody which binds to the C-terminal, extracellular/intraluminal sequence of otoferlin (Appendix Fig S1). Also here, the otoferlin immunofluorescence at the plasma membrane appears much weaker in $Otof^{Pga/Pga}$ than in $Otof^{I515T/I515T}$ IHCs. A quantitative analysis of the membrane staining, however, was not possible because the C-terminal antibody is a polyclonal rabbit antibody just as the anti-Vglut3 antibody.
Second, in Figure 1, we added insets of the line scan analysis for better visualization on how we quantified the plasma membrane staining at the basal poles of the inner hair cells. In addition, we increased the number of analyzed $Otof^{Pga/Pga}$ IHCs in Figure 1.
Third, in order to confirm that otoferlin is indeed localized at plasma membrane of the basal IHC region we added a new supplementary figure (Appendix Fig S5) showing more images of the otoferlin immunogold labeling together with a labeling for Vglut3 in wild type inner hair cells. Here, the presence of a remarkable fraction of gold particles right at the plasma membrane endorse that otoferlin is indeed an integral protein of the plasma membrane. These data are in agreement with a recent study confirming otoferlin immunogold labelling at the plasma membrane from the lab of C. Wichmann (Jung *et al*, 2015).
Our findings are further supported by earlier studies from other labs using the HCS-1 antibody (Goodyear *et al*, 2010). In chicken hair cells, they found a co-localization of otoferlin with the plasma membrane marker PMCA. In addition, they demonstrate that otoferlin cannot be solubilized from membranes unless 0.1% Triton X-100 was present. This supports the prediction that otoferlin is indeed an integral membrane protein as predicted from the amino acid sequence.

In the manuscript, we added the information about the new immunostaining (Appendix Figure S1) and the additional images of the immunogold labelling (Appendix Figure S5).

If I am not mistaken, the behavioral tests the authors used for testing the effects of the otof mutation are also commonly used to test for tinnitus. The authors need to discuss if they distinguish between hearing loss and the possibility that the otof mutation results in tinnitus.

Indeed, tests assessing the perception of silent gaps in a background sound are widely used in animal experiments trying to assess putative tinnitus, the rationale being that any sound perception in the auditory system would mask the silent gap. Though the presence of tinnitus in our animal model cannot be excluded, there is no specific reason to assume that the mutant mice suffer from tinnitus. Unlike in most tinnitus studies where tinnitus is elicited by noise trauma or ototoxic drugs, the hearing dysfunction in our mutant mice appears to be uniform across the entire tonotopic range and affects low and high spontaneous rate fibers in a similar fashion; we have no indication for a disturbed equilibrium of auditory coding which is supposed to be one basis for tinnitus development. Consistent with this argumentation, in the published reports on human otoferlin-mutation associated hearing loss with residual hearing, there is no mention of tinnitus.
In our study, the impairment of synaptic sound encoding correlates very nicely with the gap detection deficit. To us, it seems plausible that impaired coding of the offset of a sustained background sound (due to the reduction of adapted spike rates) in combination with delayed recovery from forward masking due to a vesicle reformation deficit (as seen in the forward masking

experiments) would reduce the salience of the gap. We thus favor the hypothesis that the IHC ribbon synapse dysfunction is the main reason for the behavioral results.

We modified the discussion accordingly (page 19): “In contrast, none of the patient reports mention tinnitus as an additional symptom. We thus consider it unlikely that impairment of the perception of silent gaps in noise in our mice is due to tinnitus (Turner *et al*, 2006). Instead, it is likely explained by the combination of the reduction of adapted spike rates and the delayed recovery of the sound onset response.”

Given the many different types of statistical analysis used in the different experiments, it is necessary that the authors list the specific tests used in the figure legend for each panel. There are several points in the paper in which statistical information is missing.

We added the type of statistical analysis for every experiment in the figure legends and added statistical information in the manuscript wherever it was missing.

Page 5, Appendix Fig S1C and S1D were cited erroneously.
Corrected.

Page 10, "...the elevated cochlear temperature did not fully abolish sound encoding in OtofI515T/I515T mouse mutants, as it seems to be the case in human OtofI515T/R1116X patients. Thus, heat-induced cell physiological or ultrastructural effect of fever found in our mouse model might be even stronger in human patients." These two sentences are confusing and seemingly contradictory, the authors should elaborate.

We rephrased the second sentence to make clear that the phenotype in humans is stronger than in mice:
Thus, as the heat-induced phenotype seems to be weaker in mice compared to human patients, the cell physiological or ultrastructural effect of fever found in our mouse model might also be weaker than in human patients.

Figure 6H-J, the apical and basal localization of Otoferlin was difficult to observe, particularly for the mutant, the authors should have additional panels with enhanced contrast.
We revised Figure 6 H-J accordingly.

Figure EV1, it would be helpful to include the DPOAE thresholds.
Done, see Fig EV1C.

Figure EV2A, D and F: the traces are very confusing. I wonder if there is another way to show this data, may be as in Figure 4I?

In Figure EV2A, we now reduced the number of examples shown to simplify the graph. To simplify figures EV2D and F, we now changed the dB scale and plotted the rate level function relative to the threshold of each individual SGN. Threshold was interpolated from the rate level function as the intensity at which the spike rate increased by 20 Hz above spontaneous rate. We chose not to further normalize the rate level functions further because in this expanded view figure we wanted to illustrate the original data and its variability as well as the impact of the spike rate reduction on intensity coding in the auditory nerve as a whole.

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Overall, this is a comprehensive report describing the mechanisms associated with the Ile515Thr mutation in mice, which serves as a model for hearing impairment due to OTOF mutations and the phenomenom of auditory fatigue. As heat exposure and increased hearing loss is quite unusual, it is fascinating that the reason behind this finding is now known. This work also highlights a crucial difference between humans and mice, with a plausible explanation for this difference on a molecular level.
Thank you very much for the appreciation of our study.

Only change recommended:

Change I515T to Ile515Thr when describing the mutation, to adhere to nomenclature rules for mutations. When referring to the name of the mouse, can be kept at I515T for brevity - OtofI515T/I515T.
We changed the description into Ile515Thr and the description of the other point mutations accordingly.

References

Goodyear RJ, Legan PK, Christiansen JR, Xia B, Korchagina J, Gale JE, Warchol ME, Corwin JT & Richardson GP (2010) Identification of the hair cell soma-1 antigen, HCS-1, as otoferlin. *J. Assoc. Res. Otolaryngol.* **11:** 573–586

Jung S, Maritzen T, Wichmann C, Jing Z, Neef A, Revelo NH, Al-Moyed H, Meese S, Wojcik SM, Panou I, Bulut H, Schu P, Ficner R, Reisinger E, Rizzoli SO, Neef J, Strenzke N, Haucke V & Moser T (2015) Disruption of adaptor protein 2μ (AP-2μ) in cochlear hair cells impairs vesicle reloading of synaptic release sites and hearing. *EMBO J.* **34:** 2686–2702

Robertson NG, Khetarpal U, Gutiérrez-Espeleta GA, Bieber FR & Morton CC (1994) Isolation of novel and known genes from a human fetal cochlear cDNA library using subtractive hybridization and differential screening. *Genomics* **23:** 42–50

Turner JG, Brozoski TJ, Bauer CA, Parrish JL, Myers K, Hughes LF & Caspary DM (2006) Gap detection deficits in rats with tinnitus: A potential novel screening tool. *Behavioral Neuroscience* **120:** 188–195

Accepted 13 September 2016

Thank you for submitting your revised manuscript. Your study has now been re-reviewed by the two referees and as you can see below both appreciate the introduced changes.

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

REFEREE REPORTS

Referee #1:

The authors have done a good job in responding to the comments and the paper is much better. The link between the RXR motif and heat-susceptibility remains a possibility, not a fact, but the authors discuss this issue adequately in the revised manuscript.

Referee #2:

All requests by reviewers made.

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: Ellen Reisinger
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2016-94564

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 $\chi 2$ 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. 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.
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 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 always compared/processed in parallel to littermate controls.
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 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?	All data are provided with standard error of the mean, as specified in the respective section in the text. For small sample sizes, the individual values are presented in addition.

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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 antibodies we used are commercially available. We provided detailed information on every antibody in the supplementary methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK293T cells have been ordered from ATCC in 2012.

* 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 Otof I515T/I515T mice is described in the supplementary material. Animals were backcrossed on C57/Bl6N background or on CBA/J background (the latter for single unit recordings and startle responses). Otoferlin Knock-out mice are described in (Reisinger et al., 2011). Animals were kept in small groups with food and water access. Housing and husbandry was performed in accordance with national guidelines and approved by the animal welfare committees if the University of Göttingen and the State of Lower Saxony.
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.	N/A
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.	N/A
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	The cDNA of mouse otoferlin used in our experiments was subcloned from inner ear cDNA. The sequence is deposited under GeneBank accession No KX060996
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).	N/A
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.