nature portfolio

Peer Review File

Cochlear gene therapy restores hearing and auditory processing in an autosomal recessive deafness 9 mouse model

Corresponding Author: Dr Saaïd Safieddine

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors are contributing interesting results to a current hot-topic; that of using gene therapy to treat Otof-related hearing impairment. The AAV-treatment gives a clear result in that it prevents the mutant mice from exhibiting the expected hearing impairment. An interesting addition to the normal cochlear and brainstem assays of hearing function is the behavioural test that indicates that central auditory function is maintained and still operational after AAV treatment. The results presented here provide further experimental evidence supporting the clinical observations presented earlier this year that AAV-treatment in human sufferers with Otof mutations can help to bring forth their hearing.

Introduction. The authors have presented a rounded introduction. The statement on lines 68/69 could perhaps be tempered a little; it was shown earlier this year that gene-therapy for human Otof-based hearing loss can be effective, which the authors do later acknowledge.

They discuss that 15% of DFNB9 deafness patients are "atypical" and show atypical, fluctuating deafness, such as mild-to-profound, progressive or temperature sensitive. They state that their aim is assess if AAV gene therapy could be effective in treating such patients. They generated a humanized mouse model, albeit one that does not carry the exact same human mutation, but instead carries a mutation, Otof E1799del corresponding to the human OTOF E1804del variant. The authors claim that their mouse model (the E1799del Otof mutation) mirrors the human E1804del variant linked to DFNB9, BUT the mouse does NOT appear to show either fluctuating deafness or deafness during febrile episodes. This has to raise the question of how good a model this particular mouse is for the human condition.

Ln 143. Homozygous "1779" mutants were given gene therapy as neonates (P2) or as adults (P30).
Ln 149. P15-P30 mice ... this contradicts line 143, where P30 is given as the adult age.
The ages of mice used throughout the study seems to be a little inconsistent & therefore confusing. If the authors could somehow clarify / simplify the descriptions the ages tested in different aspects of the work, it would be much appreciate. Perhaps the exact ages could be defined in the methods and given in the figure legends, but referred to in the test as neonatal, juvenile, adult, etc as appropriate?

The statistical approach taken needs further consideration by the authors. The reader has to assume that all datasets were normally distributed, allowing parametric testing to be used throughout. Given the thresholds allocated to the auditory brainstem responses of the mutant mice, normality looks unlikely. It would be appropriate to give results of a normality test (eg the Shaprio-Wilk test) for each dataset and then treat them accordingly as parametric or non-parametric data.

Ln 285 - "regardless of body temperature" all homozygous mice were profoundly deaf. Only 8 mice tested. In Humans, if only 15% have atypical hearing loss, then even fewer may show the temp-sensitive hearing loss. Given the low prevalence in humans, would many more than 8 mutant mice need to be examined to see any thermo-sensitivity? What do the authors mean by "body temperature"? Temperature at the time of recording? If so, was this monitored & recorded. Or do they mean if the mice had previously been subjected to elevated body temperature to simulate a febrile episode? If so, has this been studied fully? They say it's an aim of the study to assess if AAV gene therapy would be effective in treating atypical DFNB9 patients, but do not appear to have tested if their mouse mutant is a good model to reproduce this part of human disease phenotype.

Ln 288. Typo error? should it read ", as were the distortion product otoacoustic emissions" ??

Ln 303. Should "posttranscriptional" be hypenhated?

Ln 317, 330, 332 and other places through the manuscript & figures. The authors use "on" P8, etc, whereas "at" P8, etc would be better. Please could this be amended throughout?

Throughout the manuscript, the authors use the word restore / restoration, etc. I wonder if this is the ideal word / phrase to use in this case. "Restore" can be defined as "to bring back to or put back into a former or original state". Since the "1799" mutant mice have never had a normally functioning Otof protein, and would never have been able to generate normal hearing, the positive effects of AAV treatment should not be termed restore / restoration of hearing in the mutants, as this would effectively mean "no change". This word & phraseology is commonly used in the literature, but I do not feel it is strictly correct. Call me pedantic if you will!
In effect what is happening is that the AAV gene therapy is giving the system an alternative gene to use which produces a normally-functioning protein. The presence of the normal protein then allows the system to work as would be expected in a "normal" wildtype animal. To me, a better word to use might be "alternate" or "replacement". However, I will leave it to the editors and authors to quibble over this suggestion.

Ln 397. The subtitle suggests that the "1799" mutant mice have cortical deficits as a direct results of the mutation, and that addition of a normal gene / protein to the inner ear somehow induces cortical changes to allow normal processing in pre-pulse inhibition tests and the frequency discrimination tasks. Surely the authors do not believe this is the case? It is simply by reinstating normal inner function and synaptic input onto auditory nerve fibres that allows the central system to function in a normal (relative to wildtype, at least) way ? Please could the authors rephrase this title to be more appropriate?

The useful of PPI has always been debatable, I understand, however, I feel that the behavioural Go/NoGo test of frequency discrimination is a very nice additional assay. I feel that is such a test could be taken up more widely by other groups, if would add more "power" to the exciting findings that are emerging in the hunt for therapeutic approaches to the treatment of genetic deafness.

In the discussion, again the authors say that deafness phenotype of their mouse model is not affected by body temperature and compare this to a different Otof mutation which also does not mimic human thermosensitivity. Here also, they offer reasoned explanations as to why this may be the case.

I suggest that the authors should downplay the connection they make between this mouse model and the atypical fluctuating deafness associated with febrile episodes, as their "1799" mutant does NOT demonstrate this aspect of the phenotype. That said, clearly the AAV gene therapy approach is effective in allowing IHC synapses to function and bring about functional hearing, but it is an overstatement to compare their model (as with other models they mention) to that particular phenotypic aspect in humans. And they do indeed state that the AAV approach can be broadened to cover other forms of Otof mutation. It is likely that addition of the normal Otof gene and resultant normal Otof protein would help overcome the deafness of any form of mutant Otof-based deafness.

Reviewer #2

(Remarks to the Author)
Benamer et al - Peripheral gene therapy restores hearing and central auditory processing in an atypical DFNB9 mouse model.

Following the tremendous success of OTOF gene therapy last year, the team tries to investigate if OTOF gene delivery can also be effective for non-protein-truncating variants in OTOF, such as the in-frame deletion that they investigated. These patients present with atypical DFNB9, likely caused by the presence of mutant proteins with residual function. An important question, which could have a big impact on patients carrying this variant. Especially if any of the OTOF gene therapies currently under investigation reach market approval.

The introduction is appropriate for the research, and I appreciate the level of detail in the methods section. Also, the behavioral studies to provide insights in interpretation (or processing) of auditory signal is an important and elegant addition to the work.

The mouse model presents a challenge, as the authors cannot discriminate between mutant otoferlin and AAV-delivered otoferlin. The functional readouts all indicate a therapeutic effect, and otoferlin return to the basal region of the cells. But the assays used do not prove that this is AAV-delivered otoferlin. The authors are careful in their writing about this, and I appreciate that no false claims are made (except for one line in the discussion).

Overall, the data are strongly suggestive of this approach being effective for people with this variant. And probably all variants with a loss-of-function mechanism (which can be anticipated given the recessive inheritance pattern). I think the work is very interesting and worth publishing. Few study in the field of gene therapy address the efficacy for different

pathogenic variants, so in that we the study also stands out.

There are some textual and conceptual changes that I think the authors should consider to (further) improve the work before publication.

1) The title is misleading: The placing of the word “peripheral” suggests that it relates to the gene therapy. But in does not (RWM/PSCC delivery). In fact, they authors investigate if peripheral signal transduction of auditory function is conserved through behavioral assays. Great studies, but not a “peripheral gene therapy” as the title suggests. Similarly, the placing of the word “atypical” suggests the mouse model itself is atypical. However, it aims to model the atypical presentation of DFNB9. In fact, the phenotype of the mouse is rather typical for DFNB9.
2) The mouse is profoundly deaf, whereas the atypical DFBN9 patients are not (otherwise they would be typical). In the introduction patients are claimed to be moderate-to-profoundly deaf, while in the discussion they are severe-to-profound. The original paper by Sandrine Marlin shows this variant to be temperature sensitive and resulting in normal hearing to mild HL at normal body temperature. This brings me to two questions:
a. Is the mouse variant more devoid of function that the orthologous human variant. Are there perhaps human-specific alternative transcripts isoforms in which this residue is not present. Or does the mouse variant (also) effect pre-mRNA splicing, resulting in a proportion of stop-gain transcripts that differs between human and mice. Could it me mouse body temperature? Is the mouse variant even temperature sensitive? This is assumed (not explicitly) but not studied.
b. Secondly, the rescue is not perfect. If the human phenotype is mild, is the gain in auditory function resulting from AAV-OTOF sufficient to result in a meaning improvement for patients?
If the variant(s) in question are proven to be loss-of-function, which I believe they are, I have no question that supplementing functional otoforlin can rescue the phenotype. Especially in mice, which are almost devoid of otoferlin. But how would this translate to human with mild HL in non-fibrile state? would this improve upon gene therapy? or is the mild HL caused by the another molecular mechanism, and only the moderate to profound HL by loss of otoferlin (due to temperature instability). I wonder in this model can answer this. For the purpose of this publication, I think the authors do not have to give a final answer, but I would strongly encourage them to discuss the descrepancy between human and mouse phenotype in more detail, and how this would affect the translation of their results.

In addition, I have some specific comments and suggestions:

1) The quality of English writing can be improved. Already in the title and abstract there are some unfortunate orders of words. And throughout the manuscript is see many more (some indicated below as minor comment).
2) The use of the term protein-truncating mutations for the DFNB9 mouse models is suggestive. As far as I know, nobody has ever shown the presence of a truncated protein. Null variant, null allele or stop-gain variant is more appropriate and describes that happens at the genetic and functional level without making unsupported assumptions about the consequences on protein level.
3) The authors should be more presence with genetic and protein nomenclature. In many situations is see mutation annotated at protein level being indicates as “in the OTOF gene”. While I can accept the name of the OtofE1799del mouse, a written statement regarding a variant in patients should be correct in nomenclature.
4) can the authors please give the product numbers from the (primary) antibodies used?
5) The anti-otoferlin immunoreactivity (blue signal) in figures 1 and 2 is overexposed to a level that it is difficult to interpret subcellular localization at the synaptic region. Can the author adjust this? And/or add detailed visualizations? The images in S2 are much better.
6) Immunohistochemistry methods section should include a brief description on how quantification was done (even if manual counting was used). Preferably including the number of cells (and slides/images) per animal.
7) Lines 358-360 “This suggests that gene therapy not only preserved the existing ribbons from degeneration but also restored additional ribbon, as previously reported in Otof null mutant mice”. In the text before this line, the authors claim to see a difference between WT and treated. Firstly, this is not visualized in the graphs, which is suggestive. Secondly, this does not match the claim of restoration.

Minor comments and typos:
Line 17 and 41: it would help the reader to indicate that you have previously show the effect of AAV OTOF delivery on LOF mice.
Line 56: Environmental (or simply remove the word). Same line: OHCs (plural asyou’re your initial abbreviation for outer hair cells)
Line 64: it is up to 80% (60-80 in the cited work, but usually around 50% in other papers)
Line 66-68: grammer not correct, and the otoferlin gene should be called OTOF
Line 69: Adeno without capital A
Line 72: truncated variants -> protein-truncating variants
Line 80: IHCs (plural)
Line 86: and can restore (instead of and restoring)
Line 88: in which in-frame -> an in-frame
Line 91: Otof E1799del mutation -> mixture of gene and protein nomenclature.
Line 92: same as above, please also consider the use of italics for gene names
Line 185: were the cochleas really perfused? If so, some details would be appreciated. We usually just submerge them in PFA…

Line 287-289: order of describing morphology and DPOEA should be reversed to match the order in figure 1
Line 287-289: data show only presence of hair cells and ribbon synapses, not that “The morphological structure of the organ of Corti in Otof E1799del/E1799del mice was normal” as the authors claim.
Line 288: did -> was
Line 301-303: rephrase, it suggests that there is no otoferlin visible, but it looks different.
Line 355-358: to which of the 4 panels do the described statistics refer: please indicate this in the text. Also, the text claims a difference between WT and treated, which is not shown in any of the graphs in panel 2E.
Line 359: restored additional ribbon synapses -> restored & additional do not go together. I would say (partially) increased the number of ribbons
Line 460: mix of gene (Otof) and protein (E1799del) nomenclature
Line 466-468: bit suggestive, as there is protein left in other regions of the cells which is not mentioned.
Line 474: progressive is not a level of severity but indicates worsening over time.
Line 487: erased -> the mutant otoferin is probably still at its original position as in the untreated mice. So nothing is erased. Supplemented WT just makes the little amount of mutant otoferlin invisible.
Lines 492-494: “Finally, our results provide key evidences that were previously
missing regarding the restoration of normal auditory perception and the impact of the
critical period (P10-P30)” The results are virtually the same at p2 and p30. The make conclusions about the impact of the critical period, it is needed to also have data from gene delivery after the critical period. Especially for OTOF, where congenital HL patients are dosed up to (at least) age of 7 in some studies.

497: misuse of the term peripheral gene therapy (see major comment above)

Reviewer #3

(Remarks to the Author)
Please see below my proposed edits to the manuscript that I have provided to the authors. I prefer to be anonymized when you send my comments to the authors. I believe my edits are fair and reasonable and won't take the authors much time should you as the editor decide to offer that opportunity.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Thank you to the authors for a comprehensive rebuttal letter to address the referee's comments & suggestions.

Reviewer #2

(Remarks to the Author)
Dear Authors,

I appreciate the improvements and clarification made to the manuscript.
I agree that you clearly show that AAV-OTOF can be beneficial for the missense variant.
Overall, improvements in auditory phenotype are strong and robust. I want to clarify my comment "Secondly, the rescue is not perfect. If the human phenotype is mild, is the gain in auditory function resulting from AAV-OTOF sufficient to result in a meaning improvement for patients?"

this related to the result of figures 5, where the effect on a functional hearing parameter was robust (but not perfect). I made me wonder if the gap was due to the specifics of the mutation, and if in patients with a milder, thermosensitve phenotype, this small gap could mean that the benefit would be limited. Perhaps even making it difficult to measure improvement on outcome measures. This will start a discussion beyond the scope of your work, that will probably only be resolved when patients with this variant receive AAV-OTOF.

Either way, your conclusions are supported by the results, so I have no additional comments that have to be addressed before publication.

Reviewer #3

(Remarks to the Author)
In the revised manuscript, the authors should provide the number of males and the number of females for each experiment since they claim in their response that there is no difference in outcome for males and females. Thus, they must know the sex of the animals in their study.

Reviewer #1 (Remarks to the Author):

The authors are contributing interesting results to a current hot-topic; that of using gene therapy to treat Otof-related hearing impairment. The AAV-treatment gives a clear result in that it prevents the mutant mice from exhibiting the expected hearing impairment. An interesting addition to the normal cochlear and brainstem assays of hearing function is the behavioural test that indicates that central auditory function is maintained and still operational after AAV treatment. The results presented here provide further experimental evidence supporting the clinical observations presented earlier this year that AAV-treatment in human sufferers with Otof mutations can help to bring forth their hearing.

Introduction. The authors have presented a rounded introduction. The statement on lines 68/69 could perhaps be tempered a little; it was shown earlier this year that gene-therapy for human Otof-based hearing loss can be effective, which the authors do later acknowledge.
The statement "However, no curative therapy for hearing loss is currently available." was removed in the revised manuscript page 3.

They discuss that 15% of DFNB9 deafness patients are "atypical" and show atypical, fluctuating deafness, such as mild-to-profound, progressive or temperature sensitive. They state that their aim is assess if AAV gene therapy could be effective in treating such patients. They generated a humanized mouse model, albeit one that does not carry the exact same human mutation, but instead carries a mutation, Otof E1799del corresponding to the human OTOF E1804del variant. The authors claim that their mouse model (the E1799del Otof mutation) mirrors the human E1804del variant linked to DFNB9, BUT the mouse does NOT appear to show either fluctuating deafness or deafness during febrile episodes. This has to raise the question of how good a model this particular mouse is for the human condition.
While the Otof E1799del mutation does not cause fluctuating deafness in the knock-in mouse model, it results in abnormal cellular distribution and likely instability of otoferlin, ultimately resulting in profound deafness. It is highly probable that a similar abnormality of otoferlin cell distribution occurs in humans, contributing to fluctuating hearing loss. We can only speculate on the differences in phenotype between humans and mice. In response to the reviewer's question, the following sentence has now been added to the discussion section on page 20: "The most plausible explanation for the absence of a temperature effect is that murine otoferlin, unlike human OTOF, lacks the arginine-rich motif responsible for the temperature-dependent expression of various membrane-associated proteins [13]." The key point we wish to emphasize is that gene therapy has demonstrated its ability to correct abnormal cell distribution, a finding not previously shown. This result is essential for expanding current clinical applications to include all otoferlin mutations, which primarily involve nonsense mutations that are generally predicted to lead to truncated protein. Of note we have clearly stated in the subtitle result section page 13 that "*Otof*$^{E1799del/E1799del}$ mice are profoundly deaf"

Ln 143. Homozygous "1779" mutants were given gene therapy as neonates (P2) or as adults (P30).
Ln 149. P15-P30 mice ... this contradicts line 143, where P30 is given as the adult age.
The ages of mice used throughout the study seems to be a little inconsistent & therefore confusing. If the authors could somehow clarify / simplify the descriptions the ages tested in different aspects of the work, it would be much appreciate. Perhaps the exact ages could be defined in the methods and given in the figure legends, but referred to in the test as neonatal, juvenile, adult, etc as appropriate?
We agree with the reviewer that this was a confusing sentence that we clarified in the revised version page 6 by the following statement: "Gene therapy was administered to two groups of *Otof*$^{E1799del/E1799del}$ mice: one group was treated during the neonatal period, before the onset of hearing (at P2), and the other group was treated in adulthood, after the onset of hearing (at P30)".

The statistical approach taken needs further consideration by the authors. The reader has to assume that all datasets were normally distributed, allowing parametric testing to be used throughout. Given the thresholds allocated to the auditory brainstem responses of the mutant mice, normality looks unlikely. It would be appropriate to give results of a normality test (eg the Shaprio-Wilk test) for each dataset and then treat them accordingly as parametric or non-parametric data.

To address the reviewer's suggestions, the following details have been incorporated into the methods section of the revised manuscript page 12 to ensure transparency.

"The normality of each dataset was tested using the D'Agostino–Pearson normality test to ensure the appropriate statistical methods were applied. According to the data structure, for two-group comparisons, we used a two-tailed Student's t-test when the data followed a normal distribution. For multiple group comparisons, we applied either a parametric one-way ANOVA followed by Tukey's post-hoc test for normally distributed data or a non-parametric Kruskal–Wallis test followed by Dunn's post-hoc test for non-normally distributed data and two-way ANOVA test followed by a Bonferroni's multiple comparison post hoc test. This approach ensures robust and appropriate statistical analysis tailored to the characteristics of each dataset."

Ln 285 - "regardless of body temperature" all homozygous mice were profoundly deaf. Only 8 mice tested. In Humans, if only 15% have atypical hearing loss, then even fewer may show the temp-sensitive hearing loss. Given the low prevalence in humans, would many more than 8 mutant mice need to be examined to see any thermo-sensitivity?

Fifteen percent of atypical DFNB9 deafness cases are caused by various non-truncating mutations, including the *OTOF E1804del* mutation.

What do the authors mean by "body temperature"? Temperature at the time of recording? If so, was this monitored & recorded. Or do they mean if the mice had previously been subjected to elevated body temperature to simulate a febrile episode? If so, has this been studied fully? They say it's an aim of the study to assess if AAV gene therapy would be effective in treating atypical DFNB9 patients, but do not appear to have tested if their mouse mutant is a good model to reproduce this part of human disease phenotype.

We agree with the reviewer's remarks and have amended the text accordingly in the methods section page 7 "Mice body temperature was monitored and maintained within physiological ranges (36-37 °C) during the time of recording."

Ln 288. Typo error? should it read ", as were the distortion product otoacoustic emissions" ??

Done

Ln 303. Should "posttranscriptional" be hypenhated?

Done

Ln 317, 330, 332 and other places through the manuscript & figures. The authors use "on" P8, etc, whereas "at" P8, etc would be better. Please could this be amended throughout?

This manuscript, like all of our articles, has been proofread and cross-edited by experts whose native language is English. The expert confirmed that 'on' is the correct preposition for this context, including references such as "on P8".

Throughout the manuscript, the authors use the word restore / restoration, etc. I wonder if this is the ideal word / phrase to use in this case. "Restore" can be defined as "to bring back to or put back into a former or original state". Since the "1799" mutant mice have never had a normally functioning Otof protein, and would never have been able to generate normal hearing, the positive effects of AAV treatment should not be termed restore / restoration of hearing in the mutants, as this would effectively mean "no change". This word & phraseology is commonly used in the literature, but I do not feel it is

strictly correct. Call me pedantic if you will! In effect what is happening is that the AAV gene therapy is giving the system an alternative gene to use which produces a normally-functioning protein. The presence of the normal protein then allows the system to work as would be expected in a "normal" wildtype animal. To me, a better word to use might be "alternate" or "replacement". However, I will leave it to the editors and authors to quibble over this suggestion.

The terms used in the field of gene therapy for deafness are "prevent deafness" when therapy is administered before the onset of hearing, and "restore hearing" or "reinstate hearing" when therapy is administered in adulthood, after the onset of hearing. We have adopted these terms to keep the explanation simple.

Ln 397. The subtitle suggests that the "1799" mutant mice have cortical deficits as a direct results of the mutation, and that addition of a normal gene / protein to the inner ear somehow induces cortical changes to allow normal processing in pre-pulse inhibition tests and the frequency discrimination tasks. Surely the authors do not believe this is the case? It is simply by reinstating normal inner function and synaptic input onto auditory nerve fibres that allows the central system to function in a normal (relative to wildtype, at least) way ? Please could the authors rephrase this title to be more appropriate?

We did not state that gene therapy causes cortical changes, but it does reestablish connections in the ascending auditory pathways that were deprived of sound stimuli for almost the entire critical period. This is a key finding of the study.

The useful of PPI has always been debatable, I understand, however, I feel that the behavioural Go/NoGo test of frequency discrimination is a very nice additional assay. I feel that is such a test could be taken up more widely by other groups, if would add more "power" to the exciting findings that are emerging in the hunt for therapeutic approaches to the treatment of genetic deafness.

We appreciate the reviewer's comment on the Go/NoGo test for frequency discrimination and thank him for highlighting this important aspect. We agree that while the utility of PPI has been debated, the Go/NoGo test provides a more direct and relevant measure of auditory discrimination capacity of the treated mice. As the reviewer notes, this test has broader potential, and if adopted by other research groups, could enhance the robustness of findings in genetic deafness therapies.

In the discussion, again the authors say that deafness phenotype of their mouse model is not affected by body temperature and compare this to a different Otof mutation which also does not mimic human thermosensitivity. Here also, they offer reasoned explanations as to why this may be the case.
I suggest that the authors should downplay the connection they make between this mouse model and the atypical fluctuating deafness associated with febrile episodes, as their "1799" mutant does NOT demonstrate this aspect of the phenotype. That said, clearly the AAV gene therapy approach is effective in allowing IHC synapses to function and bring about functional hearing, but it is an overstatement to compare their model (as with other models they mention) to that particular phenotypic aspect in humans. And they do indeed state that the AAV approach can be broadened to cover other forms of Otof mutation. It is likely that addition of the normal Otof gene and resultant normal Otof protein would help overcome the deafness of any form of mutant Otof-based deafness.

We believe that although the *Otof 1799del* mutation does not cause thermosensitive deafness in mice, OTOF protein in patients with the equivalent mutation is likely to be similarly affected, exhibiting abnormal distribution and significant reduction at IHC synapse. Our results strongly suggest that transferring a healthy copy of the human otoferlin coding sequence could potentially correct these defects and restore normal hearing in these patients.

Reviewer #2 (Remarks to the Author):

Benamer et al - Peripheral gene therapy restores hearing and central auditory processing in an atypical DFNB9 mouse model.

Following the tremendous success of OTOF gene therapy last year, the team tries to investigate if OTOF gene delivery can also be effective for non-protein-truncating variants in OTOF, such as the in-frame deletion that they investigated. These patients present with atypical DFNB9, likely caused by the presence of mutant proteins with residual function. An important question, which could have a big impact on patients carrying this variant. Especially if any of the OTOF gene therapies currently under investigation reach market approval.

The introduction is appropriate for the research, and I appreciate the level of detail in the methods section. Also, the behavioral studies to provide insights in interpretation (or processing) of auditory signal is an important and elegant addition to the work.

The mouse model presents a challenge, as the authors cannot discriminate between mutant otoferlin and AAV-delivered otoferlin. The functional readouts all indicate a therapeutic effect, and otoferlin return to the basal region of the cells. But the assays used do not prove that this is AAV-delivered otoferlin. The authors are careful in their writing about this, and I appreciate that no false claims are made (except for one line in the discussion).

Overall, the data are strongly suggestive of this approach being effective for people with this variant. And probably all variants with a loss-of-function mechanism (which can be anticipated given the recessive inheritance pattern). I think the work is very interesting and worth publishing. Few study in the field of gene therapy address the efficacy for different pathogenic variants, so in that we the study also stands out.

There are some textual and conceptual changes that I think the authors should consider to (further) improve the work before publication.

1) The title is misleading: The placing of the word "peripheral" suggests that it relates to the gene therapy. But in does not (RWM/PSCC delivery). In fact, they authors investigate if peripheral signal transduction of auditory function is conserved through behavioral assays. Great studies, but not a "peripheral gene therapy" as the title suggests.

The term 'peripheral' in the title refers to the site of gene therapy delivery to the inner ear, which is part of the peripheral auditory system. To prevent any potential confusion, we have revised the title as follows: "Cochlear gene therapy restores both hearing and auditory processing in a novel DFNB9 mouse model."

For further clarity, we have added the following sentence on page 2: "…gene therapy administered to the cochlea, which is part of the peripheral auditory system…"

Similarly, the placing of the word "atypical" suggests the mouse model itself is atypical. However, it aims to model the atypical presentation of DFNB9. In fact, the phenotype of the mouse is rather typical for DFNB9.

We agree that the phenotype of the mouse is rather typical for DFNB9. The word "atypical" is deleted throughout the revised version of the manuscript.

2) The mouse is profoundly deaf, whereas the atypical DFBN9 patients are not (otherwise they would be typical). In the introduction patients are claimed to be moderate-to-profoundly deaf, while in the discussion they are severe-to-profound. The original paper by Sandrine Marlin shows this variant to be temperature sensitive and resulting in normal hearing to mild HL at normal body temperature.

The reviewer is correct in stating that human thermosensitive mutations in *OTOF* gene can cause hearing loss, ranging from moderate to profound. We have amended the text accordingly.

This brings me to two questions:
a. Is the mouse variant more devoid of function that the orthologous human variant. Are there perhaps human-specific alternative transcripts isoforms in which this residue is not present. Or does the mouse variant (also) effect pre-mRNA splicing, resulting in a proportion of stop-gain transcripts that differs between human and mice. Could it me mouse body temperature? Is the mouse variant even temperature sensitive? This is assumed (not explicitly) but not studied.

Reviewer 1 raised the same question, to which we responded by adding the following sentence in the discussion on page 20 : "The most plausible explanation for the absence of a temperature effect is that murine otoferlin, unlike human OTOF, lacks the arginine-rich motif responsible for the temperature-dependent expression of various membrane-associated proteins[13]."

b. Secondly, the rescue is not perfect. If the human phenotype is mild, is the gain in auditory function resulting from AAV-OTOF sufficient to result in a meaning improvement for patients?
We disagree with the reviewer's comment, as the hearing thresholds restored following gene therapy is near to wild-type levels. It is important to note that patients harboring thermosensitive mutations, examined during febrile periods, exhibit high pure tone thresholds and deteriorated speech comprehension. This phenotype may worsen with exposure to sound. Our data provide evidence that gene therapy may improve the quality of life for these patients.

If the variant(s) in question are proven to be loss-of-function, which I believe they are, I have no question that supplementing functional otoforlin can rescue the phenotype. Especially in mice, which are almost devoid of otoferlin. But how would this translate to human with mild HL in non-fibrile state? would this improve upon gene therapy? or is the mild HL caused by the another molecular mechanism, and only the moderate to profound HL by loss of otoferlin (due to temperature instability). I wonder in this model can answer this. For the purpose of this publication, I think the authors do not have to give a final answer, but I would strongly encourage them to discuss the descrepancy between human and mouse phenotype in more detail, and how this would affect the translation of their results.
The most compelling argument to address the reviewer's question is that heterozygous patients with temperature-sensitive mutations, where both forms of otoferlin (mutated and wild-type) coexist in IHCs, display normal hearing. The gene therapy described in our study aims to replicate this configuration in homozygous patients with hearing loss.

In addition, I have some specific comments and suggestions:

1) The quality of English writing can be improved. Already in the title and abstract there are some unfortunate orders of words. And throughout the manuscript is see many more (some indicated below as minor comment).
The manuscript has been reviewed and corrected by field experts whose native language is English. We appreciate the reviewer for highlighting specific examples, and we have incorporated the suggestions in the revised version of the manuscript.

2) The use of the term protein-truncating mutations for the DFNB9 mouse models is suggestive. As far as I know, nobody has ever shown the presence of a truncated protein. Null variant, null allele or stop-gain variant is more appropriate and describes that happens at the genetic and functional level without making unsupported assumptions about the consequences on protein level.

We agree with the reviewer's remark and have rephrased this sentence on page 4 as: "mutations that are predicted to result in truncated forms of otoferlin."

3) The authors should be more presence with genetic and protein nomenclature. In many situations is see mutation annotated at protein level being indicates as "in the OTOF gene". While I can accept the name of the OtofE1799del mouse, a written statement regarding a variant in patients should be correct in nomenclature.

We agree with reviewer suggestion and corrected the nomenclature accordingly.

4) can the authors please give the product numbers from the (primary) antibodies used?

We have added the relevant details in the revised manuscript page 9. These product numbers and the corresponding antibody information are now included in the Methods section, specifically in the immunofluorescence paragraph.

5) The anti-otoferlin immunoreactivity (blue signal) in figures 1 and 2 is overexposed to a level that it is difficult to interpret subcellular localization at the synaptic region. Can the author adjust this? And/or add detailed visualizations? The images in S2 are much better.

Done.

6) Immunohistochemistry methods section should include a brief description on how quantification was done (even if manual counting was used). Preferably including the number of cells (and slides/images) per animal.

As suggested by the reviewer, the revised version of the Methods section page 9 now reads: "Quantification of ribbons per IHC was performed manually by counting CtBP2-positively stained cells using z-stack images acquired under a confocal microscope encompassing the entire volume of IHCs. The results were obtained from a total of 4 animals per group."

7) Lines 358-360 "This suggests that gene therapy not only preserved the existing ribbons from degeneration but also restored additional ribbon, as previously reported in Otof null mutant mice". In the text before this line, the authors claim to see a difference between WT and treated. Firstly, this is not visualized in the graphs, which is suggestive. Secondly, this does not match the claim of restoration.

In the revised version of the manuscript, we have addressed this confusion by including the observed statistical difference between the WT and treated groups.

Minor comments and typos:

We thank the reviewer for their helpful comments regarding minor issues and typos. These have been carefully addressed and corrected in the revised manuscript.

Line 17 and 41: it would help the reader to indicate that you have previously show the effect of AAV OTOF delivery on LOF mice.

We have mentioned this result in the manuscript. For clarity, we have rephrased it in the text in this revised version, page 3 the sentence now reads "Notably, we have proven in a previous work that adeno associated virus (AAV)-mediated gene replacement therapy can reverse established deafness in a mouse model that faithfully replicates the profound prelingual human

DFNB9 deafness caused by frameshift mutations that are predicted to result in truncated forms of otoferlin[6].”

Line 56: Environmental (or simply remove the word). Same line: OHCs (plural as you’re your initial abbreviation for outer hair cells).
We have made the correction.

Line 64: it is up to 80% (60-80 in the cited work, but usually around 50% in other papers).
We agree with the reviewer’s comment. In the revised version, on page 3, the sentence now reads "Genetic factors account for up to 80% of these deafness cases."

Line 66-68: grammer not correct, and the otoferlin gene should be called OTOF.
We have made the correction.

Line 69: Adeno without capital A.
Done.

Line 72: truncated variants -> protein-truncating variants.
We have made the correction.

Line 80: IHCs (plural).
Done.

Line 86: and can restore (instead of and restoring).
Done.

Line 88: in which in-frame -> an in-frame.
Done.

Line 91: Otof E1799del mutation -> mixture of gene and protein nomenclature.
Done.

Line 92: same as above, please also consider the use of italics for gene names.
Done.

Line 185: were the cochleas really perfused? If so, some details would be appreciated. We usually just submerge them in PFA….
As suggested by the reviewer we have added the details below in the revised version page 8
“Cochleas were first gently and slowly perfused with 4% paraformaldehyde in PBS through the round window until the solution emerged from small hole at the apex. They were then postfixed with 4% paraformaldehyde in PBS for 40 min at room temperature. The cochleas were processed for whole-mount dissection of the organs of Corti followed by immunofluorescence analysis.”

Line 287-289: order of describing morphology and DPOEA should be reversed to match the order in figure 1.
While we understand the rationale behind this suggestion, we have deliberately chosen to describe morphology first and DPOAE second in the text. This order allows us to build a logical narrative where the morphological findings provide context and set the stage for the functional assessment through DPOAE. We believe this sequence strengthens the impact of the DPOAE

results, as it follows naturally from the structural observations. Thus, we prefer to retain the current order in the text.

Line 287-289: data show only presence of hair cells and ribbon synapses, not that "The morphological structure of the organ of Corti in Otof E1799del/E1799del mice was normal" as the authors claim.
While our data primarily show the presence of hair cells and ribbon synapses, we also examined cells and organ of Corti morphology, confirming our previous finding showing that the organ of Corti morphology is normal in our DFNB9 mutant mice.

Line 288: did -> was.
Done.

Line 301-303: rephrase, it suggests that there is no otoferlin visible, but it looks different.
For clarity, we have rephrased this sentence, which now reads on page 14: "otoferlin was no longer expressed in the OHCs of the mutant similarly to the wild-type mouse" suggesting a normal regulation of otoferlin expression during cochlear development.

Line 355-358: to which of the 4 panels do the described statistics refer: please indicate this in the text. Also, the text claims a difference between WT and treated, which is not shown in any of the graphs in panel 2E.
We have rewritten the paragraph about the statistical difference between the WT and treated groups, which are now clearly indicated in panel 2E.
The statistical analyses have been added to the figure. In addition, we have clarified page 16 that the statistical analysis refers to the entire cochlea, and this is now explicitly mentioned in the revised text.

Line 359: restored additional ribbon synapses -> restored & additional do not go together. I would say (partially) increased the number of ribbons.
To provide a more accurate description, we have revised the text page 16 to ″significantly increased the number of ribbon synapses.″

Line 460: mix of gene (Otof) and protein (E1799del) nomenclature
Corrected

Line 466-468: bit suggestive, as there is protein left in other regions of the cells which is not mentioned.
As mentioned in our manuscript, the synaptic active zone is the only region completely lacking otoferlin.

Line 474: progressive is not a level of severity but indicates worsening over time.
We agree with the reviewer and have changed "progressive" to "mild" in the revised manuscript page 21.

Line 487: erased -> the mutant otoferin is probably still at its original position as in the untreated mice. So nothing is erased. Supplemented WT just makes the little amount of mutant otoferlin invisible.
We would like to clarify that the term "erase" was not used to describe the mutant form of the otoferlin protein itself but rather to refer to the correction of the abnormal subcellular distribution of the protein.

Lines 492-494: "Finally, our results provide key evidences that were previously

missing regarding the restoration of normal auditory perception and the impact of the critical period (P10-P30)" The results are virtually the same at p2 and p30. The make conclusions about the impact of the critical period, it is needed to also have data from gene delivery after the critical period. Especially for OTOF, where congenital HL patients are dosed up to (at least) age of 7 in some studies.

We examined a time window that is widely applicable to patients, as a P30 mouse corresponds to a child aged 2.5 to 3.5 years. During this period, cochlear implants provide good outcomes for hearing and speech perception. Similarly, for gene therapy to be most effective, it should be administered within this timeframe. Beyond this window, there is a risk of limited restoration of speech perception, even if hearing is recovered. However, determining the time limit beyond which gene therapy becomes ineffective is a fascinating question but represents an entire research project on its own.

497: misuse of the term peripheral gene therapy (see major comment above).

addressed in the 1st point

Reviewer #3 (Remarks to the Author):

The authors manuscript entitled "Peripheral gene therapy restores hearing and central auditory processing in an atypical DFNB9 mouse model" explores the phenotype of another mutant Otof mouse that recapitulates a human variant of OTOF. The gene-treated deaf Otof E1799del/E1799del mouse has a near-wild-type phenotype and restoration of normal auditory perception. In general, the manuscript is nicely crafted, and figures are professionally illustrated. As human variants of OTOF have been the first successful gene therapy in humans, another Otof mouse model indicating success of gene therapy is not novel but is important and will be of both general interest and special interests to human gene therapists.

Consider the concerns below when editing the manuscript.

The word "atypicals" on page 1 in the title and on pages 2, 4, 11, 19 and 20 is incompletely defined as to what is typical and what exactly is atypical about the human DFNB9 deafness being studied in their new mouse model.

We understand that the term "atypical" may be confusing. For us, it is considered atypical because otoferlin is present in IHCs but abnormally distributed, leading to profound deafness. To prevent any confusion, we have removed the term from the text.

Fluctuating means increasing and decreasing, usually irregularly, in quantity and quality. Fluctuating hearing loss is mentioned on pages 1 and 4 of the manuscript without describing the criterion for what "fluctuation" means precisely as the authors are using this term. Are the authors talking about temperature induced changes in hearing loss of a human OTOF variant? For example see (https://pubmed.ncbi.nlm.nih.gov/34692690/). How does the fluctuating relate to the hearing phenotype.

When we mention 'fluctuating hearing loss', we are specifically referring to changes in hearing thresholds that occur when the affected patient's body temperature changes, as correctly points out by the citation and reviewer. To provide additional clarity, we have included the following sentence on page 4"…in about 15% of cases, pathogenic otoferlin mutations lead to DFNB9 deafness with varying levels of severity, such as mild-to-profound, progressive, or temperature-sensitive...".

Line 40, The authors claim, as do many others in numerous reports, that “Deafness is the most prevalent inherited sensory disorders in humans.” Is that true? Color blindness is present in 1 in 12 men and 1 in 200 women. Is color blindness not a sensory disorder or has the authors forgotten about the prevalence of color blindness?

We modified the sentence page 2 to say that “Deafness is one of the most prevalent inherited sensory disorders in humans”.

Line 90, the authors state, “We created a humanized mouse model by introducing into the mouse genome the Otof E1799del mutation (Otof E1799del/E1799del) corresponding to the human OTOF E1804del variant.” All that was engineered in the mouse was to introduce an amino acid substitution that was previously reported for human OTOF. The author’s statement quoted above about a humanized mouse is an exaggeration, at the very least. Introducing a point mutation in one mouse gene does not humanize a mouse and certainly doesn’t conform to what is meant by “humanized mouse” by the JAX or in an example paper on this topic (S. Fujiwara, J Cell Physiol doi: 10.1002/jcp.26022, Humanized mice: A brief overview on their diverse applications in biomedical research). Delete “humanized in the manuscript in reference to your Otof mouse.

We used the term "humanized" to indicate that the mouse model carries the *Otof E1799del* mutation, which corresponds to the human *OTOF E1804del* variant. However, we understand that the term "humanized" is more commonly used to describe models involving the introduction of larger portions of human DNA or entire human genes. To avoid any confusion, we revised the manuscript to reflect a more accurate description of our model and removed the term humanized.

Line 190, Indicate how each antibody [rabbit anti-otoferlin (1:200), mouse anti-CTBP2 (1:100), and/or anti-myosin 7a-biotin 1:50] used in the study was validated as to specificity. If there is a reference to a study where the antibody was validated (i.e. same antibody, same source and same epitope), provide specifically how each antibody used in your study was validated and relevant references. Indicate the source of the antibody with catalog numbers and company names. If any of the antibodies used in the author’s manuscript were not validated, indicate why a reader should assume the antisera is specific, not multispecific and not nonspecific.

The specificity of the antibodies used in this study was established over 20 years ago. Moreover, whenever there was any doubt, we systematically included positive controls and verified the absence of nonspecific signals in the corresponding KO mouse. We have now included these references and catalog numbers in the revised manuscript page 9.

Provide the catalog number and company for all reagents and equipment. For example, this reviewer can’t find information online for the Audiobox, TSE, Bad Homburg, Germany mentioned on line 236. Provide the URL and item number for the Audiobox (TSE, Bad Homburg, Germany) and do this for all items of equipment and reagents mentioned in the manuscript. Please think about making it easier for another investigator to replicate your study.

We agree with the reviewer remarks and we have revised the manuscript accordingly. In the method section we provided detailed information about reagents and equipment to facilitate reproducibility page 11.

In this regard, state explicitly and pledge that once published, the communicating author will make their engineered Otof mouse available without requiring co-authorship for requests for the mouse or requirements other than a usual Material Transfer Agreement or equivalent. One of the coauthors on this manuscript has a long-standing reputation of withholding published resources from some appropriate young scientists despite a journal’s guidelines for sharing published resources without entangled requirements.

We have consistently made our published mouse models accessible to researchers around the world, viewing this as a responsibility to advance basic research. We are committed to promote transparency about our model and ensure this resource can be broadly used to advance the field.

Line 235-236, Overall, there are missing details in the Methods section that authors need to provide. For example, the authors' state "When the mouse "nosepoked" during the NoGo tone, it received an aversive air puff." Elsewhere it says, "a light puff of air". What exactly was the chemical(s) in the "aversive air puff"?

The 'aversive air puff' mentioned in the manuscript refers to a gentle puff of air, with no chemicals involved. It was used as a non-chemical, physical aversive stimulus during the Go/NoGo task. We have revised the manuscript accordingly in the Methods section page 11 to clarify that the air puff was solely a physical stimulus.

Line 283, The authors state, "At one month of age, Otof +/E1799del mice (n = 7)." How many males and females among the seven mice? Indicate male and female numbers for ALL mice used and add this information either in the legends or better yet in each figure.

We have never observed any differences in gene therapy outcome between males and females in the four otoferlin models we have created and studied extensively to date.

In the "Author Contributions" section line 595, there is a statement that "C.P., M-J.L., Y.N. reviewed the manuscript". Does reviewing a manuscript warrant co-authorship? Does reviewing meet the Pasteur Institut's minimum criteria for co-authorship. What is the journal's view about justification for co-authorship for just reviewing a manuscript, assuming the journal has one?

C. P., M-J L., and Y. N. devoted time to offer constructive and frequent feedback, which was crucial to the success of the project. In addition, Y. N., as an ENT specialist, provided valuable tips to improve the surgical approach. We believe their involvement justifies co-authorship, in line with the Pasteur Institute's criteria. We have updated the 'Author Contributions' section page 26 to clarify their roles.

Is the methodology for AAV delivery to the inner ear as described in the Methods section of the manuscript being reported for the first time? Or is there a published study or studies that describes a similar or the same method that the authors used but haven't cited? If so, reference the published study or studies.

This is an approach we have been using and publishing for a long time, including peer-reviewed articles published in JCI, JCI insight, PNAS, and iScience. We revised the manuscript method part page 6 to include relevant references that describe similar methods.

The title of Figure 3 is "AAV gene therapy in Otof E1799del/E1799del mice rescues IHC Ca2+-dependent exocytosis and durably restores hearing function." The authors only followed the hearing ability of treated mice for a few months. That's good. But are a few months of corrected function really "durable" restoration of hearing? Seems like an exaggeration. Consider removing the word "durable" or replace it with the length of time the mice were actually checked for hearing ability, so a reader doesn't misinterpret the data and think the mouse will be hearing for the remainder of its life.

We agree that the term 'durable' might be misleading, as the hearing ability of treated mice was monitored until 4 months. However, four months is considered an advanced age for a mouse, where restored hearing remains stable following a single administration, suggesting that the hearing recovery is long-lasting. However, to stay consistent with our results, we removed 'long-lasting' in the title of Figure 3 page 16.

Figure S1 panel B, for the gel, show and label the molecular weight standards on either side of the experimental bands.

We did update Figure S1 panel B to include and label the molecular weight standards on either side of the experimental bands, as recommended.