GFI1 Functions to Repress Neuronal Gene Expression in the Developing Inner Ear Hair Cells

Maggie S. Matern, Beatrice Milon, Erika L. Lipford, Mark McMurray, Yoko Ogawa, Andrew Tkaczuk, Yang Song, Ran Elkon and Ronna Hertzano
DOI: 10.1242/dev.186015

Editor: Paola Arlotta

Review timeline

Original submission:	11 November 2019
Editorial decision:	11 February 2020
Rebuttal received:	18 March 2020
Editorial decision:	18 May 2020
First revision received:	2 June 2020
Accepted:	24 July 2020

Original submission

First decision letter

MS ID#: DEVELOP/2019/186015

MS TITLE: GFI1 Functions to Repress Neuronal Gene Expression in the Developing Inner Ear Hair Cells

AUTHORS: Maggie S Matern, Beatrice Milon, Erika L Lipford, Mark McMurray, Yoko Ogawa, Andrew Tkaczuk, Yang Song, Ran Elkon, and Ronna Hertzano

Dear Dr. Hertzano,

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see from their reports, the referees recognise the potential of your work, but they also raise significant concerns about it. Given the nature of these concerns, I am afraid I have little choice other than to reject the paper at this stage.

However, having evaluated the paper, I do recognise the potential importance of this work. I would therefore be prepared to consider as a new submission an extension of this study that contains new experiments, data and discussions and that address fully the major concerns of the referees. The work required goes beyond a standard revision of the paper. Please bear in mind that the referees (who may be different from the present reviewers) will assess the novelty of your work in the context of all previous publications, including those published between now and the time of resubmission.

If you decide to resubmit, please go to BenchPress and click on the 'Submit a new manuscript' link within the Author Area.

Please ensure that you click the 'This is a resubmission' checkbox, and enter the manuscript identification number shown above. I would also ask you to provide in the cover letter an

explanation of they key ways in which the manuscript differs from the current submission, followed by a point-by-point response to the referees' concerns.

We do understand that the work entailed in a potential new submission is significant, and that you may prefer to submit elsewhere without further delay. Please do let us know if you decide not to resubmit to Development, so that we can close our file.

Many thanks for sending your work to Development.

Reviewer 1

Advance summary and potential significance to field

The paper by Matern and colleagues entitled “GFI1 functions to repress neuronal gene expression in the developing inner ear hair cells” analyses the function of GFI1 in developing hair cells by analysing the transcriptional changes upon GFI1 deletion.
The main novel conclusion of the authors is that Gfi1 represses a neuronal program present in early differentiating hair cells.

The manuscript is based on the analysis of the hair cell translatome using the RiboTag technique. Changes in gene expression are identified by RNA-seq and then some differentially expressed mRNA analysed by ISH and immunohistochemistry. Their gene expression analysis leads them to conclude that Gfi1 represses a neuronal program since several neuronal genes are overexpressed in cochlear hair cells (NeuroD, Lhx2, St18, tubb3).
The changes in gene expression leads to hair cell death and hair cell degeneration mainly in Outer Hair Cells. Inner Hair Cells appear more immature, while vestibular hair cells are mainly affected at the stereociliary bundles. The phenotype seems stronger in cochlea that vestibular regions (differently than the Gfi1 KO).

While the experiment is well designed and well performed, their conclusions are overstated and their final conclusion that Gfi1 represses a neuronal program is pushed too far without full demonstration. Several neuronal genes are upregulated but its relevance is not clear. No ectopic neurons seem to be generated in the epithelium, the upregulation might be highly transient or represent an immature hair cell state more than a neuronal program.

Their major conclusion for a repression of a neuronal program comes from the increase of NeuroD, Insm1, Lhx2, St18 in the cochlea (St18 and Gfy also in vestibule)

NeuroD is essential for otic neuron development, but it has been shown to be expressed also in developing Hair cells. Atoh1 induces NeuroD in HC and NeuroD is then needed to repress Atoh1, loss of NeuroD not only affects otic neurons but also alters the fate from OHC to IHC
(Jahan et al., 2010, Pan et al., 2012). Thus, NeuroD is not only a marker of neuronal fate.

A similar phenomena happens with Insm1 which is not only a neuronal gene but also regulates outer hair cell fates. Loss of Insm1 changes the fate of OHC to Inner Hair Cells
(Wiwatpanit et al. 2018). Therefore, is incorrect to tag Insm1 and NeuroD as neuronal genes.

For Lhx2, although is expressed in the central nervous system and olfactory placode, there are no evidences that is expressed in the inner ear not in otic sensory neurons. Lhx2 is also expressed in other tissues outside the neurons (hair follicle, limb, pancreas), thus it cannot be considered an exclusive neuronal transcription factor.

St18, has also been associated with apoptosis as an proapoptotic factor, thus it might be upregulated upon apoptotic induction after GFI1 deletion.

To further validate the upregulation of some of the genes found by RNa-seq, the authors did ISH. Upregulation of Lhx2 is unconclusive from the images, since the background is higher in the Gfi1cre/cre and hair cells always accumulate more background as they are more superficial.

Downregulation of Sema5b is also unconclusive since in the still present HCs, staining seems quite similar in both genotypes.

The staining of POU4f1 in HC is not clear as the background is strong. Being a transcription factor, only nuclear staining should be observed.

The upregulation of DCX, is shown as a clear demonstration of a neuronal program activation. The staining is observed in neuronal fibers innervating the hair cells. In the mutant of GFi1, increased axogenesis is observable. The staining in the HC area seems to be growing neurites rather than specific HC staining. The same seems to happen for tubb3. These immunostainings must be shown at high magnification and in separate channels. In addition mRNA expression must be shown in hair cells cytoplasm to be convincing.

Overall, while the downregulation of HC differentiation genes is more clear, the upregulation of a specific neuronal program is not evident and can be also explained by a HC differentiation arrest and immaturity of HC.

Finally, the authors suggest that GFI1 might be doing different functions in the cochlea and vestibule due to the different effects on the regulation of the neuronal program. However since the vestibular phenotype is less penetrant in the Gfi1 cre/cre mouse model than the Gfi1-/- , these differences might be that the arrest of differentiation is stronger in the cochlea than in the vestibular regions in their model. The authors they should analyse changes of gene expression at later timepoints (further away than PO for the vestibular patches.

Comments for the author

While the main experiment and RNa-seq analysis is well designed and well performed, their final conclusion that Gfi1 represses a neuronal program is pushed too far without full demonstration. Several neuronal genes are upregulated but its relevance is not clear. No ectopic neurons seem to be generated in the epithelium, the upregulation might be highly transient or/and represent an immature hair cell state more than a neuronal program.

Their major conclusion for a repression of a neuronal program comes from the upregulation of NeuroD, Insm1, Lhx2, St18 in the cochlea (St18 and Gfy also in vestibule)

NeuroD is essential for otic neuron development, but it has been shown to be expressed also in developing Hair cells. Atoh1 induces NeuroD in HC and NeuroD is then needed to repress Atoh1, loss of NeuroD not only affects otic neurons but also alters the fate from OHC to IHC (Jahan et al., 2010, Pan et al., 2012). Thus, NeuroD is not only a marker of neuronal fate.

A similar phenomena happens with Insm1 which is not only a neuronal gene but also regulates outer hair cell fates. Loss of Insm1 changes the fate of OHC to Inner Hair Cells (Wiwatpanit et al. 2018). Therefore, is incorrect to tag Insm1 and NeuroD as neuronal genes.

For Lhx2, although is expressed in the central nervous system and olfactory placode, there are no evidences that is expressed in the inner ear not in otic sensory neurons. Lhx2 is also expressed in other tissues outside the neurons (hair follicle, limb, pancreas), thus it cannot be considered an exclusive neuronal transcription factor.

St18, has also been associated with apoptosis as an proapoptotic factor, thus it might be upregulated upon apoptotic induction after GFI1 deletion.

To further validate the upregulation of some of the genes found by RNA-seq, the authors performed ISH.
Upregulation of Lhx2 is unconclusive from the images, since the background is higher in the Gfi1 cre/cre and hair cells always accumulate more background as they are more superficial.
Downregulation of Sema5b is also unconclusive since in the still present HCs, staining seems quite similar in both genotypes.

The immunostaining of POU4f1 in HC is not clear as the background is strong. Being a transcription factor, only nuclear staining should be observed.

The upregulation of DCX, is shown as a clear demonstration of a neuronal program activation. The staining is observed in neuronal fibers innervating the hair cells. In the mutant of GFi1, increased axogenesis is detected. The staining in the HC area seems to be growing neurites rather than specific HC staining. The same seems to happen for tubb3. Thus, the upregulation in HC is not clear but instead an axongenesis phenotype seems to be present.
For better conclusions, immunostainings must be shown at high magnification and in separate channels. In addition, mRNA expression must be shown in hair cells cytoplasm to be convincing.

Overall, while the downregulation of HC differentiation genes is more clear, the upregulation of a specific neuronal program is not evident and can be also explained by a HC differentiation arrest and immaturity of HC. In overall, the results are not very striking since the mutant phenotypes and HC differentiation arrest after GFI1 loss were already published (Wallis et al., 2003).

Finally, the authors suggest that GFI1 might be doing different functions in the cochlea and vestibule due to the different effects on the regulation of the neuronal program. However since the vestibular phenotype is less penetrant in the Gfi1 cre/cre mouse model than the Gfi1-/- , these differences might be that the arrest of differentiation is stronger in the cochlea than in the vestibular regions in their model. The authors they should analyse changes of gene expression at later time-points (further away than PO for the vestibular patches.

Reviewer 2

Advance summary and potential significance to field

This short-format manuscript by the Hertzano lab reports that in cochlear hair cells, the Gfi1 transcriptional repressor plays a key role in repressing a neuronal fate, allowing for the upregulation of key hair cell differentiation genes. Ribosome pull-downs from Gfi1-cre/cre; RiboTag cochlear tissues were used to identify differentially expressed “translatomes” compared to Gfi1-cre/+. Most notable is a very large increase in expression of Neurod1 and St18 in response to the loss of Gfi1. Data are provided to show that the Neurod1 basic helix-loop-helix transcription factor is normally transcribed early during hair cell development (E16.5), but is downregulated by the day of birth, in cochlear hair cells. As a result of prolonged expression of Neurod1, other neuronal genes remain expressed by cochlear hair cells. There appear to be some key differences in the genes affected in inner hair cells versus outer hair cells. Moreover, in vestibular hair cells, a different (and smaller) set of genes are mis-regulated in response to the loss of Gfi1. These findings provide some important context to our understanding not only of hair cell development (inners vs. outers; auditory vs. vestibular), but also adds to an understanding the evolution of the hair cell/otic sensory ganglion neuron dichotomy. Previous studies that varied expression patterns during inner ear development of the hair cell transcription factor, Atoh1, and the neuronal factor, Neurod1, reported the induction of ectopic hair cells in the otic sensory ganglion. Here, we have something like the opposite effect, whereby there is ectopic expression of some neuronal proteins (like Tubb3; also shown by Wallis et al., (2003) for the Gfi1-null mouse) in cochlear hair cells, due to an absence of Gfi1 in those hair cells. This paper will be influential in the hair cell field, and may have broader interest to those who study neuronal fate specification, as well as the evolution of sensory organs. The manuscript is well-written, well-illustrated, and succinct.

Comments for the author

Minor comments:

1. Figure 4. This is a difficult figure to understand and could be improved by adding important details like keys for the panels, and improving the figure legend description. Please state for panels A-B what the “BG” abbreviation means when this panel is first reference in the legend. Please consider to use a key to describe the difference represented by the grey vs. white bars (white = BG = controls?). How do the two “BG” bar & whisker boxes differ from each other? For

panels C-D, please clearly identify the color coding scheme for the green vs. purple lines/symbols, ideally using a key on the figures.

2. The ectopic expression in Gfi1-Cre/Cre hair cells, of the neuronal marker DCX, is shown in a series of panels in Figure S3 and also in Figure 3G/H. In general., these images could benefit by adding insets to show the hair cell expression, which is generally very weak in comparison to the neuronal expression, and thus difficult to appreciate at these lower power images. Higher powers should be provided both for the control (Gfi1-cre/+) and null (Gfi1-cre/cre) hair cells, to emphasize that this weak labeling is indeed a reliable and detectable difference.

3. The authors have previously shown that the Gfi1-cre heterozygotic mice show age-related progressive hearing loss (Matern et al., 2017). It is these hets, carrying the RiboTag allele, that were used as the controls for the transcriptomic analysis. They reported the Gfi1 expression of the altered exons was reduced about 30-40% in postnatal day 8 heterozygote cochleas. A further 4 cochlear genes, all on the Y-chromosome (so probably an artifact of the genders chosen) passed their criterion for differential expression in the Gfi1-Cre/+ mice compared to wildtype. This potential caveat (that there could be subtle changes in Gfi1 transcript levels in the “controls”) should be explicitly stated. Also, the Metern et al. (2017) also found that cochlear blood cells, particularly macrophages, were expressing Cre in the Gfi1-cre mice. Again, just a mention of this would be helpful.

4. Figure 1H. A single TUNEL-positive cell is offered as evidence that outer hair cells are lost through a mechanism of apoptosis. Some indication of how many such cells were observed, across some reasonable number of sections, across the 3 animals examined, should be stated.

Reviewer 3

Advance summary and potential significance to field

In the paper by Hertzano et al., authors utilized the Gfi1cre;RiboTag mice to evaluate changes to the hair cell translatome in the absence of Gfi1.

The authors report downregulation of hair cell differentiation genes, concomitant with a robust upregulation of neuronal genes in the Gfi1 deficient hair cells. This includes increased expression of the canonical neuronal transcription factors Neurod1, Lhx2, St18, Pou4f1 and Insm1 in the cochlea, and Atoh1 and St18 in the vestibular system, respectively.

Comments for the author

It has been previously shown that the transcription factor Gfi1 is key to the development of both the clochlea and the vestibular system; in the Gfi1-/- mouse inner ears, the cochlear outer HCs (OHCs) and the inner HCs (IHCs) degenerate in a basal to apical gradient and also display disorganized vestibular HCs resulting in both auditory and vestibular 45 dysfunction (Fiolka et al., 2006; Wallis et al., 2003).

In this paper the authors used as a model the Gfi1Cre mouse which is commonly used for conditional hair cell-specific gene deletion/reporter gene activation in the inner ear. In the Gfi1cre knock-in mouse exons 1-5 of Gfi1 have been replaced with a construct that encodes for Cre recombinase. This mouse model has been used in the present work and by other researchers to drive the expression of Cre to hair cells and also to study the function of Gfi1 since this mouse lack Gfi1 expression.
In the use of this model resides one of the problems of this paper. In a recent report by the same group (even the same first author) using immunofluorescence and flow cytometry, it was shown that the Gfi1Cre mice produce a pattern of recombination that is not strictly limited to hair cells within the inner ear. The authors reported "…a broad expression of Cre recombinase in the Gfi1Cre mouse neonatal inner ear primarily in inner ear resident macrophages, which outnumber the hair cells."(Matern, 2017). Moreover, the authors reported that "…we also observe broad recombination in other cells throughout the inner ear resulting in the Cre-expressing HCs being outnumbered by Cre-expressing non-HCs."(Matern, et al., 2017).

In this paper the authors used the RiboTag mouse that allows for Cre dependent expression of hemagglutinin-tagged (HA) in ribosomes that can then be used to immunoprecipitate actively translated mRNA from cell types of interest (Sanz et al., 2009). So, if cre is expressed in HC and non-HC and also HA is expressed in these two cell populations, authors are analyzing other cell types in addition to HCs. Or HA is differentially expressed in HC vs other cell types expressing cre?. This shoud be clarified. In the first results section subtitled "Enrichment of the inner ear HC translatomes using RiboTag" the authors show differences in expression in the inmunoprocipitated sample vs the input sample (IP vs IN in Fig 2) of a HC marker Myo6 and the mesenchymal-expressed gene Pou3f4. However, these results show only enrichment of HC, it is not clear that there are no other cell types in the IP sample analyzed and this should be demonstrated to really say that they are analyzing the HC translatome as read the title of the manuscript.
Since they previously showed that Cre recombinase expressing non-HCs are primarily immune cells they should use a immune cell marker as they already used to characterize the cre expressing cells. I have observed some immune cells markers like CD24a and CD163 being upregulated in the list of genes obtained by RNAseq (Dataset S1).

The second problem with the mouse model and the experimental design is the use of the control mice. Thus, in this paper the translatome (RNAseq) comparison is performed between Gfi1cre/+;Rpl22HA/HA and Gfi1cre/cre;Rpl22HA/HA. In the previous report by Matern et al 2017 that characterizes the Gfi1cre model it was shown that the heterozygous mice also display a phenotype; in fact Gfi1cre/+ heterozygotes (that have one functional copy of Gfi1), display an early onset progressive hearing loss. In this previous report the authors also analyzed if some differences in expression are observed in the heterozygous mice.
Thus, to determine if the Gfi1Cre/+ mice have statistically significant changes in cochlear gene expression that could contribute to their hearing loss phenotype, the authors extracted RNA from cochlear ducts of eight day old (P8) Gfi1Cre/+ and Gfi1+/+ mice and measured gene expression using RNA-seq. The authors showed that HC-enriched transcripts were found to be overall slightly downregulated in Gfi1Cre heterozygotes (Matern et al 2017).
They also found that functional Gfi1 transcript abundance measured in newborn Gfi1Cre/+ cochleae by RT-qPCR was decreased by 30–40%, consistent with a possible gene-dosage effect (Matern et al 2017).
Thus, in this paper it is necessary to show how expression is affected in heterozygous mice in comparison to wild type mice Gf1+/+ at the developmental stage analyzed. It is noticeable that even though this heterozygous mice has a demonstrated phenotype and also the previous results obtained by the same authors at P8 showed that gene expression is altered in the used control mice, these facts are not mentioned or discussed at any place of the manuscript.

Minor points

1- Some important experimental details are not described with enough detail in the Methods section:
a-what is a biological replicate in this design for RNAseq one cochlea or two cochleae per mice?
b- In Figure 3, A to F panels looks like IHQ and ISH have been performed in the same slice. If this is true the protocol to perform IHQ after ISH in the same sample is not described.

References

Matern, M., Vijayakumar, S., Margulies, Z., Milon, B., Song, Y., Elkon, R., Zhang, X., Jones, S. M. and Hertzano, R. (2017). Gfi1Cre mice have early onset progressive hearing loss and induce recombination in numerous inner ear non-hair cells. Sci. Rep. 7, 42079.

Fiolka, K., Hertzano, R., Vassen, L., Zeng, H., Hermesh, O., Avraham, K. B., Duhrsen, U. and Moroy, T. (2006). Gfi1 and Gfi1b act equivalently in haematopoiesis, but have distinct, non-overlapping functions in inner ear development. EMBO Rep. 2006 Mar;7(3):326-33.

Wallis, D., Hamblen, M., Zhou, Y., Venken, K. J. T., Schumacher, A., Grimes, H. L., Zoghbi, H. Y., Orkin, S. H. and Bellen, H. J. (2003). The zinc finger transcription factor Gfi1, implicated in lymphomagenesis, is required for inner ear hair cell differentiation and survival. Development 130, 221–232.

Sanz, E., Yang, L., Su, T., Morris, D. R., Mcknight, G. S. and Amieux, P. S. (2009). Cell-type-specific isolation of ribosome-associated mRNA from complex tissues. PNAS 106, 13939–13944.

Reviewer 4

Advance summary and potential significance to field

The manuscript by Matern and colleagues represents a carefully detailed effort to identify the translatome of developing hair cells and thereby establish the transcriptional targets of Gfi1 which is required for hair cell differentiation and OHC survival. It is a careful and thorough technical approach that demonstrates the strengths of the ribosome precipitation technique and enhanced sensitivity compared to bulk sequencing approaches.
These experimental observations are consistent with those from others of transient neural gene expression in developing hair cells, and that hair cell maturation requires this program to be repressed. Unfortunately, the study does not shed light on why transient expression of these genes might be necessary nor do the translatome analyses reveal why outer hair cells die in Gfi mutant. Nonetheless, if published as a resources/techniques piece it is a valuable contribution to the inner ear research field.

Comments for the author

Items that would improve the manuscript if addressed upon resubmission:
(1) The organization of Figure 2 is confusing. Foremost, the pairwise comparisons that are made in 2D are not obvious, and at first glance the data
(including legends) appear the same as that presented in 2B and 2C. The text describing this section (lines 94 – 98) could also be improved to better describe the pairwise comparisons that are being made and to distinguish this analysis form 2B and 2C. In addition, the red highlighted graphs in 2B&2C easily confused with the graph in 2D.

(2) It is unclear what is meant by ‘disorganized vestibular hair cells’
when describing the GFi mutant phenotype. This warrants a clearer description in the text or better examples in 1F.

(3) It is unclear what is different between ‘dysregulated gene expression’ (fig.2i)versus genes that are upregulated or downregulated in the Gfi mutant (fig.2j,k).

(4) Difference between purple and green lines in Fig.4 are not clearly defined in the text or legend.

(5) The Gfi mutant phenotype resembles that of Emx2 mutants because in both lines the OHCs are lost and IHC tend to cluster in pairs. It would be interesting to know if these transcription factors regulate similar targets or if Emx2 is required for Gfi1 expression. This does not warrant new experimentation but might be a worthwhile talking point for the discussion.

Author rebuttal letter

Thank you for serving as the editor for our manuscript. We read the reviewers’ critiques and believe that several fundamental misunderstandings led to the major comments of reviewers 1 and 3 leading to the decision to reject the manuscript. In particular:

Reviewer 1 felt that (**a**) our conclusion that GFI1 represses a neuronal program in hair cells is too strong; that (**b**) some of the tissue validation were inconclusive; and that (**c**) the vestibular phenotype of our GFI1-Cre mutant is not identical to the original GFI1 published knockouts. We show, unequivocally, that neuronal-expressed genes are upregulated in the GFI1 mutant hair cells

as discussed below. Thus, we think that much of the Reviewer 1 comments were driven from semantic differences that are easily resolvable. With regards to the tissue validation, we provide additional figures as well as separated channels, and also point to validation by real time PCR. Below we also show that the vestibular phenotype of our GFI1 mutant and the two published GFI1 mutants (KO and KI-GFP, both of which were also characterized by the last author) are identical. We do not know why the reviewer thought that this is not the case, or that the Cre-expression is not fully penetrant. We also previously published data from vestibular systems of Gfi1-Cre;Ai14 showing that all vestibular hair cells in the Gfi1-Cre mice express the Cre-recombinase instead of GFI1[1].

1. Reviewer 3 was concerned that (**a**) the GFI1-het hair cells may have a different pattern of expression compared with wild type hair cells, and (**b**) that the additional recombination of GFI1 in immune cells may cause a systematic bias in our results. We previously published a comparison of gene expression of hair cells between the GFI1 heterozygous and wild type animals, which shows that no significant differences are detected in any one hair cell-expressed gene between the Gfi1-het and wild type ears, and that when considering all cochlear expressed genes – only 4 genes were differentially expressed, all of which are encoded on the Y chromosome and reflect the differences in the number of male and females in our samples[1].Thus, the use of heterozygous mice as controls in our experiment is both appropriate and necessary (to drive the Cre-recombinase expression). Finally, we provide additional analyses to relieve the reviewer's second concern.

Therefore, several minor modifications to the manuscript should fully satisfy the reviewers. Taken together, we believe that this short and focused report, looking at the immediate molecular consequences of loss of expression of the critical hair cell-fate gene, GFI1, *in vivo*, will make a significant addition to the literature and will have a significant impact. I hope that the letter below along with our clarifications would lead to an opportunity to revise and resubmit our manuscript to *Development*.

Detailed response to the reviewers' comments:

Reviewer 1 – Overall, Reviewer 1 was impressed by the appropriate study design and analyses but brought up three concerns all of which we can address as listed below:

Major comment 1 – difference in opinion about the overall interpretation of the hair cell gene expression data. The Reviewer points out that most of the canonical neuronal genes that we identified as upregulated in our dataset are also expressed either in early developing hair cells (e.g., Neurod1 and Insm1), other tissues (Lhx2) or other cell states (St18). We agree with the Reviewer that (**a**) some of the neuronal genes are also expressed in nascent hair cells (a finding we stress as part of the manuscript, see multiple components of **Figure 4**) and that (**b**) genes expressed in neurons are not necessarily exclusively neuronal. However, we would like to point out that (**a**) neuronal genes and programs are strongly statistically over-represented in the upregulated genes in our dataset (see **Figure 2G,H** shown also below); (**b**) as shown in **Figure 4F**, genes that we discovered as repressed by GFI1 in hair cells, as a group, are induced in experiments of neurodifferentiation of mESCs towards neuronal fate by overexpression of Neurod1; and (**c**) a direct role for GFI1 in repressing these 'neuronal genes' is also supported by a recent paper from Lee et al., 20192. In this 2019 manuscript, mis-expressed GFI1 was shown to "inhibit genes involved in neuronal commitment and differentiation" in neuronal precursors via cooperation with the GFI1 cofactor LSD1[2]. ***Taken together***, we propose to change the conclusion from "a neuronal program fails to be repressed without Gfi1" to: "an early hair cell program, which is similar to a neuronal program, fails to be repressed without Gfi1".

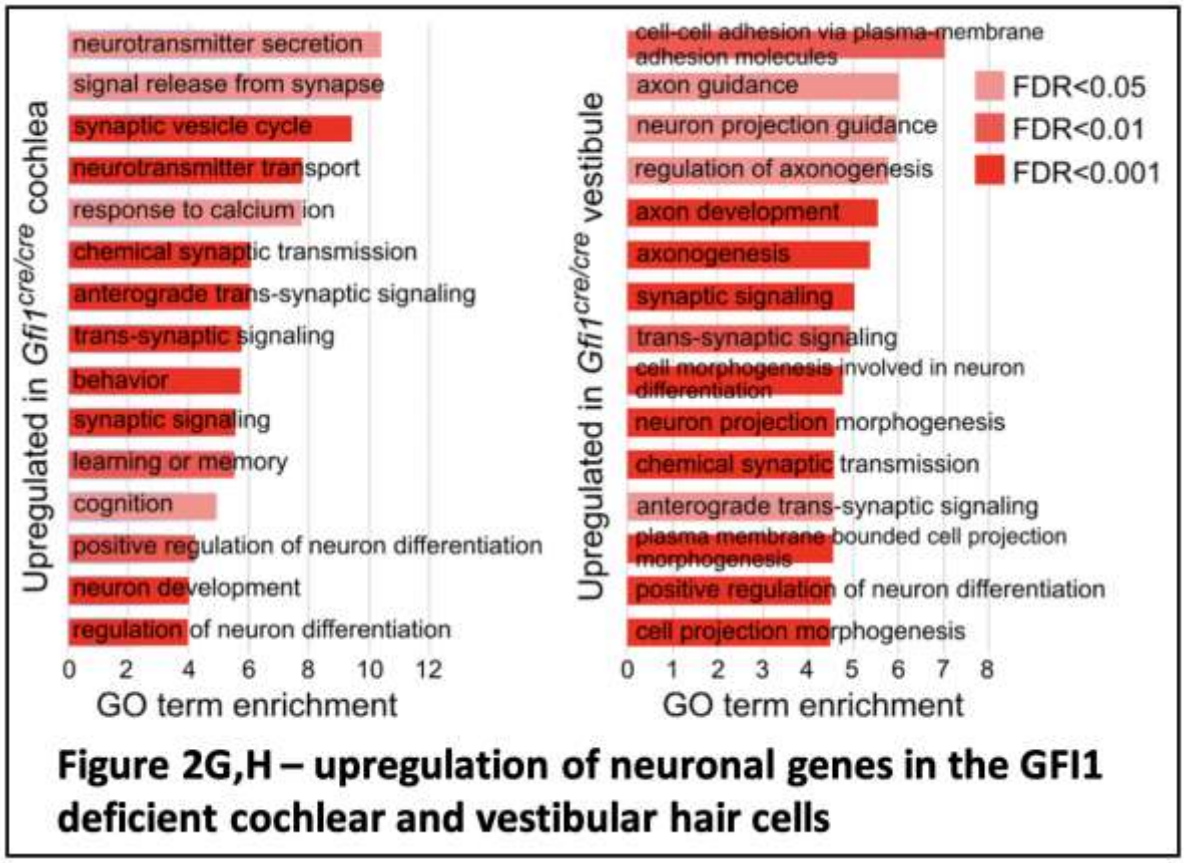


Figure 2G,H – upregulation of neuronal genes in the GFI1 deficient cochlear and vestibular hair cells

Major comment 2 – inconclusive appearance of in situ staining for two genes (Sema5b and Lhx2) and one of the immunohistochemistry staining for one protein (POU4F1). While in situ hybridization as well as immunohistochemistry can be sometimes challenging, we respectfully disagree with at least some of the criticism and would like to point out the figures in question. Downregulation of Sema5b is shown in **Figure 3B** (also shown here): while the outer hair cells are dying, they still express the hair cell marker MYO6 and a near complete loss of expression of **Sema5b** is easily observed in the mutant hair cells. With regards to **Lhx2** upregulation, we agree that the in situ staining is relatively faint and can easily provide new images to support it. Finally, while the POU4F1 staining has some

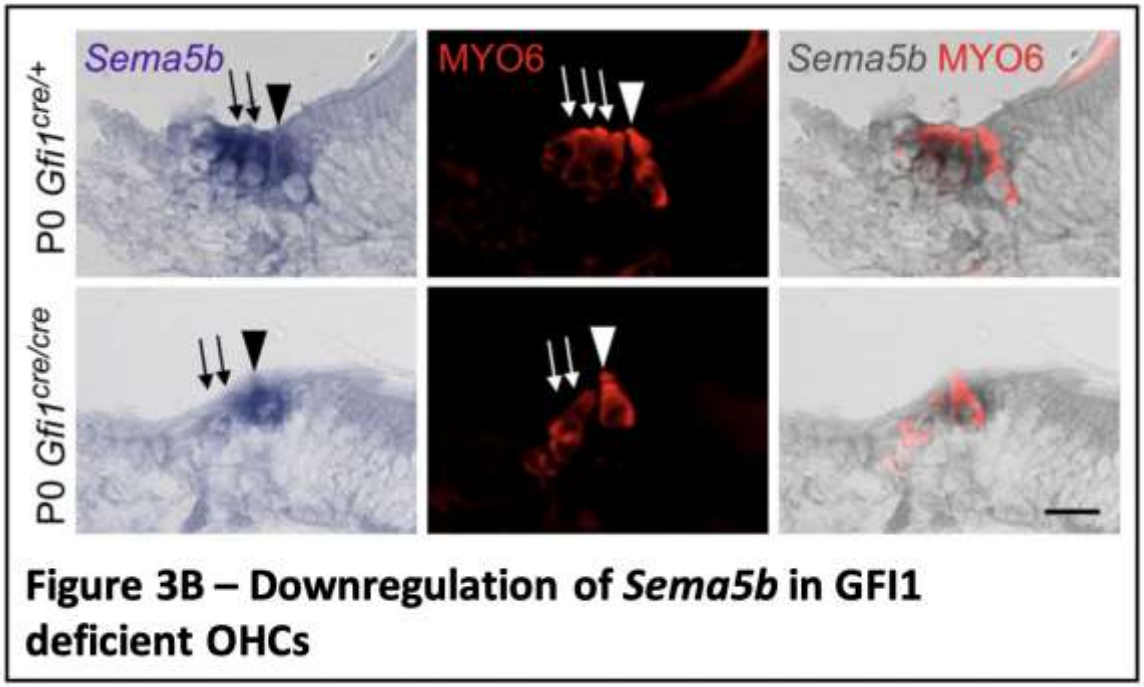


Figure 3B – Downregulation of *Sema5b* in GFI1 deficient OHCs

background, as show in **Figure 3F'** (see below), a nuclear staining which is not present in the wild type hair cells is clearly and strongly present in the mutant hair cells. We propose to add asterisks to point out the nuclear staining in the revised version of the manuscript.

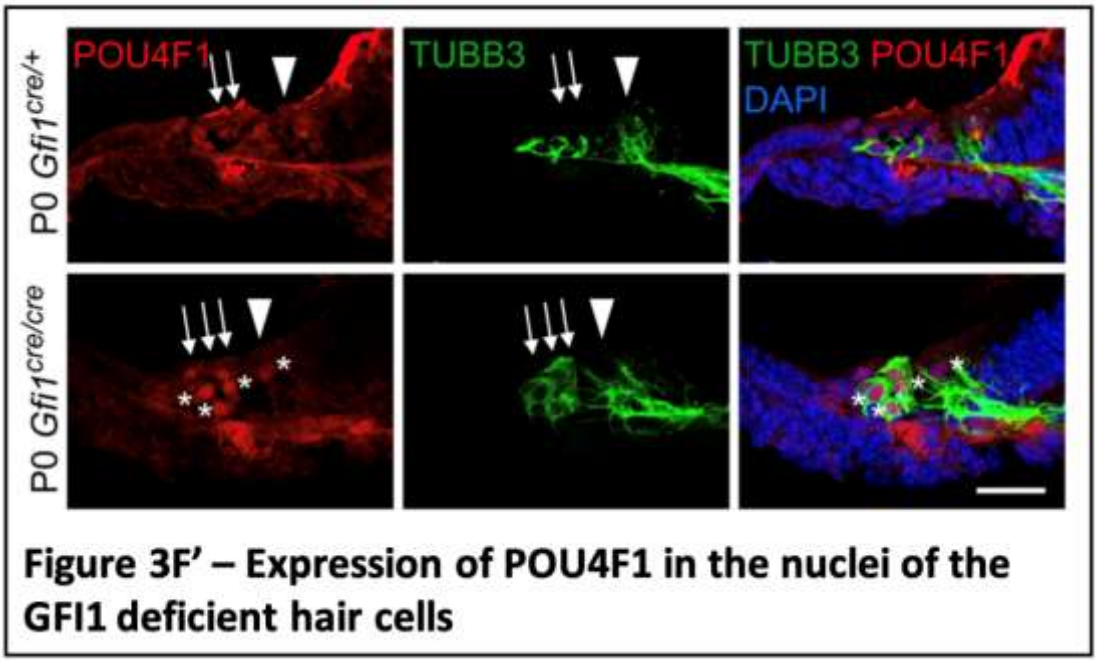
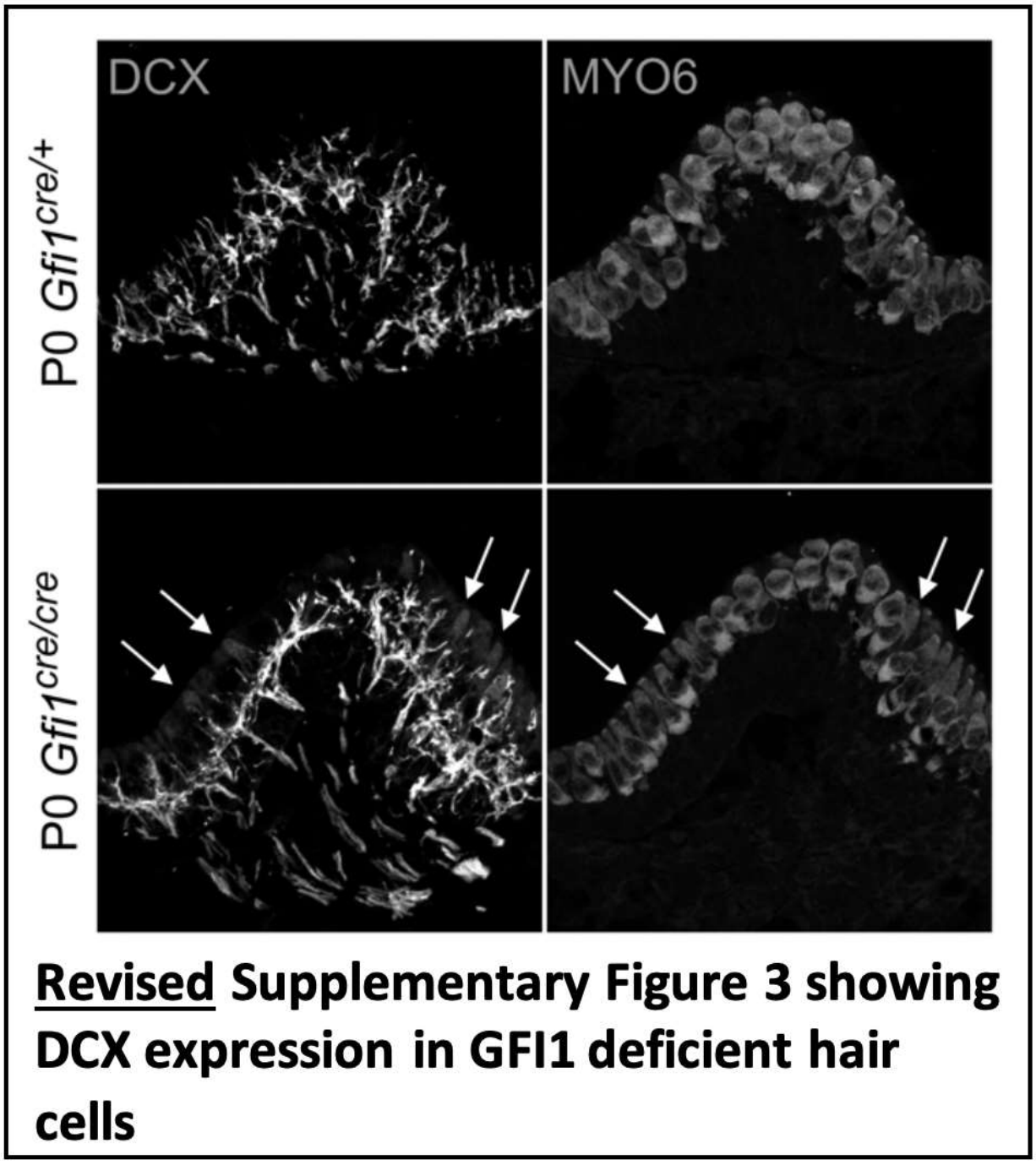


Figure 3F' – Expression of POU4F1 in the nuclei of the GFI1 deficient hair cells

Finally, and critically important, the expression patterns of many of the genes/proteins were carefully validated and confirmed with 2-3 independent methods (RNA-seq, RT-qPCR (Figure 2I-K, including **Sema5b** and **Lhx2**), and in situ/immunostaining) throughout the manuscript. ***Taken together***, we propose to better annotate the nuclear staining, present new in situ data for Lhx2 and present magnified immunostaining images for DCX, as suggested by both Reviewers 1 and 2. The DCX images separated by channel requested by Reviewer 1 were provided in the submitted manuscript as part of **Supplemental Figure 3** (partially shown below, now in gray scale). We agree with the reviewer that when separated by channel the data are easier to interpret.



Major comment 3 – differences in the Gfi1$^{cre/cre}$ vestibular phenotype compared to Gfi1$^{-/-}$. We were sorry to see this comment and are unclear of its origin. To the best of our knowledge, there is not a difference in the vestibular phenotype of the two mutant lines. I (the last author) have personally studied three mouse models for loss of Gfi1 expression (Gfi1-null[3], Gfi1-KI-GFP[4] and Gfi1-Cre[1]). All three lines have identical vestibular phenotypes – where they have a severe functional vestibular defect with disorganized stereocilia but no loss of hair cells. In addition, the published vestibular phenotype of the Gfi1-null mice by Wallis and colleagues is identical to the data shown in this manuscript, however the characterization in the Wallis and colleagues 2003 paper was only partial[5]. ***Taken together***, to our knowledge, our manuscript contains the most detailed description of Gfi1 mutant vestibular hair cells that has been performed and does not conflict with the phenotype of the published Gfi1-null. Furthermore, the deletion of Gfi1 expression is fully penetrant, as is the Gfi1-driven Cre expression (as shown in several manuscripts including ours[1]).

Reviewer 2 – We are grateful for Reviewer 2's positive comments about the impact and quality of the work presented in our short-format manuscript. We believe that their minor comments are

easily addressed with changes to the text and figures as listed below.

Minor comment 1 – improve the annotation of Figure 4. We now realize that we mistakenly omitted the legend for **Figure 4 panels C-D**. The green lines represent changes to gene expression in hair cells (GFP-positive cells), and the purple lines represent changes to gene expression in non-hair cells (all other cochlear or utricular cells) from E16 to P7. This will be fixed in the next version of the manuscript (see corrected **Figure 4C** shown here).

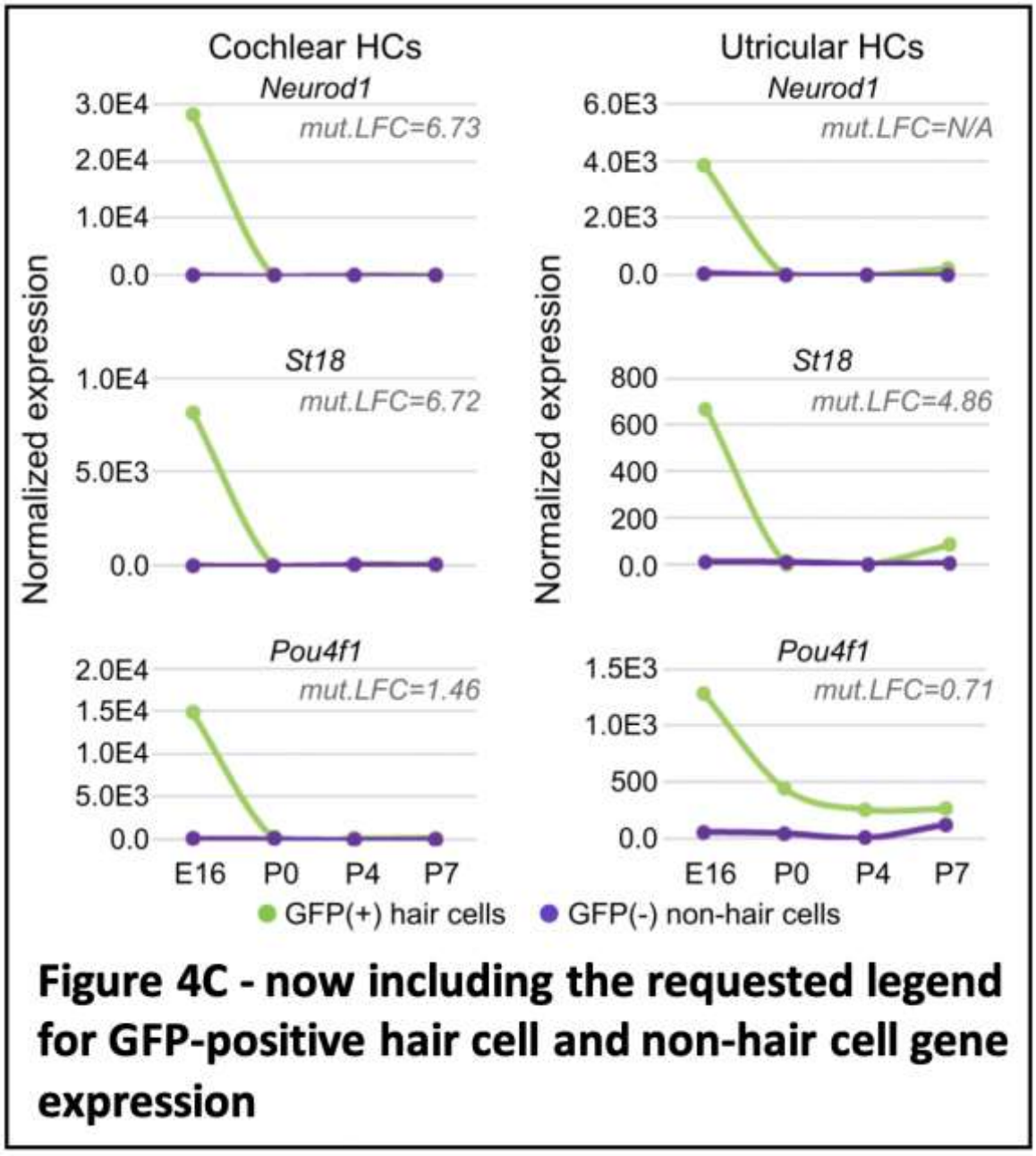


Figure 4C - now including the requested legend for GFP-positive hair cell and non-hair cell gene expression

For the box and whisker plots, the grey boxes represent the fold change differences in either normal developing hair cells (**Figure 4A-B**) or neurodifferentiating mESCs (**Figure 4F**) of the groups of genes identified as upregulated or downregulated in the Gfi1 mutant hair cells. These values are compared to the background (BG) gene expression (BG, white boxes), which is the dataset specific fold change values of all other genes expressed in Gfi1 mutant hair cells that are not included in the grey box and whisker. Since the two white BG bar and whisker plots within **Figure 4A-B** and **4F** are indeed different, we propose to separate each comparison into individual plots to improve clarity.

Minor comment 2 – DCX expression in mutant vestibular hair cells could benefit by adding insets at higher magnification. This comment was also brought up by Reviewer 1, and we agree that these

images would greatly benefit from increased magnification. We propose to include this in the next version of this manuscript.

Minor comment 3 – the caveat of using Gfi1$^{cre/+}$ animals as controls should be explicitly stated. This comment was also stated by Reviewer 3, and a discussion of this was removed from a previous version of the manuscript to conserve space. We now recognize that this is a critical caveat to discuss and propose to not only include it in the text of the next version of the manuscript, but to address it with additional analyses as outlined in our response to Reviewer 3.

Minor comment 4 – Figure 1H: an indication of how many TUNEL positive cells were observed should be stated. We propose to include the following text in the next version of the manuscript: "0-1 TUNEL positive HC nuclei were observed per Gfi1$^{cre/cre}$ cochlear cross section (approximately 12 cross sections analyzed per animal, 3 animals per genotype), always within the middle and basal turns where OHCs are actively degenerating."

Reviewer 3 – Below is a point by point discussion of Reviewer 3's critiques. We believe these concerns can be addressed by revising the discussion along with additional analysis of the already existing data, as outlined below:

Major comment 1 - Additional analysis of the immunoprecipitated mRNA from Gfi1$^{cre/+}$ inner ear should be done to show enrichment of macrophage genes. Indeed, in our paper published in 20171, we showed that Gfi1-Cre mediated recombination also occurs in the resident macrophages of the inner ear. It is for this reason that we performed in situ hybridization and immunostaining experiments to localize changes in gene/protein expression that we identified through RNA-seq. As expected, we have only observed differences within hair cells. The Reviewer suggested experiment to assess enrichment of macrophage genes in the immunoprecipitated mRNA is valid, and indeed we would likely see enrichment of macrophage genes. However, we would also expect this enrichment to be similar between samples, regardless of genotype, without a large number of macrophage-specific transcripts detected as differentially expressed when comparing the immunoprecipitated (IP) mRNA. To further assess this point, we queried the IP samples for differential expression of 360 macrophage-expressed genes identified in our 2017 manuscript. From this list, we detected 304 as expressed in the cochlear IP samples and 357 in the vestibular IP samples. Of these, only 4 genes were misregulated in the mutant IP (log2 fold change >1 or <-1, FDR<0.001): 2 significantly downregulated in the Gfi1$^{cre/cre}$ cochlea (**Nceh1** and **Rnf128**), and one upregulated (**Fgd3**) and one downregulated (**Spp1**) in the Gfi1$^{cre/cre}$ vestibule. Importantly, each of these genes show some appreciable expression in hair cells according to deposited datasets in umgear.org, and **Spp1** has recently been identified as a specific Type I vestibular hair cell marker[6,7] indicating that its downregulation is more likely the result of a change in hair cell gene expression. ***Taken together***, this analysis rules out the possibility that major changes in gene expression are driven by macrophage expressed genes.

Major comment 2 - the possibility of differences between Gfi1$^{+/+}$ and Gfi1$^{cre/+}$ gene expression at P0. We appreciate this concern and would take this opportunity to discuss how this issue was previously addressed in our 2017 publication[1]. As stated within the comments of Reviewer 3, our 2017 results showed that differences in gene expression between Gfi1$^{cre/+}$ and Gfi1$^{+/+}$ cochlea at P8 was minimal, with only 4 genes reaching statistical significance (log2 fold change >1 or <-1, FDR <0.05), all of which were located on the Y chromosome mirroring the sex imbalance in our analyzed samples. Slight differences in hair cell gene expression could only be detected when analyzing hair cell genes as a group (a more sensitive analysis), and not when comparing expression of individual genes. The use of Gfi1$^{cre/+}$ as the controls in our RiboTag approach was necessary to induce recombination, and we believe that any slight decreases in hair cell gene expression in the Gfi1 heterozygotes would decrease the sensitivity of detecting downregulation of these same genes in the mutants. Notably, we were still able to detect robust downregulation of hair cell genes in the mutants. Importantly, as an additional control, the majority of our in situ and immunostaining experiments were done using both wildtype and heterozygous littermates for controls (for example, **Tubb3** staining in **Figure 1I** - wildtype, and **Figure 3F-G** - heterozygote). We have not observed any differences between wildtypes and heterozygotes in these validations. This will be clarified in the text of the revised manuscript.

Minor comment 1 – additional information required in the experimental methods: what is a

biological replicate for the RNA-seq experiment? Include methods for immunostaining following in situ hybridization (Figure 3A-F). Information on the number of mice used for each replicate was included in the methods of the submitted manuscript under the section **RiboTag immunoprecipitations**: "For each biological sample, cochlear ducts or vestibular tissues (utricles, saccules and cristae) from five P0 Gfi1$^{cre/+}$;Rpl22$^{HA/HA}$ or Gfi1$^{cre/cre}$;Rpl22$^{HA/HA}$ mice were pooled..." Immunostaining was indeed performed following in situ hybridization for the images presented in **Figure 3A-E**. We propose to add the following sentence to the methods section **In situ hybridization**: "The colorimetric reaction was halted by soaking slides in 1X TBS, after which slides were reblocked and immunostained with the rabbit anti-myosin-VI antibody (1:1,000, Proteus BioSciences) and a corresponding Alexa Fluor 488 or 546 secondary (1:800, Invitrogen)."

We thank you for your time in considering this appeal letter and look forward to hearing from you. We hope that you would agree that this manuscript contributes significantly to our understanding of what are now considered the 'Yamanaka factors' of hair cells, and particularly the role of GFI1 in hair cell development. We therefore believe that this manuscript will have broad interest and a significant impact.

References:

1. Matern, M. S., Vijayakumar, S., Margulies, Z., Milon, B., Song, Y., Elkon, R., … Hertzano, R. (2017). Gfi1Cre mice have early onset progressive hearing loss and induce recombination in numerous inner ear non-hair cells. Scientific Reports, 7, 42079. https://doi.org/10.1038/srep42079
2. Lee, C., Rudneva, V. A., Erkek, S., Zapatka, M., Chau, L. Q., Tacheva-Grigorova, S. K., … Wechsler-Reya, R. J. (2019). Lsd1 as a therapeutic target in Gfi1-activated medulloblastoma. Nature Communications, 10(1), 332. https://doi.org/10.1038/s41467-018-08269-5
3. Hertzano, R., Montcouquiol, M., Rashi-elkeles, S., Elkon, R., Friedman, T. B., Frankel, W. N., … Avraham, K. B. (2004). Transcription profiling of inner ears from Pou4f3 ddl / ddl identifies Gfi1 as a target of the Pou4f3 deafness gene. Human Molecular Genetics, 13(18), 2143–2153. https://doi.org/10.1093/hmg/ddh218
4. Fiolka, K., Hertzano, R., Vassen, L., Zeng, H., Hermesh, O., Avraham, K. B., … Moroy, T. (2006). Gfi1 and Gfi1b act equivalently in haematopoiesis, but have distinct, non-overlapping functions in inner ear development. EMBO Reports, 7(3). https://doi.org/10.1038/sj.embor.7400618
5. Wallis, D., Hamblen, M., Zhou, Y., Venken, K. J. T., Schumacher, A., Grimes, H. L., … Bellen, H. J. (2003). The zinc finger transcription factor Gfi1, implicated in lymphomagenesis, is required for inner ear hair cell differentiation and survival. Development, 130, 221–232. https://doi.org/10.1242/dev.00190
6. McInturff, S., Burns, J. C., & Kelley, M. W. (2018). Characterization of spatial and temporal development of Type I and Type II hair cells in the mouse utricle using new cell-type-specific markers. Biology Open, 7(11), bio038083. https://doi.org/10.1242/bio.038083
7. Wang, T., Niwa, M., Sayyid, Z. N., Hosseini, D. K., Pham, N., Jones, S. M., … Cheng, A. G. (2019). Uncoordinated maturation of developing and regenerating postnatal mammalian vestibular hair cells. PLOS Biology, 17(7), e3000326. https://doi.org/10.1371/journal.pbio.3000326
8.

Reviewers' Response to Rebuttal

Reviewer 2

I think the author's have made a credible argument that their data strongly support the idea that the changed state of the hair cell is towards a neuronal phenotype, while also encompassing Reviewer #1's idea that the hair cells may be stalled in an early state in the mutants. That this early frozen state reflects something like a pro-neural state in young hair cells goes a long way towards resolving the differences in interpretation between the authors and Reviewer #1. All the other comments have or will be adequately addressed in the proposed revision. I still think the

paper is worthy of publication in Development, especially considering it is short format and they have fit quite a lot of work into it, despite its brevity.

Reviewer 4

I have read the other reviewers comments, and while I think that they are accurate, I do not share the opinion that the points raised justify rejecting this manuscript. The most significant argument is that the Gf1-Cre heterozygous mouse has a partially penetrant phenotype and this is not adequately discussed. However given the technical requirements of ribosome precipitation another genotype cannot be used as the control. Moreover, despite the mild phenotype in this control genotype, the authors are still able to identify significant changes in gene expression between heterozygotes and Gfi mutants. For this reason the experiment was a success and I believe it would be sufficient for the authors to cite their previous study and relate the het phenotype as a caveat or unavoidable limitation.

The greater concern, which likely triggered the detailed gene by gene itemization provided by reviewer one , is the dependence upon Gene Ontology (GO) analysis. However the limitations and assumptions inherent to this approach are largely understood and taken with a grain of salt by those outside of the bioinformatics specialty. Whether the pathway suppressed by Gfi is a neuralization gene regulator network (GRN) or not does not detract from the broader observations that auditory hair cells must repress a transiently active GRN in order to complete differentiation and maintain viability. Furthermore the observation that distinct cohorts of genes are repressed during auditory versus vestibular development is a useful contribution that should catalyze additional research in this area.

Finally as you pointed out, it is not realistic to request additional experimentation at this time. Fortunately I believe that these issues may be adequately addressed with a thought revision of the current manuscript.

Rebuttal response letter

I have asked the reviewers to look at your rebuttal letter and I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees agree with some of your points and think that their concerns can be addressed by text modification, edits to the figures and some minimal experimentation. I know that at this time new experiments may be hard to include so I would be willing to receive a revised manuscript with substantial text modification that addresses directly the shortcoming, tones down the claims (especially in the abstract and conclusions) according to the comments of the reviewers. Your revised paper will be re-reviewed by me, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.
First revision

Author response to reviewers' comments

We thank you for the opportunity to address the reviewers comments. We have implemented all of their suggestions in the text of the revised manuscript and below is a point by point response to all comments, listing all of the changes included in the manuscript. We feel that these changes have indeed improved the manuscript and are grateful for your help.

Reviewer 1

Major comment 1 – difference in opinion about the overall interpretation of the hair cell gene expression data.

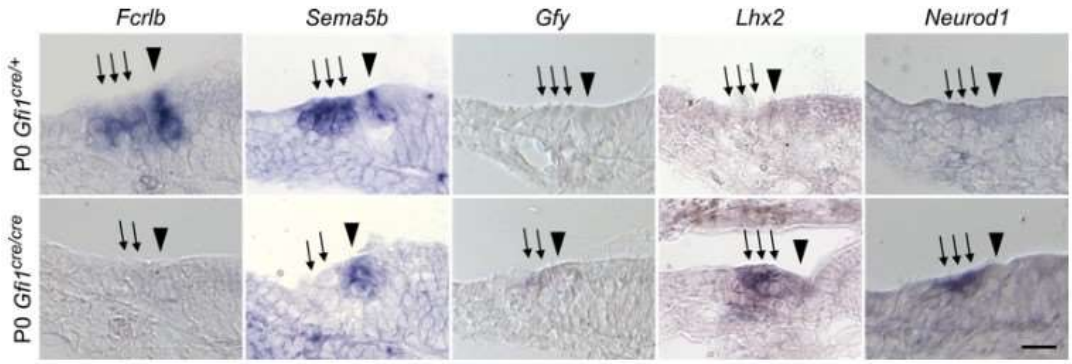
The Reviewer points out that most of the canonical neuronal genes that we identified as upregulated in our dataset are also expressed either in early developing hair cells (e.g., Neurod1 and Insm1), other tissues (Lhx2) or other cell states (St18). We agree with the Reviewer that (a) some of the neuronal genes are also expressed in nascent hair cells (a finding we stress as part of the manuscript, see multiple components of Figure 4) and that (b) genes expressed in neurons are not necessarily exclusively neuronal. However, we would like to point out that (a) neuronal genes and programs are strongly statistically over-represented in the upregulated genes in our dataset (see Figure 2G,H); (b) as shown in Figure 4F, genes that we discovered as repressed by GFI1 in hair cells, as a group, are induced in experiments of neurodifferentiation of mESCs towards neuronal fate by overexpression of Neurod1; and (c) a direct role for GFI1 in repressing these 'neuronal genes' is also supported by a recent paper from Lee et al., 2019[1]. In this 2019 manuscript, mis-expressed GFI1 was shown to "inhibit genes involved in neuronal commitment and differentiation" in neuronal precursors via cooperation with the GFI1 cofactor LSD1[1].

As suggested by the reviewer, we have made an effort to tone down the conclusions of GFI1 mediated repression of a neuronal program throughout the manuscript (changes highlighted in yellow in the text, including in the summary statement, abstract, introduction and conclusion paragraphs). We have instead focused on the conclusion that an early hair cell transcriptional profile, which is similar to a neuronal profile, fails to be repressed without GFI1. We have also added the Lee et al., 2019 reference to the conclusion, stating that our results are "adding to the accumulating evidence of GFI1s ability to serve this function (see Lee et al., 2019)" (Lines 183-184, highlighted).

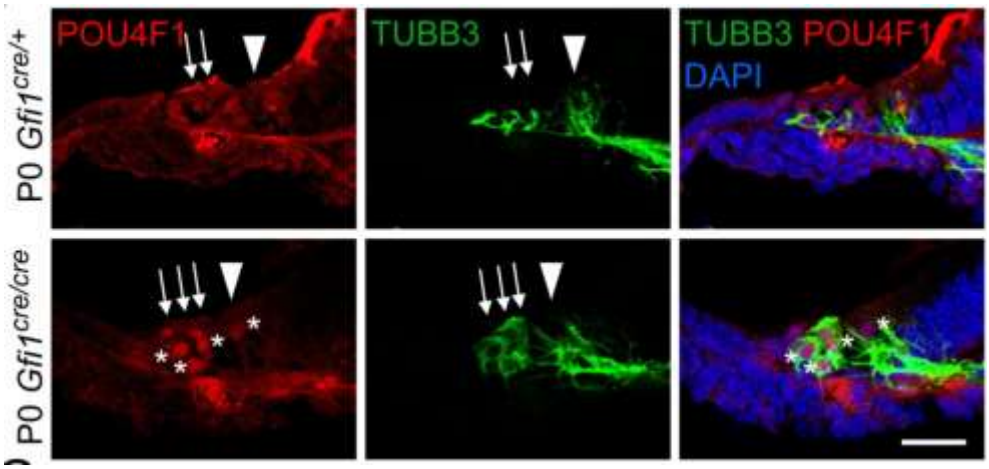
Major comment 2 – inconclusive appearance of in situ staining for two genes (Sema5b and Lhx2) and one of the immunohistochemistry staining for one protein (POU4F1).

While in situ hybridization as well as immunohistochemistry can be sometimes challenging, we respectfully disagree with at least some of the criticism and would like to point out the figures in question.

Downregulation of Sema5b is shown in Figure 3B: while the outer hair cells are dying, they still express the hair cell marker MYO6 and a near complete loss of expression of Sema5b is easily observed in the mutant hair cells. With regards to Lhx2 upregulation, we agree that the in situ staining is relatively faint, and have now provided additional images as part of a new Supplemental Figure 3 to support it.

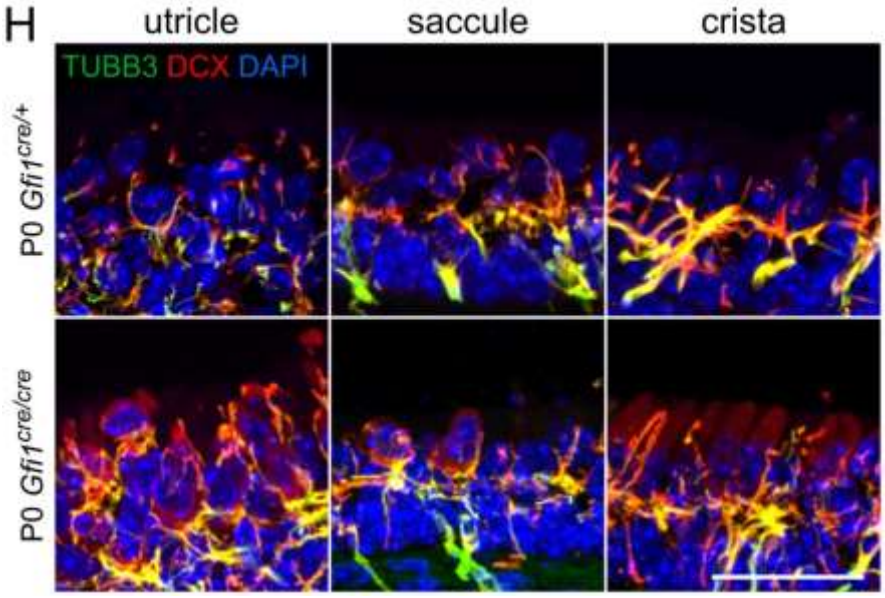


While the POU4F1 staining has some background, as show in Figure 3F', a nuclear staining which is not present in the wild type hair cells is clearly and strongly present in the mutant hair cells. In this revised version of the manuscript we have added asterisks to point out the nuclear staining (shown below).

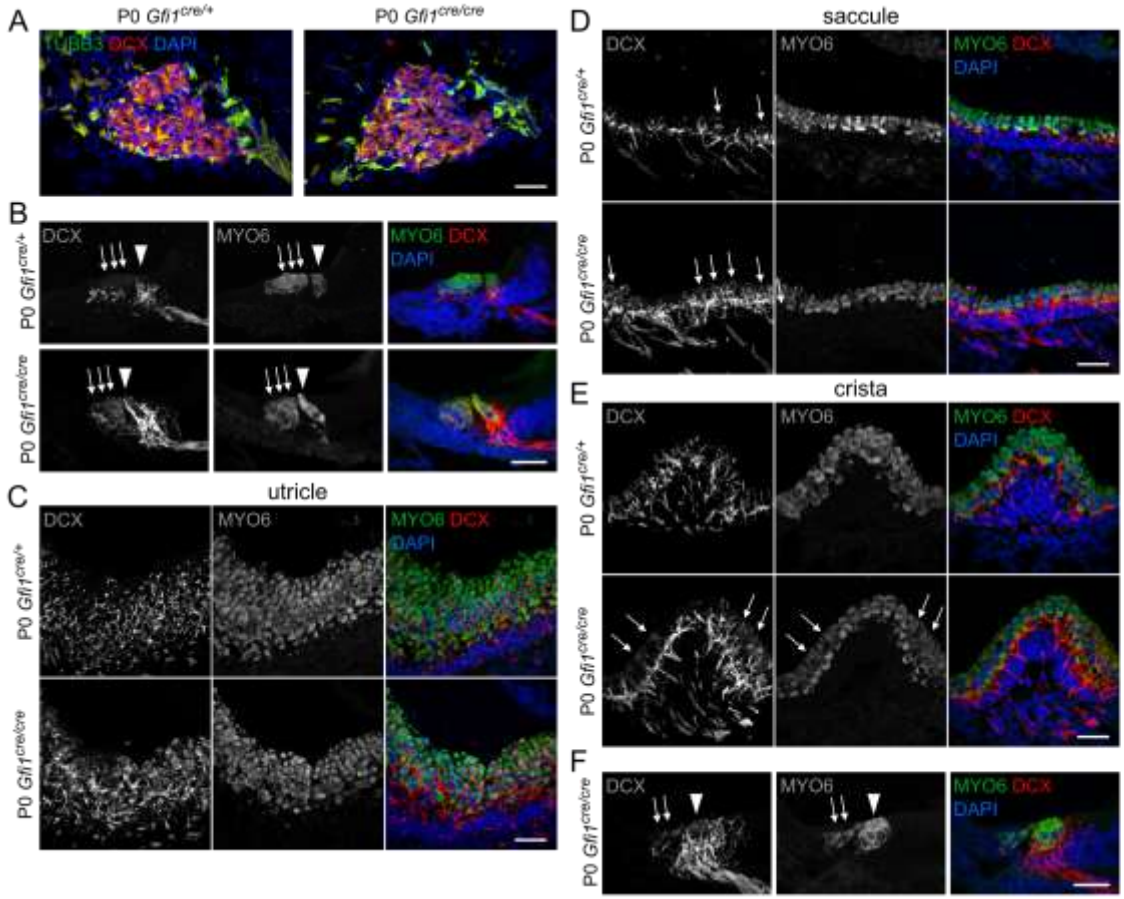


Finally, and critically important, the expression patterns of many of the genes/proteins were carefully validated and confirmed with 2-3 independent methods (RNA-seq, RT-qPCR (Figure 2I-K, including Sema5b and Lhx2), in situ/immunostaining) throughout the manuscript.

Altogether, we have now better annotated the nuclear staining of POU4F1 in Figure 3F', presented new data for all in situ experiments in a new Supplemental Figure 3, and have present magnified immunostaining images for DCX in Figure 3H, as suggested by both Reviewers 1 and 2 (shown below).



The DCX images separated by channel requested by Reviewer 1 were provided in the original submitted manuscript as part of Supplemental Figure 3 (now Supplemental Figure 4) and are now in gray scale to better appreciate hair cell staining in the mutants (shown below). We agree with the reviewer that when separated by channel the data are easier to interpret.



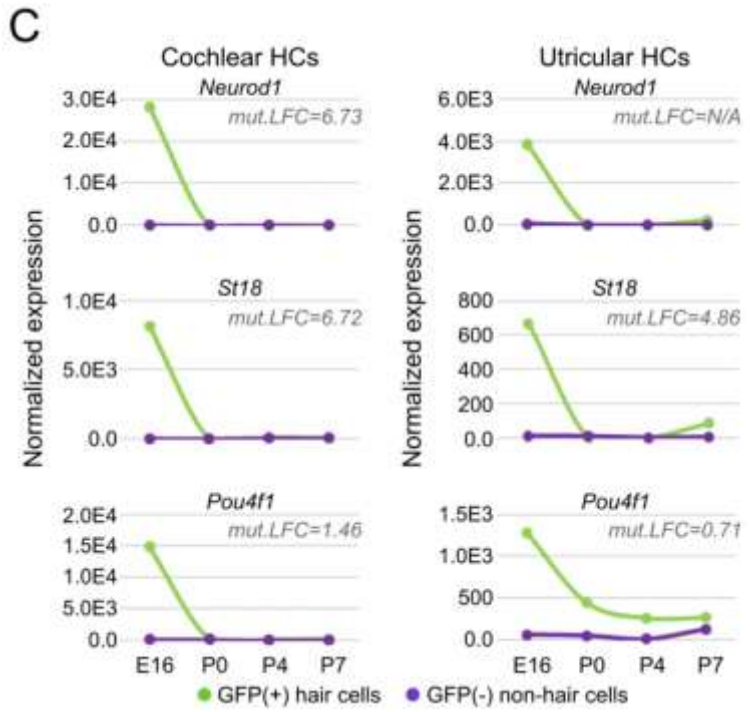
Major comment 3 – differences in the Gfi1$^{cre/cre}$ vestibular phenotype compared to Gfi1$^{-/-}$.

We were sorry to see this comment and are unclear of its origin. To the best of our knowledge, there is not a difference in the vestibular phenotype of the two mutant lines. I (the last author)

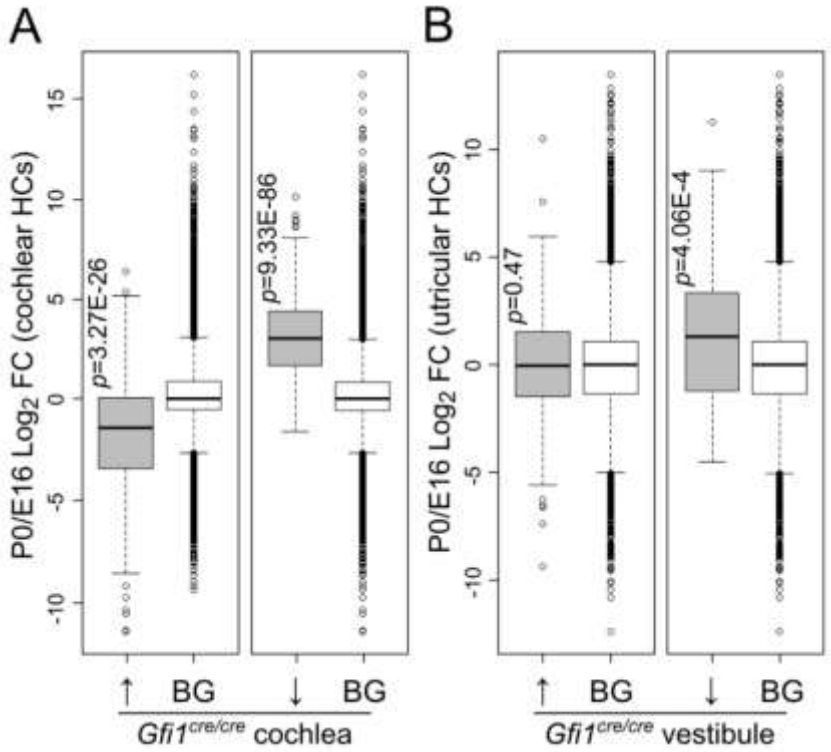
have personally studied three mouse models for loss of Gfi1 expression (Gfi1-Cre[2],Gfi1-null[3], and Gfi1-KI-GFP[4]). All three lines have identical vestibular phenotypes – where they have a severe functional vestibular defect with disorganized stereocilia but no loss of hair cells. In addition, the published vestibular phenotype of the Gfi1-null mice by Wallis and colleagues is identical to the data shown in this manuscript, however the characterization in the Wallis and colleagues 2003 paper was only partial[5]. To our knowledge, our manuscript contains the most detailed description of Gfi1 mutant vestibular hair cells that has been performed and does not conflict with the phenotype of the published Gfi1-null. Furthermore, the deletion of Gfi1 expression is fully penetrant, as is the Gfi1-driven Cre expression (as shown in several manuscripts including ours[2]).

Reviewer 2
Minor comment 1 – improve the annotation of Figure 4.

We now realize that we mistakenly omitted the legend for Figure 4 panels C-D. The green lines represent changes to gene expression in hair cells (GFP-positive cells), and the purple lines represent changes to gene expression in non-hair cells (all other cochlear or utricular cells) from E16 to P7. This has now been fixed in this version of the manuscript (shown below).



For the box and whisker plots, the grey boxes represent the fold change differences in either normal developing hair cells (Figure 4A-B) or neurodifferentiating mESCs (Figure 4F) of the groups of genes identified as upregulated or downregulated in the Gfi1 mutant hair cells. These values are compared to the background (BG) gene expression (BG, white boxes), which is the dataset specific fold change values of all other genes expressed in Gfi1 mutant hair cells that are not included in the grey box and whisker. Since the two white BG bar and whisker plots within Figure 4A-B and 4F are indeed different, we have now separated each comparison into individual plots to improve clarity (partially shown below). We have also updated the figure legend to better explain the comparisons: “Statistical significance for A-C, F was assessed by Wilcoxon's test, comparing the Log2 fold change (FC) values of each gene group to the Log2 FC values of all other genes expressed (background, BG)” (lines 324-325, also highlighted in yellow).



Minor comment 2 – DCX expression in mutant vestibular hair cells could benefit by adding insets at higher magnification.

This comment was also brought up by Reviewer 1, and we agree that these images greatly benefit from increased magnification. We have now added higher magnification images to Figure 4, and images separated by channel are provided as part of Supplemental Figure 4.

Minor comment 3 – the caveat of using Gfi1$^{cre/+}$ animals as controls should be explicitly stated.

This comment was also brought up by Reviewer 3. We have now stated this caveat in the text and further address it with additional analyses as outlined in our response to Reviewer 3.

Minor comment 4 – Figure 1H: an indication of how many TUNEL positive cells were observed should be stated.

We have now added the following text to the manuscript (lines 209-211, highlighted): "0-1 TUNEL positive HC nuclei were observed per Gfi1$^{cre/cre}$ cochlear cross section (approximately 12 cross sections analyzed per animal, 3 animals per genotype), always within the middle and basal turns where OHCs are actively degenerating."

Reviewer 3

Major comment 1 - Additional analysis of the immunoprecipitated mRNA from Gfi1$^{cre/+}$ inner ear should be done to show enrichment of macrophage genes.

Indeed, in our paper published in 2017[2], we showed that Gfi1-Cre mediated recombination also occurs in the resident macrophages of the inner ear. This is now stated in the text (lines 59 and 107, highlighted), and it is for this reason that we performed in situ hybridization and immunostaining experiments to localize changes in gene/protein expression that we identified through RNA-seq. As expected, we have only observed differences within hair cells. The Reviewer suggested experiment to assess enrichment of macrophage genes in the immunoprecipitated mRNA is valid, and indeed we would likely see enrichment of macrophage genes. However, we would also expect this enrichment to be similar between samples, regardless of genotype,

without a large number of macrophage-specific transcripts detected as differentially expressed when comparing the immunoprecipitated (IP) mRNA.

To assess this point, we queried the IP samples for differential expression of 360 macrophage-expressed genes identified in our 2017 manuscript. From this list, we detected 304 as expressed in the cochlear IP samples and 357 in the vestibular IP samples. Of these, only 4 genes were misregulated in the mutant IP (log2 fold change >1 or <-1, FDR<0.01): 2 significantly downregulated in the $Gfi1^{cre/cre}$ cochlea (Nceh1 and Rnf128), and one upregulated (Fgd3) and one downregulated (Spp1) in the $Gfi1^{cre/cre}$ vestibule. Importantly, each of these genes show some appreciable expression in hair cells according to deposited datasets in umgear.org, and Spp1 has recently been identified as a specific Type I vestibular hair cell marker[6,7] indicating that its downregulation is more likely the result of a change in hair cell gene expression. Taken together, this analysis rules out the possibility that major changes in gene expression are driven by macrophage expressed genes, and we have added the following text to this version of the manuscript (lines 107-111, highlighted):

"Finally, as *Gfi1^{cre}* also drives recombination in inner ear macrophages, we further interrogated our dataset for changes to 360 previously defined macrophage-expressed genes (Matern et al., 2017). Of these, only 4 genes (*Nceh1, Rnf128, Fgd3* and *Spp1*) were dysregulated in either the *Gfi1$^{cre/cre}$* cochlear or vestibular samples, suggesting that the observed differences in gene expression are likely a result of global changes to HCs rather than macrophages."

Major comment 2 - the possibility of differences between $Gfi1^{+/+}$ and $Gfi1^{cre/+}$ gene expression at P0.

We appreciate this concern and would take this opportunity to discuss how this issue was previously addressed in our 2017 publication[2]. As stated within the comments of Reviewer 3, our 2017 results showed that differences in gene expression between $Gfi1^{cre/+}$ and $Gfi1^{+/+}$ cochlea at P8 was minimal, with only 4 genes reaching statistical significance (log2 fold change >1 or <-1, FDR <0.05), all of which were located on the Y chromosome mirroring the sex imbalance of our analyzed samples. Slight differences in hair cell gene expression could only be detected when analyzing hair cell genes as a group (a more sensitive analysis), and not when comparing expression of individual genes. The use of $Gfi1^{cre/+}$ as the controls in our RiboTag approach was necessary to induce recombination, and we believe that any slight decreases in hair cell gene expression in the Gfi1 heterozygotes would decrease the sensitivity of detecting downregulation of these same genes in the mutants. Notably, we were still able to detect robust downregulation of hair cell genes in the mutants. As an additional control, the majority of our in situ and immunostaining experiments were done using both wildtype and heterozygous littermates for controls (for example, Tubb3 staining in Figure 1I- wildtype, and Figure 3F- G- heterozygote). We have not observed any differences in wildtypes and heterozygotes in these validations. This has now been clarified in the text of the revised manuscript (lines 217-220, highlighted).

"Of note, *Gfi1$^{cre/+}$* animals were used as controls in this experiment out of necessity to drive Cre recombination, despite their age-related hearing loss phenotype (Matern et al., 2017). Only minimal differences in gene expression have been previously noted between *Gfi1$^{+/+}$* and *Gfi1$^{cre/+}$* at P8 (all attributed to potential sex bias in samples analyzed), suggesting that *Gfi1$^{cre/+}$* can serve as proxy for *Gfi1$^{+/+}$* (Matern et al., 2017)."

<u>Minor comment 1 – additional information required in the experimental methods: what is a biological replicate for the RNA-seq experiment? Include methods for immunohistochemistry following in situ hybridization (Figure 3A-F).</u>

Information on the number of mice used for each replicate was included in the methods of the original submitted manuscript under the section RiboTag immunoprecipitations (lines 220-222): "For each biological sample, cochlear ducts or vestibular tissues (utricles, saccules and cristae) from five P0 *Gfi1$^{cre/+}$;Rpl22$^{HA/HA}$* or *Gfi1$^{cre/cre}$;Rpl22$^{HA/HA}$* mice were pooled..."

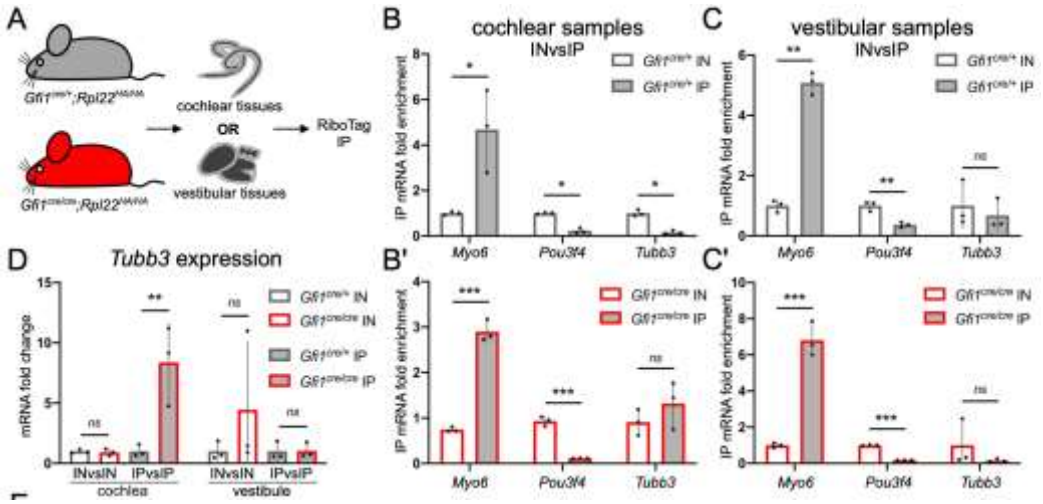
Immunohistochemistry was indeed performed following in situ hybridization for the images presented in Figure 3A-E. We have now added the following sentence to the methods section (lines 268-271, highlighted): "The colorimetric reaction was halted by soaking slides in 1X TBS,

after which slides were reblocked and immunostained with the rabbit anti-myosin-VI antibody (1:1,000, Proteus BioSciences) and a corresponding Alexa Fluor 488 or 546 secondary (1:800, Invitrogen)."

Reviewer 4

Comment 1 – improve the organization and description of Figure 2B-D, pairwise comparisons in 2D are not obvious and appear the same as that presented in 2B and 2C.

Thank you for the suggestion. We have improved the organization of Figure 2B-D by adding additional labels (B' and C'), adding the type of pairwise comparison that is made to each graph (INvsIP and IPvsIP where applicable), and changing the colors of the bars to be less busy.



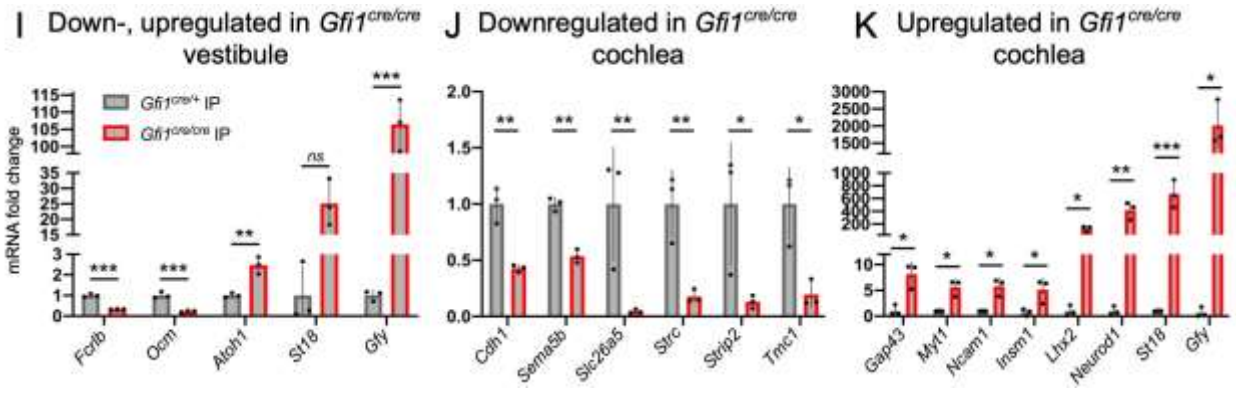
The results in 2D are related to 2B-C, however in 2B-C the IP expression values are being compared to IN, and in 2D the Gfi1$^{cre/cre}$ expression values are being compared to Gfi1$^{cre/+}$. This comparison shows that upregulation of Tubb3 was only be detected when comparing IP to IP and not IN to IN, thus demonstrating the usefulness of enriching for cell type-specific transcripts with RiboTag. We have altered the figure legend and text (lines 88-91, 299, highlighted) to make this comparison more clear.

Comment 2 - It is unclear what is meant by 'disorganized vestibular hair cells' when describing the Gfi1 mutant phenotype. This warrants clearer description in the text or better examples.

In the Gfi1 mutant vestibular system, hair bundles appear to be thinner compared to controls (shown at three time points in Figures 1, S1 and S2 – P1, P5 and P32). We agree that the term "disorganized" is not informative, and this wording is mainly a carryover from its extensive use to describe the Gfi1-KO vestibular hair cells in Wallis et al., 2003. We have therefore removed this wording throughout the manuscript, while keeping the description of "thinner" mutant vestibular hair bundles (highlighted throughout the text).

Comment 3 - It is unclear what is different between 'dysregulated gene expression' (Fig.2I) versus genes that are upregulated or downregulated in the Gfi1 mutant (Fig.2J,K).

In Figure 2J and 2K, validation of either upregulated or downregulated genes in the cochlea are shown, whereas in Figure 2I, validation of both up and downregulated genes in the vestibule are shown. We have changed the title of Figure 2I to "Down-, upregulated in Gfi1$^{cre/cre}$ vestibule" to make this more clear (shown below).



Comment 4 - Difference between purple and green lines in Fig.4 are not clearly defined in the text or legend.

This was also brought to our attention by Reviewer 1 and has been fixed within the figure. We apologize for the original omission.

Comment 5 - The Gfi1 mutant phenotype resembles that of Emx2 mutants. Do these transcription factors regulate similar targets, or is Emx2 required for Gfi1 expression? This does not warrant new experimentation but might be a worthwhile talking point for the discussion.

This is an interesting observation, and one that we had not considered. In our Gfi1 mutant RNA-seq dataset, Emx2 is not differentially expressed in either the cochlear or vestibular hair cells, and we are not aware of any gene expression data showing downregulation of Gfi1 in the Emx2 mutant hair cells. Additionally, looking more closely into the Emx2 mutant cochlear phenotype, it appears that defects in the development of the outer hair cells present even before the onset of Gfi1, whereby 2-3 rows of the outer hair cells do not develop by E16.5 in Emx2 mutants compared to controls[8,9]. This contrasts somewhat with the Gfi1 mutant phenotypes, whereby outer hair cells initially form but degrade postnatally. The frequency of inner hair cell doublets in Emx2 mutants is also much higher than that of Gfi1 mutants, with 50-55% of inner hair cells presenting as doublets throughout the cochlea of Emx2 mutants[8] compared to only a slight but significant increase in inner hair cell doublets in Gfi1 mutants at 16kHz. These observations suggest that if Gfi1 expression were indeed decreased in Emx2 mutants, it may not fully explain the severity of the cochlear hair cell defects present.

Within the vestibular system, Emx2 mutant utricular and saccular hair cells and bundles appear to develop normally but present with absent polarity reversal across the striolar region[8,9]. This again contrasts somewhat with the Gfi1 mutant, whereby we see thinner hair bundles in all three sensory organs of the vestibular system. It would however be interesting to perform immunostaining for beta spectrin in the Gfi1 mutants to assess polarity reversal. Immunostaining of OCM in Emx2 mutant hair cells also reveals no apparent decrease in the striolar region compared to controls[9], again contrasting to the Gfi1 mutants where OCM is greatly reduced. Finally, analysis of Emx2 mutant hair cells in the cristae revealed no apparent defects, consistent with an observed lack of Emx2 expression in the cristae[8,9]. Therefore, if Gfi1 expression indeed relies on Emx2 in the utricle and saccule, activation of Gfi1 in the cristae would rely on a separate mechanism.

While this discussion is of interest and suggests that further investigation of Gfi1 expression in Emx2 mutant hair cells is warranted, we respectfully request to not include it in the text of the manuscript purely due to the text limitations of this short report.

References:

1. Lee, C., Rudneva, V. A., Erkek, S., Zapatka, M., Chau, L. Q., Tacheva-Grigorova, S. K., … Wechsler-Reya, R. J. (2019). Lsd1 as a therapeutic target in Gfi1-activated

medulloblastoma. Nature Communications, 10(1), 332. https://doi.org/10.1038/s41467-018-08269-5
2. Matern, M. S., Vijayakumar, S., Margulies, Z., Milon, B., Song, Y., Elkon, R., … Hertzano, R. (2017). Gfi1Cre mice have early onset progressive hearing loss and induce recombination in numerous inner ear non-hair cells. Scientific Reports, 7, 42079. https://doi.org/10.1038/srep42079
3. Hertzano, R., Montcouquiol, M., Rashi-elkeles, S., Elkon, R., Friedman, T. B., Frankel, W. N., … Avraham, K. B. (2004). Transcription profiling of inner ears from Pou4f3 ddl / ddl identifies Gfi1 as a target of the Pou4f3 deafness gene. Human Molecular Genetics, 13(18), 2143–2153. https://doi.org/10.1093/hmg/ddh218
4. Fiolka, K., Hertzano, R., Vassen, L., Zeng, H., Hermesh, O., Avraham, K. B., … Moroy, T. (2006). Gfi1 and Gfi1b act equivalently in haematopoiesis, but have distinct, non-overlapping functions in inner ear development. EMBO Reports, 7(3). https://doi.org/10.1038/sj.embor.7400618
5. Wallis, D., Hamblen, M., Zhou, Y., Venken, K. J. T., Schumacher, A., Grimes, H. L., … Bellen,
H. J. (2003). The zinc finger transcription factor Gfi1, implicated in lymphomagenesis, is required for inner ear hair cell differentiation and survival. *Development*, *130*, 221–232. https://doi.org/10.1242/dev.00190
6. McInturff, S., Burns, J. C., & Kelley, M. W. (2018). Characterization of spatial and temporal development of Type I and Type II hair cells in the mouse utricle using new cell-type-specific markers. *Biology Open*, *7*(11), bio038083.https://doi.org/10.1242/bio.038083
7. Wang, T., Niwa, M., Sayyid, Z. N., Hosseini, D. K., Pham, N., Jones, S. M., … Cheng, A.G. (2019). Uncoordinated maturation of developing and regenerating postnatal mammalian vestibular hair cells. *PLOS Biology*, *17*(7), e3000326. https://doi.org/10.1371/journal.pbio.3000326
8. Holley, M., Rhodes, C., Kneebone, A., Herde, M. K., Fleming, M., & Steel, K. P. (2010). Emx2 and early hair cell development in the mouse inner ear. *Developmental biology*, *340*(2), 547– 556. https://doi.org/10.1016/j.ydbio.2010.02.004
9. Jiang, T., Kindt, K., & Wu, D. K. (2017). Transcription factor Emx2 controls stereociliary bundle orientation of sensory hair cells. *eLife*, *6*, e23661. https://doi.org/10.7554/eLife.23661

Second decision letter

MS ID#: DEVELOP/2019/186015

MS TITLE: GFI1 Functions to Repress Neuronal Gene Expression in the Developing Inner Ear Hair Cells

AUTHORS: Maggie S Matern, Beatrice Milon, Erika L Lipford, Mark McMurray, Yoko Ogawa, Andrew Tkaczuk, Yang Song, Ran Elkon, and Ronna Hertzano
ARTICLE TYPE: Research Report

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks. I am sorry for the prolonged review period but delighted of the outcome.

Reviewer 1

Advance summary and potential significance to field

The manuscript by Matem and colleagues entitled "GFI1 Functions to Repress Neuronal Gene Expression in the Developing Inner Ear Hair Cells" addresses the role of the transcription factor Gfi1 in vivo by analysing the Hair Cell specific translatome in Gfi1 mouse mutants. In accordance with other reports of hair cell differentiation in vitro and with a recent report in medulloblastoma, they

propose that Gfi1 represses an neuronal program to stabilize hair cell differentiation. This work is interesting and adds information regarding the gene regulatory events taking place during hair cell differentiation in the inner ear . Moreover, the authors observe that the Gfi1 gene targets are not exactly identical in vestibular and cochlear hair cells. This might be due to temporal differences in hair cell differentiation between both sensory patches or to co-factors working together with Gfi1, not addressed here. The owrk is thourough and the Figures have improved after the revision.

The authors have modified the text and added novel panels to incorporate my concerns and the paper has been improved substantially.

Comments for the author

The authors have modified the text and added novel panels to incorporate my concerns and the paper has been improved substantially.

Reviewer 2

Advance summary and potential significance to field

It has been known for some time that Gfi1 is an essential transcription factor, along with several others, to establish and maintain a hair cell fate. This manuscript reveals for the first time that this transcriptional regulator is primarily repressing a set of neuronal genes, and especially the neuroD1 transcription factor, and thus also the genes downstream of NeuroD1. This regulatory control presumably allows the hair cell to progress through its differentiation program. This offers valuable new insight about this important sensory cell, and can help to inform the numerous efforts underway currently to stimulate hair cell regeneration as a therapeutic approach to hearing loss due to the loss of hair cells.

Comments for the author

The author has made revisions that satisfy all of my comments arising from the first submission. This is a well executed study