Manuscript Title: Tbx2 is a master regulator of inner vs outer hair cell differentiation and maintenance

Editorial Note: Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

The authors present here beautiful follow-up work on a 2018 Nature paper in which they identified a regulator of outer vs inner hair cell differentiation, called Insm1. Insm1 is expressed in and required for outer hair cells and it its absence, outer hair cells transdifferentiate into inner hair cells. In the present paper, the authors now fill in a critical, previously unknown gap – how do the inner hair cells become specified? The authors show that they require another TF, tbx2, which is again sufficient and required for inner hair cell fate. Through rigorous and very laudable double mutant analysis (not often done in vertebrates), the authors show that Tbx2 is epistatic to Insm1. What makes this paper of general interest is not just a better understanding of hair cell differentiation, but also the validation of the “master regulatory” concept of cell differentiation (which of course is context-dependent, but this goes without saying these days); but more importantly, the evolutionary concept of a default state of OHC from which IHCs then branched off. The paper is very crisply written, there's not much comparison of concepts to other cell types, but frankly, that's totally OK. I can recommend publication as is.

Referee #2 (Remarks to the Author):

This is an interesting follow up study to the authors' manuscript on the role of Insm1 in hair cell development. In that earlier study, they found that Insm1 loss led to the conversion of OHC to IHC. Tbx2 was one of the genes whose expression changed upon Insm1 loss. Here they investigated the role of Tbx2 in the development of these two cell types. The study relies on ko mouse strains and immunohistochemical markers, which define these cell types. The work is well done and interesting. Those in the field will appreciate the fact that Insm1 appears to make cells able to ignore inductive cues that can lead to Tbx2 expression, and that Tbx2 expression is both necessary and sufficient for IHC formation. It is particularly interesting that Tbx2 can enact this determination event even in postnatal OHC's when Tbx2 is delivered via an AAV in an explant.

Recommended additional experiment:
In lines 49-54, the authors claim that less than half of Insm1-/- OHCs misexpress Tbx2, which is not expressed in wild-type OHCs, and only those cells transdifferentiate into IHCs (Extended Fig 1b,c). However, Extended Fig 1b,c simply show the in situ images of OHCs with or without Tbx2 expression. Also, in lines 473-474, the legend says “Tbx2 is expressed in about half of the OHCs, presumably those transdifferentiating into IHCs”. The underlying premise of this study is that Tbx2 is the critical

factor among the core set of early IHC-specific genes misregulated during the homeotic transformation of Insm1-/- OHCs into IHCs. Can the authors demonstrate that only Insm1-/- OHCs expressing Tbx2 transdifferentiate into IHCs while Insm1-/- OHCs without Tbx2 expression do not? This will also strengthen the finding that Tbx2 is epistatic to Insm1.

Minor points

• The authors could do a better job in the opening paragraph to lay out their rationale earlier on.

• The authors should include the quantification of each immunofluorescence image.

• Adding a schematic figure for the mechanisms of the transdifferentiation between IHC and OHC would be helpful to readers.

Referee #3 (Remarks to the Author):

Hair cells are required for hearing and balance function. In the mammalian cochlea, inner hair cells are the primary receptors of sound and outer hair cells serve as amplifiers, and thus understanding determinants of their cell fate is highly significant. The current manuscript has elegantly characterized the expression and function of Tbx2 as a master regulator that is necessary and sufficient for inducing hair cell fate. The claim that Tb2 is required for inner hair cell fate in the neonatal cochlea is convincing, supported by transgenic models, in situ hybridization, and rendering the IHC-deficient cochlea non-functional, and interestingly leading to outer hair cell-like cell formation in the inner hair cell position. This is provocative and supported by some molecular markers, measurement of nucleus size, and electrophysiology experiments, and can be improved upon by further characterization and quantification (see below). Furthermore to build on what they have shown that OHC can convert to IHC-like cells in the absence of Insm1, Insm1/tb2 dcKO OHCs fail to convert. Both early neonatal and (P1-8) IHCs can still convert but P8 IHCs are still maturing (see below) so the claim of fate maintenance is only partly supported. Lastly, viral transduction of Tbx2 to OHCs can promote and IHC fate, but the extent is not fully characterized and the claim of sufficiency also will benefit some addition work. Overall, the manuscript is well written and some of the claims supported, but will benefit from having the following points addressed. I recommend a revision.

Major

1) Direct switch between IHC and OHC-this seems to be lack sufficient support. First one would expect some intermediate genes (coexpressing of IHC and OHC) without de-differentiation (ie hybrid IHC/OHC), prob the need to assess multiple timepoints to more thoroughly show this. Electrophysiology and bundle morphology changes are also expect to shift over time, given the partial conversion reported (Chessum et al, 2018). The current dataset is supportive of a fate switch, but how and the extent of this happening is not clear.

2) hair cell characterization: IHC and OHC are distinguished by numerous other factors in addition to molecular markers and nucleus size, it is important to also characterize bundle morphology (e.g. high res f-actin staining or SEM) and innervation (similar this group's previous paper, Webber et al., 2021). The data on the claim that tbx2 is sufficient in inducing an IHC fate is not well supported. One

would expect a battery of molecular markers labeling IHC in the OHC region, bundle changes, ephys changes similar to what has been shown in Wiwatpanit et al.,
3) IHC fate maintenance-IHCs continue to mature in the postnatal period at least till P21-30 (Payne Font Synap neurosci 2021), although some molecular markers such as Vglut3 is expressed early and mature IHCs. It is difficult to appreciate whether tbx2 maintains IHC fate in both immature and mature IHC without deleting tbx2 later. It is also difficult to tell if P8 deletion less efficiently convert cell fate than early. One possibility is to use Vglut3cre to delete tbx2 at later timepoints (Li et al., Hear Res 2018).
Minor:
1)- Line 47: "In contrast, we do not detect Tbx2 mRNA in cells of the outer compartment of the organ of Corti (OHCs and supporting Pillar, Deiters', and Hensen's cells) (Extended Fig 1a)". It does look like there was some mRNA in the outer compartment just much less than the inner compartment. Providing the positive and negative control samples and what probes were used for RNAscope would be useful.
2)- Line 68: "In these mice, the cochlear hair cells in the position of the IHCs (the inner or medial compartment)". What does inner or medial compartment mean in refer to the position of IHCs mean?
3) Line 90: "Upon maturation (P25-29), we dissociated fluorescently labeled ic-OHCs from these mice, as well as from IHCs and OHCs obtained from control mice, and performed whole-cell, patch clamp recordings": Why was this done by dissociation? Why was this not done in situ, if this is just an age issue could it not have at least been done in the apex?
4) Line 101: "We generated mice lacking both TBX2 and INSM1 in embryonic IHCs and OHCs (Atoh1Cre; Tbx2F/F; Insm1F/F) and found that Tbx2 is epistatic to Insm1: none of the OHCs transdifferentiated into IHCs, and all of the IHCs transdifferentiated into OHCs (Fig 2, extended Table 1 and extended Fig 2)": the authors should provide information on the efficacy of gene deletion of both alleles.
5) - Lines 143: "Therefore, it appears that TBX2, in addition to being necessary for initiating the specific differentiation of IHCs during embryogenesis, is also required throughout the postnatal period for IHCs to continue expressing their already acquired features and to keep them from expressing those of OHCs; in other words, to maintain their differentiating state." Interesting that even the hair cells which are not tdTomato positive seem to have lost their vglut3 and calb2 expression – why is this? Also I would like to see these in single channel images.
6) Line 158: "While non-transfected IHCs lacking TBX2 transdifferentiated into OHCs and expressed Prestin, transfected IHCs, identified by expression of mCherry, did not appear to transdifferentiate as they did not express Prestin (Fig 4a,a')": Really need another hair cell marker (e.g. myo7a) here, very hard to tell what cells were transduced in Fig 4A. Seems low efficiency.
7) typo: deficient in abstract, line 117-loosing
8) figure 1-quantitative-in situ-where was this quantified?
9) Line 124: "Hence, unlike IHCs missing TBX2 embryonically, which differentiated as OHCs (Fig 1 and extended Table 2), these transdifferentiating cells switched from IHCs to OHCs without recapitulating OHC differentiation": I want to see some actual images here to show this in more detail – cells lacking or gaining an IHC or OHC respectively. I would also like to know what the bundles look like, do you get the classic OHC "V" shaped bundle?
10) Electrophysiological characterization: There seems to be paucity of info on cells patched, and the variability of results which should be expected to occur a bit. One would also expect to have ephys

data on a cell that was not recombined displaying the properties of an IHC. Also were the ephys findings throughout the cochlea – where were these ic-OHCs from? Also should have the ages in the figure.

Author Rebuttals to Initial Comments:

Referees' comments:

Referee #1 (Remarks to the Author):

The authors present here beautiful follow-up work on a 2018 Nature paper in which they identified a regulator of outer vs inner hair cell differentiation, called Insm1. Insm1 is expressed in and required for outer hair cells and it its absence, outer hair cells transdifferentiate into inner hair cells. In the present paper, the authors now fill in a critical, previously unknown gap – how do the inner hair cells become specified? The authors show that they require another TF, tbx2, which is again sufficient and required for inner hair cell fate. Through rigorous and very laudable double mutant analysis (not often done in vertebrates), the authors show that Tbx2 is epistatic to Insm1. What makes this paper of general interest is not just a better understanding of hair cell differentiation, but also the validation of the "master regulatory" concept of cell differentiation (which of course is context-dependent, but this goes without saying these days); but more importantly, the

evolutionary concept of a default state of OHC from which IHCs then branched off. The paper is very crisply written, there's not much comparison of concepts to other cell types, but frankly, that's totally OK. I can recommend publication as is.

We agree that a comparison of our findings (the role we find for TBX2 in IHC vs OHC differentiation) with other cell types would be very interesting. However, the constrained space of a Nature article does not allow for this topic to be adequately addressed. We anticipate that this will be covered in separate reviews or commentaries, by ourselves or by experts in other developmental systems.

Referee #2 (Remarks to the Author):

This is an interesting follow up study to the authors' manuscript on the role of Insm1 in hair cell development. In that earlier study, they found that Insm1 loss led to the conversion of OHC to IHC. Tbx2 was one of the genes whose expression changed upon Insm1 loss. Here they investigated the role of Tbx2 in the development of these two cell types. The study relies on ko mouse strains and immunohistochemical markers, which define these cell types. The work is well done and interesting. Those in the field will appreciate the fact that Insm1 appears to make cells able to ignore inductive cues that can lead to Tbx2 expression, and that Tbx2 expression is both necessary and sufficient for IHC formation. It is particularly interesting that Tbx2 can enact this determination event even in postnatal OHC's when Tbx2 is delivered via an AAV in an explant.

Recommended additional experiment:

In lines 49-54, the authors claim that less than half of Insm1-/- OHCs misexpress Tbx2, which is not expressed in wild-type OHCs, and only those cells transdifferentiate into IHCs (Extended Fig 1b,c). However, Extended Fig 1b,c simply show the in situ images of OHCs with or without Tbx2 expression. Also, in lines 473-474, the legend says “Tbx2 is expressed in about half of the OHCs, presumably those transdifferentiating into IHCs”. The underlying premise of this study is that Tbx2 is the critical factor among the core set of early IHC-specific genes misregulated during the homeotic transformation of Insm1-/- OHCs into IHCs. *Can the authors demonstrate that only Insm1-/- OHCs expressing Tbx2 transdifferentiate into IHCs while Insm1-/- OHCs without Tbx2 expression do not? This will also strengthen the finding that Tbx2 is epistatic to Insm1*.

We now provide evidence that, in neonatal *Insm1* conditional KOs, all of the outer compartment hair cells expressing *Tbx2* (about half of them, as expected) already co-express the functional IHC marker *Vglut3*, whereas none of the outer compartment hair cells miss-express either *Vglut3* or *Tbx2* alone. This supports the interpretation that expression of *Tbx2* by OHCs correlates completely with transdifferentiation into IHCs. We provide this new evidence in added panels (f,g) to extended Fig. 1.

Minor points

• The authors could do a better job in the opening paragraph to lay out their rationale earlier on.

We added the following at the beginning of the opening paragraph: “With the goal of identifying a gene driving IHC-specific differentiation, we surmised that it would (1) encode a gene regulator such as a transcription factor, (2) be expressed during normal development in IHCs but not OHCs, and (3) be expressed during abnormal development (that of *Insm1* mutants) in those OHCs that transdifferentiate into IHCs. One such gene is *Tbx2*.”

• The authors should include the quantification of each immunofluorescence image.

We are unclear as to what sort of quantification is being requested (we inquired with the editor and he was also unclear). If this means the number of IHCs that express any given OHC marker, these are all of the IHCs in the various *Tbx2* cKO mutants (whether deleted by *Atoh1*-Cre, *Gfi1*-Cre or *Fgf8*-CreER with Tamoxifen at P0 to P9), and none of the IHCs in the controls, for all the cochlear tissues examined. We

now mention in each figure legend that, for the mutants, “all of the examined cells” in the position of the IHCs displayed features of OHCs and not of IHCs.

However, if what was suggested is that we quantify the intensity of each fluorescent channel, we don’t think this will be appropriate for our figures because they are confocal projections of 3-D tissues (whole mounts of the organ of Corti). Our understanding is that with confocal imaging, the intensity varies with the depth of the cell within the tissue and the distance from the objective. In addition, we think that quantification of intensity for each fluorescent marker is not necessary, because these immunofluorescences are either detected or not in IHCs vs OHCs with the confocal conditions used (the same for IHCs and OHCs as well as for experimental and control tissues). What we have quantified are nuclear volumes (extended Fig. 2) and number of cells expressing signs of early OHC differentiation (*Insm1* and *Bcl11b* expressing cells; extended Table 2).

• Adding a schematic figure for the mechanisms of the transdifferentiation between IHC and OHC would be helpful to readers.

We are adding a new Extended Fig. 4 with a schematic depiction of our results.

Referee #3 (Remarks to the Author):

Hair cells are required for hearing and balance function. In the mammalian cochlea, inner hair cells are the primary receptors of sound and outer hair cells serve as amplifiers, and thus understanding determinants of their cell fate is highly significant. The current manuscript has elegantly characterized the expression and function of Tbx2 as a master regulator that is necessary and sufficient for inducing hair cell fate. The claim that Tb2 is required for inner hair cell fate in the neonatal cochlea is convincing, supported by transgenic models, in situ hybridization, and rendering the IHC-deficient cochlea non-functional, and interestingly leading to outer hair cell-like cell formation in the inner hair cell position. This is provocative and supported by some molecular markers, measurement of nucleus size, and electrophysiology experiments, and can be improved upon by further characterization and quantification (see below). Furthermore to build on what they have shown that OHC can convert to IHC-like cells in the absence of Insm1, Insm1/tb2 dcKO OHCs fail to convert. Both early neonatal and (P1-8) IHCs can still convert but P8 IHCs are still maturing (see below) so the claim of fate maintenance is only partly supported. Lastly, viral transduction of Tbx2 to OHCs can promote and IHC fate, but the extent is not fully characterized and the claim of sufficiency also will benefit some addition work. Overall, the manuscript is well written and some of the claims supported, but will benefit from having the following points addressed. I recommend a revision.

Major

(1) Direct switch between IHC and OHC-this seems to be lack sufficient support. First one would expect some intermediate genes (coexpressing of IHC and OHC) without de-differentiation (ie hybrid IHC/OHC), prob the need to assess multiple timepoints to more thoroughly show this. Electrophysiology and bundle morphology changes are also expect to shift over time, given the partial conversion reported (Chessum et al, 2018). The current dataset is supportive of a fate switch, but how and the extent of this happening is not clear.

We do see hybrid IHC/OHC cells, expressing functional markers of both cell types at the same time points during transdifferentiation. To better demonstrate this, and as suggested by the reviewer, we have examined transdifferentiated cells daily from P1 to P8 after temporal induction of *Tbx2* ablation with Tamoxifen administration at P0. We show that after ablation of *Tbx2*, IHCs acquire expression of mature OHC markers PMCA2 and Prestin, while at the same time still expressing the IHC functional marker VGLUT3. This co-expression extends from as early as P1 and at least until P7. We also show that at no time point during this transdifferentiating period do the IHCs converting into OHCs express SOX2, which is expressed in nascent hair cells and, hence, would be expected in IHCs if they de-differentiated before differentiating as OHCs. This is in addition to our prior data showing that at no time point do these postnatal transdifferentiating cells express the early OHC markers Insm1 and Bcl11b. This is shown in a new extended Fig. 4.

As we show in Fig. 3, the postnatal cells that transdifferentiate directly from IHCs to OHCs, without de-differentiation, become in nearly all respects like OHCs, including their electrophysiological profiles. However, we are not examining the cells electrophysiologically during transdifferentiation to show intermediate/hybrid characteristics for two reasons: (1) The whole-cell currents that distinguish IHCs from OHCs are the results of multiple channels. As these switch their expression from those of IHCs to

OHCs, the cells would have altered currents, but in ways that could not be clearly interpreted as hybrids; (2) The whole-cell currents that distinguish IHCs from OHCs are still developing and are not clearly defined during postnatal days P1 to P8, when transdifferentiation is occurring. The best way to show that transdifferentiating cells go through an intermediate/hybrid state in which they express features of IHCs and OHCs is gene expression, as we show in the new extended Fig. 4.

With respect to Chessum et al., 2018, this manuscript reported that a mutation in the OHC transcription factor Helios had a mild effect on OHCs (lower expression of some markers but retention of electromotility, $I_{K,n}$ currents and stereocilia arrangement), whereas expressing it ectopically in IHCs made them lose some IHC properties and acquire a few OHC properties. Based on this partial conversion, they conclude that "Helios functions to decrease the expression of early pan-hair-cell markers […] as well as to upregulate OHC marker genes", "consistent with a partial role for Helios in regulating OHC-fate". What we find by removing TBX2 is much more striking and in keeping with a master regulator: a complete conversion of IHCs into OHCs if the removal is embryonic, and nearly complete (with the exception of stereocilia arrangement, which we suspect is irreversible once established) if the removal is postnatal. The conversion reported by Chessum is partial because the role of Helios is secondary, not because IHCs cannot completely convert into OHCs, as we show with *Tbx2* ablations.

2) hair cell characterization: IHC and OHC are distinguished by numerous other factors in addition to molecular markers and nucleus size, it is important to also characterize bundle morphology (e.g. high res f-actin staining or SEM) and innervation (similar this group's previous paper, Webber et al., 2021). The data on the claim that tbx2 is sufficient in inducing an IHC fate is not well supported. One would expect a battery of molecular markers labeling IHC in the OHC region, bundle changes, ephys changes similar to what has been shown in Wiwatpanit et al.,

This request to further characterize the transdifferentiated cells poses different demands in the conversion of TBX2-lacking IHCs to OHCs, obtained with conditional mutant mice, from the conversion of TBX2-expressing OHCs to IHCs, obtained with viral transduction and ectopic expression in cochlear explants. We are addressing both, but to a different extent due to experimental limitations.

Regarding the IHC to OHC conversions in the absence of TBX2, we have added the requested high-resolution phalloidin staining as well as immunohistochemistry of the OHC-stereociliary marker PMCA2. Altogether, we attest the conversion of IHCs into OHCs with 10 molecular markers, three physiological properties (gain of $I_{k,n}$ and electromotility and loss of $I_{k,f}$), and five anatomical features (nuclear size, nuclear position, cellular shape, number of ribbons, and stereocilia). This resulted in new panels to Figs. 1-3 and added columns to extended Table 1. In all these respects, IHCs convert into OHCs if *Tbx2* is ablated embryonically. Much of the same applies to postnatal transdifferentiation except for the stereocilia which, although expressing the OHC stereociliary-marker PMCA2, nonetheless display an overall bundling that resembles that of IHCs. We believe that this lack of conversion in stereociliary bundle arrangement after birth is due to the irreversibility of these structures once they have begun to form, as they have not been shown to reform if eliminated after development.

In the revised manuscript, the above observation and argument are stated as follows: "These postnatally transdifferentiated hair cells, however, were not like OHCs in one respect: their stereocilia, despite expressing the OHC marker PMCA2, had the overall appearance of IHC

stereocilia (extended Table 1 and Fig 3k,k'). This may be attributed to the irreversibility of stereocilia shape and arrangement once formed: in mouse cochleae hair cell stereocilia develop during a critical, embryonic to early postnatal period[12] and, if subsequently lost, are never reformed[23,24]. Hence, it is quite possible that cochlear HCs cannot alter their stereocilia size and bundling arrangement once formed, in keeping with the lack of plasticity of this organelle, even if their cellular identities switch from IHCs to OHCs, as it otherwise occurs by ablating Tbx2 in IHCs."

With respect to innervation, we have examined it (see below), but prefer not to include it in this manuscript because it is not a defining characteristic of IHCs or OHCs. What we concluded in our Webber et al., paper was precisely that the innervation received by a hair cell is determined by multiple factors and not just by whether it is an IHC or an OHC. We reported that IHCs in the position of OHCs can be innervated as IHCs, as OHCs, or not at all, depending on context. With *Tbx2* cKOs we have found that, when we delete *Tbx2* embryonically and IHCs differentiate as OHCs, the approaching Type I afferents fail to contact them. [REDACTED]. This failure is in keeping with IHCs converting into OHCs, since the latter are not innervated by Type I afferents. However, this occurs when the IHC to OHC conversion occurs prior to innervation. When it occurs afterwards (i.e., postnatally), our preliminary observations are that already contacting Type I afferents remain in contact, although they lose their functionality, i.e. they lose the characteristic apposition of presynaptic ribbon to postsynaptic GLUR2/3 and, based on ABRs, there is no signaling from hair cell to neuron. Characterizing the innervation of these hair cells at different times of transdifferentiation is a high priority for us, as it would teach us about the rules governing the establishment and maintenance of innervation and synaptic communication. However, including these analyses in this manuscript would divert from it.
Documenting and explaining all of the above (in addition to the type of efferent innervation, which our Webber et al. study predicts will be determined by the location of the hair cells and the type of afferent innervation received, regardless of whether it is an IHC or an OHC) would take as much space as this journal allows for the entire manuscript. The point of this manuscript is that TBX2 acts as a master regulator to make IHCs different from OHCs. This is true regardless of the type of innervation the cells receive.

[REDACTED]

Regarding the TBX2 ectopic expression turning developing OHCs into IHC-like cells, the limitation is that we do this in virally-infected explant cultures, which we can only perform during a very restricted time window (~ a week perinatally), before many of the definite markers of IHCs and OHCs have been expressed, and before the differences in stereociliary bundle arrangement are visible (other than expression of PMCA2, which we now include). Also, we cannot record currents from these preparations (in part because these have not developed yet). However, the point is that expression of TBX2 leads to at least a partial conversion from IHCs to OHCs, because they express VGLUT3 (the earliest mature IHC marker to be expressed, and thus suitable for these early explant cultures) but fail to express Prestin (one of the two earliest marker of mature OHCs we know of, and thus one that we can use to assess OHC fate in these cultures). As additional evidence, we are now also showing that OHCs ectopically expressing AAV-delivered TBX2 lose expression of PMCA2 (new Fig. 4d), the other marker of mature OHCs that begins to be expressed soon after birth, and hence during the lifespan of the cochlear explant cultures. From these results, however, I think we can conclude that TBX2 is sufficient for making a hair cell differentiate (at least partially, to the extent that we can assess) as an IHC and not an OHC. We have modified the text to accommodate this limitation as follows: "Hence, ectopic expression of TBX2 in perinatal OHCs caused their transdifferentiation (at least to the extent that we can assess in the timespan of these cultures) into IHCs".

3) IHC fate maintenance-IHCs continue to mature in the postnatal period at least till P21-30 (Payne Font Synap neurosci 2021), although some molecular markers such as Vglut3 is expressed early and mature IHCs. It is difficult to appreciate whether tbx2 maintains IHC fate in both immature and mature IHC without deleting tbx2 later. It is also difficult to tell if P8 deletion less efficiently convert cell fate than early. One possibility is to use Vglut3cre to delete tbx2 at later timepoints (Li et al., Hear Res 2018).

We are not claiming that *Tbx2* is required for IHCs to maintain their fully-differentiated state throughout life, but throughout development (embryonic and postnatal until at least P9). The text of the manuscript states the following: "Therefore, it appears that TBX2, in addition to being necessary for initiating the specific differentiation of IHCs during embryogenesis, is also required throughout the postnatal period (at least until P9) for IHCs to continue expressing their already acquired features and to keep them from expressing those of OHCs; in other words, to maintain their differentiating state". The words in parenthesis have been added to the prior version for clarification.

Nonetheless, we appreciate these suggestions, think these are valuable experiments, and are performing them in order to determine the role that *Tbx2* may have in adult IHCs. However, regardless of the results, they would not alter our conclusion (based on *Tbx2* ablations up to P9) that *Tbx2* is required throughout much of their development to maintain IHCs differentiating properly and not as OHCs (see below for a more complete explanation). Given that these experiments would delay us for a year (we ordered the suggested Vglut3-CreER mice from Jackson, but until this week it has been unavailable; upon arrival, it would take us 6 to 12 months to generate and examine mice with the right genotype), and that the results would not alter the conclusions of our manuscript, we consider them appropriate for a subsequent study, and beyond the scope of the present one.

Finally, for clarification, up to at least P9 (the latest we can reliably target with the available *Fgf8*-CreER line), *Tbx2* ablation (with Tamoxifen) results in all HCs in the position of the IHCs to replace nearly all of

their IHC features with those of OHCs (Table 1). The one exception is stereocilia arrangement, which once established does not revert with a postnatal transdifferentiation.

Minor:

1)- Line 47: “In contrast, we do not detect Tbx2 mRNA in cells of the outer compartment of the organ of Corti (OHCs and supporting Pillar, Deiters’, and Hensen’s cells) (Extended Fig 1a)”. It does look like there was some mRNA in the outer compartment just much less than the inner compartment. Providing the positive and negative control samples and what probes were used for RNAscope would be useful.

We suspect that these few dots detected in 1a (but not in 1d), represent non-specific background. However, to be more precise we now state that “we detect little or no *Tbx2* mRNA in cells of the outer compartment of the organ of Corti (OHCs and supporting Pillar, Deiters’, and Hensen’s cells) (Extended Fig. 1a,d,f)”. By RNAseq, we had previously found that *Tbx2* mRNA levels were 20x higher in IHCs (2400) than in OHCs (100) (Wiwatpanit et al., 20218). While it is impossible for us to know for certain whether the low level of *Tbx2* mRNA detected in OHCs and other cells of the outer compartment has any physiological significance, the lack of OHC phenotype specifically in the *Atoh1*-Cre and *Gfi1*-Cre cKOs argues against such a role.

The positive controls are the high levels of *Tbx2* mRNA detected by ISH in the adjacent IHCs and in other cells lining the scala media, while the negative controls are other non-epithelial cells in the section. A better negative control, the *Tbx2* cKO, is not possible in this case because the large probes that we used detect portions of the mRNA (nucleotides 1718 – 3076, most of exons 6 to 7; Cat No 448991) that are not deleted by the cKO. This conditional KO deletes exon2 (nucleotides 393-663). However, our intention with this figure is to show that the levels of *Tbx2* mRNA are much higher in IHCs than in OHCs, suggesting a role in IHCs, as the manuscript proceeds to reveal.

2)- Line 68: “In these mice, the cochlear hair cells in the position of the IHCs (the inner or medial compartment)”. What does inner or medial compartment mean in refer to the position of IHCs mean?

The inner or medial compartment is the part of the organ of Corti medial to the tunnel of Corti. It normally consists of IHCs and their associated supporting cells (Inner Pillar Cells, Inner Phalangeal Cells, and Inner Border cells). We clarify this in the text in the following way: “In these mice, the cochlear hair cells in the position of the IHCs (i.e., those in the inner, or medial, compartment of the organ of Corti)”.

3) Line 90: “Upon maturation (P25-29), we dissociated fluorescently labeled ic-OHCs from these mice, as well as from IHCs and OHCs obtained from control mice, and performed whole-cell, patch clamp recordings”: Why was this done by dissociation? Why was this not done in situ, if this is just an age issue could it not have at least been done in the apex?

These recordings were performed on dissociated cells in order to measure electromotility.

4) Line 101: “We generated mice lacking both TBX2 and INSM1 in embryonic IHCs and OHCs (Atoh1Cre; Tbx2F/F; Insm1F/F) and found that Tbx2 is epistatic to Insm1: none of the OHCs transdifferentiated into IHCs, and all of the IHCs transdifferentiated into OHCs (Fig 2, extended Table 1 and extended Fig 2)”: the authors should provide information on the efficacy of gene deletion of both alleles.

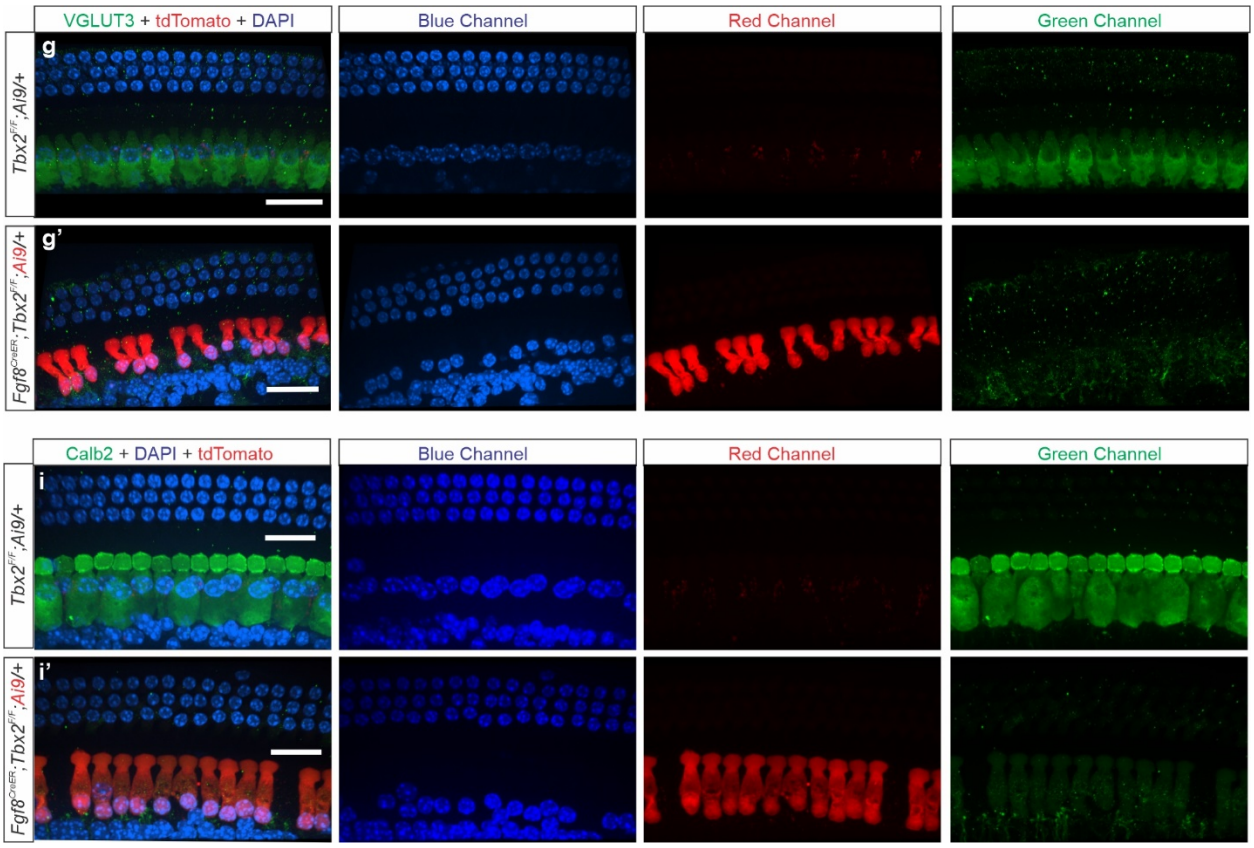
We previously reported the efficacy of deletion of *Insm1* as follows: “In $Atoh1^{Cre/+}$;$Insm1^{Flox/Flox}$ cochleae, *Insm1* mRNA is present in 43% of OHCs at E16.5, reduced to 7% (4/54) at E17.5, and entirely absent from all OHCs on day P1” (Wiwatpanit et al., 2018. Extended Data Fig. 2). In that manuscript, we explained that this transient expression at earliest stages is not the reason why only about half (42.6 ± 10.9%) of the OHCs transdifferentiate into IHCs because $TgPax2^{Cre/+}$;$Insm1^{Flox/Flox}$, in which *Insm1* was deleted before the onset of its expression, the fraction of transdifferentiated cells was indistinguishable (46.0 ± 5.64%). This, however, is not relevant for the present study because in double KOs of *Insm1* and *Tbx2* none of the OHCs transdifferentiate into IHCs. To summarize, the efficacy of deleting *Insm1* in $Atoh1^{Cre/+}$;$Insm1^{Flox/Flox}$ mice is 100% by P1.

With respect to *Tbx2*, we could not accurately perform the equivalent quantification because of the high expression on this mRNA in Inner Border and Inner Phalangeal cells, which sheath the IHCs, impedes us from unambiguously distinguishing whether some of the *in situ* signal comes from the IHC or from the IBC/IPhC. However, we can safely deduce that the efficacy of *Tbx2* deletion in IHCs of $Atoh1^{Cre/+}$;$Tbx2^{Flox/Flox}$, $Gfi1^{Cre/+}$;$Tbx2^{Flox/Flox}$, and $Fgf8^{CrETe/+}$;$Tbx2^{Flox/Flox}$ (receiving tamoxifen anytime from P0 to P9) is complete because all of them transdifferentiate into OHCs. These three drivers express Cre at very high levels. Likewise, we can deduce that the efficacy of *Tbx2* deletion in OHCs of $Atoh1^{Cre/+}$; $Insm1^{Flox/Flox}$;$Tbx2^{Flox/Flox}$ is complete because none of them transdifferentiate into IHCs.

5) - Lines 143: “Therefore, it appears that TBX2, in addition to being necessary for initiating the specific differentiation of IHCs during embryogenesis, is also required throughout the postnatal period for IHCs to continue expressing their already acquired features and to keep them from expressing those of OHCs; in other words, to maintain their differentiating state.” Interesting that even the hair cells which are not tdTomato positive seem to have lost their vglut3 and calb2 expression – why is this? Also I would like to see these in single channel images.

This refers to data shown in former Fig. 3h’ and j’ (3g’ and 3i’ in this resubmitted version). All of the cells in the positions of the IHCs are TdTomato+, there are no cells that lost VGLUT3 or Calb2 that did not express TdTomato. What may be confusing in these panels is that there are some gaps between the cells in the IHC row. As the DAPI shows, there are no cells in these gaps. These gaps may be explained because as the flask-shaped IHCs transdifferentiate into thinner, cylindrical OHCs with smaller nuclei such that some gaps appear between them. These gaps are not evenly spaced between hair cells, but seem to form randomly. It is also possible that these gaps correspond to death of some of the TBX2-defficient IHCs. Either way, all the hair cells that remain are TdTomato+, VGLUT3- and Calb2-.

As requested, we provide the single channel images of these merged images below. These reveal that the gaps with no TdTomato+ cells contain no cells at all, as there are no nuclei (imaged in the DAPI channel).



6) Line 158: "While non-transfected IHCs lacking TBX2 transdifferentiated into OHCs and expressed Prestin, transfected IHCs, identified by expression of mCherry, did not appear to transdifferentiate as they did not express Prestin (Fig 4a,a')": Really need another hair cell marker (e.g. myo7a) here, very hard to tell what cells were transduced in Fig 4A. Seems low efficiency.

In Fig. 4a,a' we show a single row of hair cells that are in the IHC position, all of which have lost endogenous TBX2 because their genotype is *Fgf8*$^{CrETe/+}$;*Tbx2*$^{Flox/Flox}$ and they were exposed to 4-Hydroxytamoxifen. Only those expressing ectopic TBX2 (mCherry+) fail to express Prestin. There are no other cells in the IHC row other than hair cells. Myosin 7a immuno would certainly confirm this, but we feel this is unnecessary. These panels (a,a') are not the significant finding of the figure, only a reassuring proof of principle consistent with the main result. The panel (Fig. 4a,a') simply shows that the viral delivery of TBX2 is functional because it rescues TBX2-defficient IHCs. However, the next panels show that ectopic expression of TBX2 in wild type OHCs makes them transdifferentiate (express VGLUT3 instead of Prestin and PMCA2). Even if the rescue in panels a,a' had failed (for example if the virally-produced TBX2 was at improper levels, or stage, for normal IHC development), the results in OHCs would be equally meaningful. What the referee asks for is conceptually easy to do, but it would be difficult for us because the antibodies to Myo7a and Prestin are both rabbit. And we need timed pregnancies to set up these cultures. We could work the conditions for a double rabbit immuno and do them in newly scheduled cultures, but it would delay us a couple of months for something that we believe is unnecessary.

Incidentally, to strengthen this figure, we have added a new panel (Fig. 4d) showing that OHCs ectopically expressing AAV-generated TBX2 do not express another OHC marker, PMCA2.

7) typo: deficient in abstract, line 117-loosing

Corrected.

8) figure 1-quantitative-in situ-where was this quantified?

This type of *in situ* hybridization from RNAscope is generally referred to by the company as "quantitative" because under certain conditions it can be quantitated (by eye counting of dots and, in my opinion, imprecisely). However, we did not quantify our *in situ* hybridization results as our intention is just to see expression of *Insm1* and *Bcl11b*, and not of *Fgf8*, *Brip1* and *Msx1*, in IHCs of *Tbx2* cKOs. Hence, we have replaced the term "quantitative" with "RNAscope" to describe the kind of *in situ* hybridization performed.

9) Line 124: "Hence, unlike IHCs missing TBX2 embryonically, which differentiated as OHCs (Fig 1 and extended Table 2), these transdifferentiating cells switched from IHCs to OHCs without recapitulating OHC differentiation": I want to see some actual images here to show this in more detail – cells lacking or gaining an IHC or OHC respectively. I would also like to know what the bundles look like, do you get the classic OHC "V" shaped bundle?

As requested, we are now adding a new figure (extended Fig. 4) showing with images and a summary table that cells that are transdifferentiating postnatally (*Fgf8*$^{CrETe/+}$;*Tbx2*$^{Flox/Flox}$ mice that received Tamoxifen at P0) express markers of both IHCs (VGLUT3) and OHCs (PMCA2 and Prestin) from as early as P1 (PMCA2) or P4 (PMCA2 and Prestin) to P7 (after which VGLUT3 subsides). PMCA2 labels the stereocilia. However, as we explained above (in addressing the major point 2 of this reviewer) and show in the revised manuscript (new panels to Fig. 3), the one feature that does not change in postnatally transdifferentiating IHCs to OHCs is their stereociliary bundle arrangement, which we think is due to the irreversibility of cochlear stereocilia arrangements once formed.

To this new extended Fig. 4 we have also added images and a summary showing no expression at any point during the transdifferentiation (from P1 to P8) of: (1) the developing OHC markers *Insm1* (mRNA) and *Bcl11b* (mRNA and protein); and (2) the nascent hair cell marker SOX2. The data on SOX2 is new and was not requested by any referee, but we feel it corroborates our conclusion that, after postnatal deletion of *Tbx2*, IHCs transdifferentiate directly from maturing IHCs into maturing OHCs without de-differentiating to recapitulate OHC development.

10) Electrophysiological characterization: There seems to be paucity of info on cells patched, and the variability of results which should be expected to occur a bit. One would also expect to have ephys data on a cell that was not recombined displaying the properties of an IHC. Also were the ephys findings throughout the cochlea – where were these ic-OHCs from? Also should have the ages in the figure.

Hair cells were obtained from the apical ¼ of the cochleae of mice at P25-P29. This information has been added to the legend of Fig. 1 in the revised manuscript.

In the mutants, all of the IHCs were recombined and transdifferentiated into ic-OHCs. Hence, we cannot record from a non-recombined IHC of *Tbx2* cKOs. What we have done is to record from the IHCs of control littermates, which naturally display the physiological properties of IHCs.

Reviewer Reports on the First Revision:

Referees' comments:

Referee #2 (Remarks to the Author):

The authors have satisfactorily addressed the critique and the paper is now acceptable.

Referee #3 (Remarks to the Author):

The authors have done admirable work to address my previous comments, and have provided new and convincing data supporting OHC-IHC switch, without dedifferentiation. The lack of bundle conversion despite fate switch in the postnatal, but not embryonic, period is striking and fascinating, and still support their overall conclusion. I also appreciate the detailed explanation on why some of the additional experiments were not done due to time constrains or details that are better served for future manuscripts. Overall I have no more concerns and wholehearted recommend acceptance of this elegant study.

Alan Cheng