Multiple PDZ domain protein maintains patterning of the apical cytoskeleton in sensory hair cells

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MS TITLE: Multiple PDZ Domain Protein Maintains Patterning of the Apical Cytoskeleton in Sensory Hair Cells

AUTHORS: Amandine Jarysta and Basile Tarchini

I sincerily apologise for the delay before being able to come back to you with a decision. 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.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript with the exception of the request of biochemical experiments under point 1 of Reviewer 2 which I tend to believe is not necessary for the manuscript to be published. Please detail your answers in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Reviewer 1

Advance summary and potential significance to field

While the majority of studies relating to planar polarity and stereociliary bundle morphogenesis in the inner ear are focused on the onset of these events this manuscript is unique and significant in that it identifies factors that are essential their maintenance. Moreover, the manuscript demonstrates that maintenance is required for auditory function as MPDZ mutants have high frequency hearing loss.

Comments for the author

This manuscript by Jarysta and Tarchini describes the function of a putative scaffolding protein, MPDZ, in the maintenance of hair cell stereociliary bundle morphology, likely by maintaining the polarized distribution of a set of intracellular polarity effectors including GNAI, GPSM2 and aPKC. MPDZ is proposed to bind MPP3 and CRB3 to assemble a unique CRMB protein complex that is proposed to maintain GNAI,GPSM2,aPKC distribution but is not required for initiating there distribution on either side of the stereocilia. A similar apical cell surface organizer mechanism is proposed to be functionally significant for choroid plexus function. A relation to hydrocephalus phenotypes is discussed though for unknown reasons this MPDZ mutant line is viable while others are not.
The manuscript is well written with clear and well-formatted figures, and the conclusions based upon these genetic studies are sound. Outstanding question is whether MPDZ directly binds to any of the proteins that comprise the molecular blueprint or CRMB complex. This is implied, but not demonstrated.
It may be beyond the scope of this study, but the identification of MPDZ binding partners would be a nice addition. Regardless, this is a strong manuscript that is suitable for publication in its current form.

Minor comments
Line 25 and 104, the phrase 'misaligned stereocilia' is ambiguous unless it is stated what anatomical reference they should be in alignment to. As written this phrase is easily misinterpreted as a PCP phenotype which is not the case.

Line 150: Why state 'a possibly reduced hair cell circumference' if surface area is reduced when measured (1E)?

MPP5 and CRB3 should be included within the summary/abstract since these molecules are a key part of the proposed MPDZ function

Reviewer 2

Advance summary and potential significance to field

The manuscript from Jarysta and Tarchini examines the role of MPDZ in development of apical polarity in hair cells. Tarchini's lab is leading the way in defining how cell polarity signals set up the location and structure of the hair bundle, and this manuscript is a nice addition to that literature. The manuscript is illustrated well with clear immunocytochemistry experiments, and key points are strengthened with convincing quantitative data and statistical inference. The manuscript is well written and the authors' key points are argued well.

That said, this manuscript is largely descriptive. Experiments with Mpdz mice definitely show that this gene plays an important role in polarity of apical proteins and structures, but we don't really know how. Likewise experiments with Gpsm2 KO mice and mice with inactivation of GNAI both demonstrate that MPZD localization depends on GNAI/GPSM2, but how?

The manuscript is very informative, but mechanistically is pretty sparse. A new player in hair cell apical polarization is introduced, but what does it do, really? While I feel that the data in the paper do justify the conclusions drawn, the advance in the field—an indication that MPZD plays a role in apical polarization—is not fully developed.

Comments for the author

Major points
1. Experiments with MPP5 and CRB3 localization suggest that these proteins are dependent on MPDZ for their location at the apical surface, but we don't know what this complex really is. It seems unlikely to be just MPDZ, MPP5, and CRB3, but that is the impression the authors give. Why does this (apparent) apical complex form, and why is INADL excluded? What other components contribute to this complex? While it is perhaps outside the scope of this work, biochemical experiments to define the composition of the complex would be interesting. Why is it localized at a non-canonical location in hair cells?

2. The supposed MPDZ-MPP5-CRB3 apical complex is definitely polarized, but it is less polarized than several proteins (e.g., aPKC and GNAI) that are dependent on this complex. I don't doubt that result—it's a key result and is well documented—but it raises interesting mechanistic questions that the present manuscript doesn't attempt to address. Is there anything to add?

3. An interesting question is how does the only-partially polarized MPDZ lead to highly polarized localization of aPKC, GPSM2, and GNAI? I know you don't have an answer.

Other points
Page 3, line 33. The antecedent for "This" is not crystal clear; perhaps replace with "Mechanical detection" or something like that. (This what?)

Page 3, line 38. No need to capitalize the "g" in "guanine" as the average reader can figure out that the capital G corresponds to the little g. Likewise, the name for GPSM2 does not need capital letters (just say "G protein signaling modulator 2).

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Page 7, line 149. Can these apical junctions be measured with the Circularity function in Fiji/ImageJ or a similar tool to quantify this?

Page 7, line 154. Given that reference [48] indicates a role for MYO7A in this process, any chance that MYO7A interacts with MPDZ?

Page 8, line 178. Be a little more clear what you mean by "longitudinal or medial sides."

Page 8, lines 187-189. This is pretty speculative; you could think of other models.

Page 10, line 234. Remove dash between "1" and "C."

Page 10, lines 257-258. We all have repeated failures of one sort or another; I don't think you need to mention them here. But given all of those failures, what makes you think that the CRB3 antibody is specific? I presume you didn't test the labeling in a Crb3 KO.

Page 37, line 2. Should be Gpsm2^DEL/DEL ("2" missing).

Reviewer 3

Advance summary and potential significance to field

This study by Jarysta and Tarchini identifies MPDZ as an important factor in development and function of cochlear hair cells. The experiments are nicely described, the results are clear and the images are outstanding. The study very nicely shows that MPDZ, MPP5 and CRB3 are generally localized to the same regions on the apical surface areas of hair cells and that these factors, which may form a complex, act to regulate different aspects of hair cell and hair bundle formation. Deletion of Mpdz also leads to changes in the distribution of GNAI and GPSM2, which reciprocally regulate MPDZ and MPP5, suggesting mutual interactions between the two complexes.

Comments for the author

I do have several concerns/questions. First, defects in bundle morphology are reported by P0, but changes in GNAI aren't observed until P4. Wouldn't this suggest that MPDZ is acting through additional pathways to influence bundle formation? The decrease in MPP5 labeling at hair cell junctions in MPDZ mutants could suggest that there are defects in junctional integrity. Was this examined at all? The hearing loss in Mpdz mutants, in particular at high frequencies, seems more

significant than one might expect considering that all hair cells are still present with hair bundles. Would it be possible to use FM143 to determine if hair bundles are even functional?

Additional specific comments
Line 116 cross-sectional views would be useful to demonstrate the position of MPDZ relative to the apex of the hair cells.

Line 128 the observation that these mutants do not die, while previous MPDZ lines do suffer from lethality raises concerns. True the antibody targeting the third PDZ no longer showed any labeling in the mutants, but what is the explanation for the differences between the different mouse lines? And does this suggest the possibility that the line used in this study retains some level of MPDZ expression/function?

Line 156 the difference in outer hair cell surface area is actually quite intriguing as this represents an increased decrease in overall surface area?

Does this suggest that MPDZ acts as a negative regulator of cell remodeling?
Of is this a secondary effect of the increased bundle size?

Line 164 GNAI distribution is normal in Mpdz mutants at P2, but defects in stereocilia are present by P0, correct? Wouldn't this suggest that MPDZ is affecting additional processes prior to GNAI distribution? What does the distribution of GNAI look like at P0?

Line 183 and figure 2C and D. There does appear to be a change in the spatial distribution of GNAI in the Mpdz mutants, but figure 2C just seems to illustrate a significant decrease in the total amount of GNAI across all three regions. Maybe this would be clearer if regions a, b and c were limited to the lateral compartment? Or is that the case already?

Line 284 figure 4E, it is not really possible to see localization of MPP5 in hair cell bundles.

Additional imaging should be provided to confirm this conclusion.

Line 289 the reduction in junctional MPP5 in Mpdz mutants is significant and could be an indication of junctional defects in the epithelium.

Line 315 there is a typo I think, should be Gpsm2 mutants not Mpdz mutants.

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:
While the majority of studies relating to planar polarity and stereociliary bundle morphogenesis in the inner ear are focused on the onset of these events, this manuscript is unique and significant in that it identifies factors that are essential their maintenance. Moreover, the manuscript demonstrates that maintenance is required for auditory function as MPDZ mutants have high frequency hearing loss.

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It may be beyond the scope of this study, but the identification of MPDZ binding partners would be a nice addition. Regardless, this is a strong manuscript that is suitable for publication in its current form.

Minor comments

Line 25 and 104, the phrase 'misaligned stereocilia' is ambiguous unless it is stated what anatomical reference they should be in alignment to. As written this phrase is easily misinterpreted as a PCP phenotype which is not the case.

We understand this concern and changed the language for "misaligned stereocilia placement" in these locations. Of note, at line 27 in the Summary, we also define stereocilia alignment as their distribution in rows.

Line 150: Why state 'a possibly reduced hair cell circumference' if surface area is reduced when measured (1E)?

We removed this part of the sentence to only hint at "abnormally round circumferences" as an introduction (line 173). This is followed by a quantification of the surface area in OHCs (and also in IHCs in the revised version). We next quantify circularity as well, to address comments from Reviewer 2.

MPP5 and CRB3 should be included within the summary/abstract since these molecules are a key part of the proposed MPDZ function

We agree and added MPP5 and CRB3 as requested.

Reviewer 2 Advance Summary and Potential Significance to Field:
The manuscript from Jarysta and Tarchini examines the role of MPDZ in development of apical polarity in hair cells. Tarchini's lab is leading the way in defining how cell polarity signals set up the location and structure of the hair bundle, and this manuscript is a nice addition to that literature. The manuscript is illustrated well with clear immunocytochemistry experiments, and key points are strengthened with convincing quantitative data and statistical inference. The manuscript is well written and the authors' key points are argued well.

That said, this manuscript is largely descriptive. Experiments with Mpdz mice definitely show that this gene plays an important role in polarity of apical proteins and structures, but we don't really know how. Likewise, experiments with Gpsm2 KO mice and mice with inactivation of GNAI both demonstrate that MPZD localization depends on GNAI/GPSM2, but how?

The manuscript is very informative, but mechanistically is pretty sparse. A new player in hair cell apical polarization is introduced, but what does it do, really? While I feel that the data in the paper do justify the conclusions drawn, the advance in the field—an indication that MPZD plays a role in apical polarization—is not fully developed.

Reviewer 2 Comments for the Author:

Major points

1. Experiments with MPP5 and CRB3 localization suggest that these proteins are dependent on MPDZ for their location at the apical surface, but we don't know what this complex really is. It seems unlikely to be just MPDZ, MPP5, and CRB3, but that is the impression the authors give. Why does this (apparent) apical complex form, and why is INADL excluded? What other components contribute to this complex? While it is perhaps outside the scope of this work, biochemical experiments to define the composition of the complex would be interesting. Why is it localized at a non-canonical location in hair cells?

We agree that biochemistry experiments testing interactions between GNAI-GPSM2 and MPDZ-MPP5-CRB3 will be informative and represent an interesting follow-up to this work. Of note however, these experiments typically involve co-IP with HEK293 cell extracts following plasmid transfection, or in vitro pull-down, as hair cells are very sparse. These approaches tend to limit the biological relevance of the interactions discovered for cytoskeleton polarization in sensory cells.

The reason we present CRB3-MPP5-MPDZ as a trio is that these proteins have been well characterized as one version of the Crumbs complex (the more canonical version being CRB3- MPP5-INADL/PatJ). We are simply referring to the literature that most frequently describes the Crumbs complex as tri-partite, but we agree that other proteins are likely enriched with CRB3- MPP5-MPDZ in hair cells. In fact, our results suggest that GNAI-GPSM2 may represent such additional components, at least on the lateral (bare zone) side. We added a paragraph in the discussion line 504 to raise this possibility and acknowledge remaining gaps in knowledge.

Of note, INADL exclusion from the MPDZ complex in hair cell is actually expected since MPDZ is a homolog of INADL with similar binding properties to MPP5 (line 83). Previous work established that MPDZ and INADL are mutually exclusive on this basis (Assemat et al., 2013 reference included in the manuscript).

Finally, one of the conclusions of this work is that considering apical junctions as a canonical location for the Crumbs complex including MPDZ may be a simplification: not only hair cells, but choroid plexus epithelial cells, show CRB3-MPP5-MPDZ at the apical membrane, and no MPDZ is detected at junctions in either model! We highlight these results and conclusions in the Results line 311 and Discussion line 479.

2. The supposed MPDZ-MPP5-CRB3 apical complex is definitely polarized, but it is less polarized than several proteins (e.g., aPKC and GNAI) that are dependent on this complex. I don't doubt that result—it's a key result and is well documented—but it raises interesting mechanistic questions that the present manuscript doesn't attempt to address. Is there anything to add?

This remains largely unclear. At line 369 in the Results, after concluding that CRB3-MPP5-MPDZ and GNAI-GPSM2 reciprocally influence each other's distribution, we propose that increased MPP5-MPDZ enrichment on both sides of the hair bundle in absence of GPSM2 may indicate that GNAI-GPSM2 somehow limits the total amount of MPDZ allowed to occupy the apical membrane.

This point is also discussed in the new paragraph line 504. We speculate that MPDZ might interact with GNAI-GPSM2, and that without anchorage at the bare zone provided by GNAI- GPSM2, CRB3-MPP5-MPDZ might be trafficked there in increased amounts as a compensation mechanism, but diffuse across the entire HC apex.

3. An interesting question is how does the only-partially polarized MPDZ lead to highly polarized localization of aPKC, GPSM2, and GNAI? I know you don't have an answer.

Indeed, any proposal would be speculation at this point. However, please note that MPDZ clearly does not drive initial GNAI-GPSM2 polarization since a loss of the GNAI-GPSM2 vs aPKC blueprint is not seen before P4. We emphasize that MPDZ is only required for GNAI- GPSM2 maintenance, and not its early polarization at the bare zone (Results line 264; Discussion line 425). To clarify this important point, we also added a simple model as Fig. 7 to capture together protein (de)localization in each group and bundle defects.

Other points

Page 3, line 33. The antecedent for "This" is not crystal clear; perhaps replace with "Mechanical detection" or something like that. (This what?)

We changed for "Detecting mechanical vibrations is the role of the hair bundle,.." to avoid any confusion. Line 34.

Page 3, line 38. No need to capitalize the "g" in "guanine" as the average reader can figure out that the capital G corresponds to the little g. Likewise, the name for GPSM2 does not need capital letters (just say "G protein signaling modulator 2).

Agreed, modified as suggested. Line 40.

Page 3, line 45. What do you mean “that coincides with the…surface”?

We changed the language line 48 to read “…by antagonizing the polarity kinase aPKC that labels the HC surface decorated with microvilli”.

Page 4, line 77. Where it says “which in turn” it is not clear what this refers to (MPP5/PALS1 or MPDZ/INADL/PATJ or both).

We changed the language line 84 to read “…, with MPP5 in turn binding the integral protein CRB3 to form the Crumbs complex”.

Page 4, line 91. Correct to “…in the choroid plexus, which secretes…” Corrected.
Page 7, line 149. Can these apical junctions be measured with the Circularity function in Fiji/ImageJ or a similar tool to quantify this?

We improved this part of the analysis in two ways: first, we extended quantifications to IHCs (which are less affected than OHCs), and second, we took this suggestion and measured circularity in both OHC and IHCs at P0 (base) and P4 (base and apex). As expected from visual inspection, the circularity is abnormally high (Fig. 1G), in particular in OHCs at P4, in line with their failure to adopt a “peanut” shape and with their reduced surface area (Figure 1F).
Circularity is represented by a 0-to-1 coefficient where 1 denotes a perfect circle.

Page 7, line 154. Given that reference [48] indicates a role for MYO7A in this process, any chance that MYO7A interacts with MPDZ?

We were curious about that possibility. To ask this question in line with approaches used in the manuscript, we immunolabeled MYO7A in *Mpdz* mutants and control littermates at P4. Using the DSHB MYO7A antibody that we routinely use and that produces a strong and specific signal in hair cells, we did not observe the medial/peripheral apex polarization shown in Etournay et al., 2010 (Figure 6A in their publication). As our MYO7A signal at the apical membrane was unchanged in the mutants, and because the antibody used by Etournay and colleagues is not readily available, we did not include these results in the revision or pursue further.

The idea that MYO7A might be working with MPDZ to exert anisotropic force on junctions to transition from a circular to a peanut-shaped outline is very interesting and warrants further investigations.

Page 8, line 178. Be a little more clear what you mean by “longitudinal or medial sides.”

We modified the explanation as follows line 207: “GNAI was variably depleted at the center of the bare zone and at stereocilia tips, but generally retained at the bare zone periphery in *Mpdz* mutants (Fig. 2C). Interestingly, GNAI was ectopically found 1) at the medial apical membrane (Fig. 2C; arrow), and 2) at the medial HC junction with the neighboring support cell (Fig. 2C; hollow arrowheads).”

Page 8, lines 187-189. This is pretty speculative; you could think of other models.

Also taking into account comments from Reviewer 3 on GNAI quantification at 3 sub-regions of the lateral apex (bare zone; regions a, b, c), we added in Supp. Fig. 2 an equivalent quantification of GNAI signals in the corresponding 3 sub-regions of the medial apex (regions d, e, f).

We found that GNAI is principally lost in the central region of the bare zone (b in Fig. 2D) in *Mpdz* mutants, and that ectopic enrichment at the medial apex is not significantly different across the 3 sub-regions. As this result do not support GNAI seeping from the peripheral sides of the hair bundle towards the medial apex, we modified the conclusion of this paragraph line
206. We now conclude more soberly that GNAI is first lost near the basal body on the lateral side, and progressively invading the medial apex, perhaps relocalizing from the lateral to the medial side.

Page 10, line 234. Remove dash between “1” and “C.” Done.
Page 10, lines 257-258. We all have repeated failures of one sort or another; I don’t think you need to mention them here. But given all of those failures, what makes you think that the CRB3 antibody is specific? I presume you didn’t test the labeling in a Crb3 KO.

Actually, Szymaniak et al., Dev Cell 2015 (reference cited) tested the CRB3 antibody used here on trachea airway tissue from *Crb3* mutants, and validated the signal observed in controls as specific (Fig. 5B in their publication). We thus removed the notion of repeated failures as recommended line 293 and also clarified that this antibody was previously validated.

Page 37, line 2. Should be Gpsm2^DEL/DEL (“2” missing). Thank you, corrected.

Reviewer 3 Advance Summary and Potential Significance to Field:
This study by Jarysta and Tarchini identifies MPDZ as an important factor in development and function of cochlear hair cells. The experiments are nicely described, the results are clear and the images are outstanding. The study very nicely shows that MPDZ, MPP5 and CRB3 are generally localized to the same regions on the apical surface areas of hair cells and that these factors, which may form a complex, act to regulate different aspects of hair cell and hair bundle formation. Deletion of Mpdz also leads to changes in the distribution of GNAI and GPSM2, which reciprocally regulate MPDZ and MPP5, suggesting mutual interactions between the two complexes.

Reviewer 3 Comments for the Author:
I do have several concerns/questions. First, defects in bundle morphology are reported by P0, but changes in GNAI aren’t observed until P4. Wouldn’t this suggest that MPDZ is acting through additional pathways to influence bundle formation?

This is correct indeed, and also our conclusion. We underscore this early in the Discussion, paragraph starting line 434 (“While delocalization of GNAI-GPSM2 is likely an important contributing factor, loss of MPDZ must interfere with other proteins required for early hair bundle development. Alternatively, hair bundle morphology might suffer from the relocalization of both MPP5 and CRB3 from the flat HC apex to stereocilia at P0 “). We also moderate at the very end of the Discussion (line 514, “MPDZ specifically patterns the HC apical membrane, in part by maintaining the proper planar segregation of blueprint proteins at neonate stages”).

As this important nuance was not made obvious enough, we modified the Results section to acknowledge early morphology defects after completing the analysis of blueprint proteins, line 268. Line 340 we also note that abnormal localization of CRB3-MPP5 in the hair bundle in *Mpdz* mutants at P0 (Fig. 4D-E; Supp. Fig. 5B-C) might be the cause of these early morphology defects. Finally, we show normal GNAI distribution but already defective hair bundles at P0 in our schematic summary in Fig. 7.

The decrease in MPP5 labeling at hair cell junctions in MPDZ
mutants could suggest that there are defects in junctional integrity. Was this examined at all?

We first worried that the decrease in MPP5 signal at HC junctions (Fig. 4G) might have been an artifact from the quantification method. Visually, we in fact do not observe a clear reduction of MPP5 at the medial junction (new Fig. 4E). Previously, we used a ~1.5x 0.5µm Region of Interest to quantify junctional signal, which encompassed a significant region of (medial) apical membrane as well, and consequently, did not strictly reflect junctional enrichment. We thus re-quantified MPP5 at the medial HC junction in the same sample set using a much narrower ROI (1.5 x 0.1µm) that more accurately encompasses the junction. The new graph in Fig. 4G still shows a mildly significant reduction of the signal.

Despite slightly reduced MPP5 junctional levels, we have no obvious evidence for defective junctional integrity: a) the enrichment of ZO1 and Afadin, used as marker for apical junctions in the study, appear normal in *Mpdz* mutants. b) Compromised junctional integrity would be expected to lead to aberrant cell-cell contacts, but we never observe a defect in the exquisite hair cell-support cell intercalation mosaic in *Mpdz* mutants. We now add this notion along with a representative illustration in Supp. Fig. 5A. This is reported line 326.

The hearing loss in Mpdz mutants, in particular at high frequencies, seems more significant than one might expect considering that all hair cells are still present with hair bundles. Would it be possible to use FM143 to determine if hair bundles are even functional?

We performed FM 1-43 at P4 to ask whether hair cells at the cochlear base with defective hair bundles can take up the dye. These results are presented in Supp. Fig. 1B and suggest that all hair cells are able to normally load the dye in mutants. However, we would argue that this is not necessarily at odd with severe hearing loss at higher frequencies. For example, *Gpsm2* and *Gnai* mutants, with much more severely stunted stereocilia and profound hearing loss (no ABR thresholds), were still able to load the dye (Tarchini et al. Development 2016).

We do appreciate this concern however, and we now remind the reader at the end of the Discussion (line 516) that constitutive *Mpdz* mutants might affect other structures and cell types required for auditory function besides hair cells and their hair bundle.

Additional specific comments

Line 116 cross-sectional views would be useful to demonstrate the position of MPDZ relative to the apex of the hair cells.

We agree, and added new panels in Fig. 2B where MPDZ enrichment is shown as a Z projection. Fig. 2B clarifies earlier in the manuscript that MPDZ is not enriched at apical junctions labeled with ZO1, and is only found at the apical membrane facing the cochlear duct past the junctions.

Line 128 the observation that these mutants do not die, while previous MPDZ lines do suffer from lethality raises concerns. True the antibody targeting the third PDZ no longer showed any labeling in the mutants, but what is the explanation for the differences between the different mouse lines? And does this suggest the possibility that the line used in this study retains some level of MPDZ expression/function?

We addressed this concern from multiple new angles.

First, we immunolabeled the choroid plexus of $Mpdz^{DEL}$ mutants with the MPDZ antibody. Reassuringly, signal at the apical membrane was missing, which is now shown in Supp. Fig. 1A and mentioned along with the loss of signal in $Mpdz^{DEL}$ hair cells in the Results (line 148).
Please note that we use the same MPDZ antibody used previously to show MPDZ accumulation in choroid plexus cells, and its absence in a distinct *Mpdz* mutant model that shows lethality and hydrocephalus (Yang et al., EMBO Mol Med 2019). This unambiguously defines lethality and hydrocephalus as distinct from the mere absence of MPDZ protein as seen in immunolabelings.

If MPDZ is uniformly lost in all mouse models, what can explain the lack of hydrocephalus and better fitness in the $Mpdz^{DEL}$ allele we use?

Second, we now clarify in the Discussion and Supp. Table 1 that the $Mpdz^{DEL}$ allele is in the C57BL/6N background used by the KOMP consortium, whereas previous mutants were in C57BL/6J (line 459). We have good reasons to believe that this is sufficient to explain the discrepancy. 1) Hydrocephalus is likely a leading cause of lethality in the previously described C57BL/6J *Mpdz* strains, and hydro is known to be highly sensitive to genetic background (Tapanes-Castillo et al. reference). 2) There is published evidence that other afflictions show different severity in C57BL/6N and C57BL/6J. For example, epilepsy linked to *Scn1a* mutation (Kang et al. reference). These notions are now reported in the Discussion with extra reference citations line 467.

Finally, we have two informal pieces of evidence that further substantiate this hypothesis. 1) We observed hydrocephalus in another mutant studied in our laboratory that was raised in the C57BL/6J background. We seem to be able to prevent hydro by breeding that mutant in the C57BL/6N background, however! (unpublished work still). 2) Dr. Arie Horowitz, who is the lead author of the (Yang et al., EMBO Mol Med 2019) study, shared with us in a call that he obtained the $Mpdz^{DEL}$ allele from KOMP as well. He failed to observe hydrocephalus in that strain as we did. This work is not published yet.

Line 156 the difference in outer hair cell surface area is actually quite intriguing as this represents an increased decrease in overall surface area? Does this suggest that MPDZ acts as a negative regulator of cell remodeling? Of is this a secondary effect of the increased bundle size?

It does represent an "increased decrease" indeed. Loss of MPDZ has two consequences on hair cell circumference remodeling: a decrease in surface area from P4, and a rounder shape compared to controls littermate. This suggests that during normal apex remodeling (including apical surface reduction), MPDZ is needed to reshape the circumference, and to positively influence surface area (i.e., limit reduction). In that light, we would argue MPDZ acts as a positive regulator of cell remodeling.

Etournay et al. 2010 showed that remodeling of the HC circumference can follow defects in the hair bundle, with in particular more flat and shorter bundles resulting in OHCs that lost their peanut shape and width. As bundles are flattened and stereocilia placement is off in *Mpdz* mutants, this could be the cause here as well. We cannot rule out however that MPDZ at the apical membrane also influences junctional remodeling directly, and these different topics are now better discussed in the paragraph starting line 488.

Line 164 GNAI distribution is normal in Mpdz mutants at P2, but defects in stereocilia are present by P0, correct? Wouldn't this suggest that MPDZ is affecting additional processes prior to GNAI distribution?

This is the same point as above (first comment). The reply made is copied here:

This is correct indeed, and also our conclusion. We underscore this early in the Discussion, paragraph starting line 434 ("While delocalization of GNAI-GPSM2 is likely an important contributing factor, loss of MPDZ must interfere with other proteins required for early hair bundle development. Alternatively, hair bundle morphology might suffer from the relocalization of both MPP5 and CRB3 from the flat HC apex to stereocilia at P0 "). We also moderate at the very end of the Discussion (line 514, "MPDZ specifically patterns the HC apical membrane, in part by maintaining the proper planar segregation of blueprint proteins at neonate stages").

As this important nuance was not made obvious enough, we modified the Results section to acknowlege early morphology defects after completing the analysis of blueprint proteins, line 268. Line 340 we also note that abnormal localization of CRB3-MPP5 in the hair bundle in *Mpdz* mutants at P0 (Fig. 4D-E; Supp. Fig. 5B-C) might be the cause of these early morphology defects. Finally, we show normal GNAI distribution but already defective hair bundles at P0 in our schematic summary in Fig. 7.

What does the distribution of GNAI look like at P0?

We did analyze GNAI in *Mpdz* mutants at P0, and GNAI distribution was unaffected, as also observed at P2 in Figure 2A (i.e GNAI was limited to the lateral bare zone and stereocilia tips). For this reason, we do not show the redundant P0 results, but we now clarify that GNAI is normal at P0 and P2 stages (line 193).

Line 183 and figure 2C and D. There does appear to be a change in the spatial distribution of GNAI in the Mpdz mutants, but figure 2C just seems to illustrate a significant decrease in the total amount of GNAI across all threeregions. Maybe this would be clearer if regions a, b and c were limited to the lateral compartment? Or is that the case already?

This is correct, GNAI is first and foremost reduced across at the bare zone (lateral flat apex), and ectopically detected at the medial apex in *Mpdz* mutants. This is first explained at line 200 and quantified in Fig. 2B. In Fig. 2D, we look in more detail at these changes by partitioning the bare zone in 3 equivalent sub-regions (a, b, c). It was the case previously, and it remains true that the graph in Fig. 2D and sub-regions a, b, c only represents the lateral bare zone.

See also reply to Reviewer 2, copied here:

Also taking into account comments from Reviewer 3 on GNAI quantification at 3 sub-regions of the lateral apex (bare zone; regions a, b, c), we added in Supp. Fig. 2 an equivalent quantification of

GNAI signals in the corresponding 3 sub-regions of the medial apex (regions d, e, f).

We found that GNAI is principally lost in the central region of the bare zone (b in Fig. 2D) in *Mpdz* mutants, and that ectopic enrichment at the medial apex is not significantly different across the 3 sub-regions. As this result do not support GNAI seeping from the peripheral sides of the hair bundle towards the medial apex, we modified the conclusion of this paragraph line 206. We now conclude more soberly that GNAI is first lost near the basal body on the lateral side, and progressively invading the medial apex, perhaps relocalizing from the lateral to the medial side.

Line 284 figure 4E, it is not really possible to see localization of MPP5 in hair cell bundles. Additional imaging should be provided to confirm this conclusion.

We agree. We now added high magnification panels in Fig. 5E where a boxed region in 5D is detailed. This shows MPP5 coinciding with F-actin in the bundle in *Mpdz* mutants (solid arrowheads).

Line 289 the reduction in junctional MPP5 in Mpdz mutants is significant and could be an indication of junctional defects in the epithelium.

This is the same point as above (second comment). The reply is copied here:

We first worried that the decrease in MPP5 signal at HC junctions (Fig. 4G) might have been an artifact from the quantification method. Visually, we in fact do not observe a clear reduction of MPP5 at the medial junction (new Fig. 4E). Previously, we used a ~1.5x 0.5µm Region of Interest to quantify junctional signal, which encompassed a significant region of (medial) apical membrane as well, and consequently, did not strictly reflect junctional enrichment. We thus re- quantified MPP5 at the medial HC junction in the same sample set using a much narrower ROI (1.5 x 0.1µm) that more accurately encompasses the junction. The new graph in Fig. 4G still shows a mildly significant reduction of the signal.

Despite slightly reduced MPP5 junctional levels, we have no obvious evidence for defective junctional integrity: a) the enrichment of ZO1 and Afadin, used as marker for apical junctions in the study, appear normal in *Mpdz* mutants. b) Compromised junctional integrity would be expected to lead to aberrant cell-cell contacts, but we never observe a defect in the exquisite hair cell-support cell intercalation mosaic in *Mpdz* mutants. We now add this notion along with a representative illustration in Supp. Fig. 5A. This is reported line 326.

Line 315 there is a typo I think, should be Gpsm2 mutants not Mpdz mutants.
Thank you, corrected.

Second decision letter

MS ID#: DEVELOP/2021/199549

MS TITLE: Multiple PDZ Domain Protein Maintains Patterning of the Apical Cytoskeleton in Sensory Hair Cells

AUTHORS: Amandine Jarysta and Basile Tarchini

ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

as previously stated in my first review

Comments for the author

The authors adequately addressed my concerns, which were minor, and appropriately responded to the concerns and questions raised by the other reviewers, which were more lengthy.
I recommend that the journal moves forward with publication.

Reviewer 2

Advance summary and potential significance to field

The responses were thorough and reasonable given what was feasible for the authors to do. I think the new text the authors added was helpful—it's a nice paper overall.

Comments for the author

No additional comments.

Reviewer 3

Advance summary and potential significance to field

This study by Jarysta and Tarchini identifies MPDZ as an important factor in development and function of cochlear hair cells. The experiments are nicely described, the results are clear and the images are outstanding. The study very nicely shows that MPDZ, MPP5 and CRB3 are generally localized to the same regions on the apical surface areas of hair cells and that these factors, which may form a complex, act to regulate different aspects of hair cell and hair bundle formation.

Comments for the author

The authors have very adequately addressed all of my concerns. I have no further comments.