Dual regulation of planar polarization by secreted Wnts and Vangl2 in the developing mouse cochlea

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DOI: 10.1242/dev.191981

Editor: Patrick Tam

Review timeline
Original submission: 22 April 2020
Editorial decision: 26 May 2020
First revision received: 28 July 2020
Accepted: 24 August 2020

Original submission

First decision letter

MS ID#: DEVELOP/2020/191981

MS TITLE: Dual regulation of planar polarization by secreted Wnts and Vangl2 in the developing mouse cochlea

AUTHORS: Alan Gi-Lun Cheng, Elvis Huarcaya Najarro, Nicolas Grillet, Adrian Jacobo, Jennifer Huang, and Lee Quiruz

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 express considerable interest in your work, but have some significant criticisms and recommend a revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, 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.

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.

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.

Reviewer 1

Advance summary and potential significance to field

In this manuscript the authors describe the effects of conditional deletion of two genes Wls and Porcn both of which are required for wnt secretion. Loss of either of these proteins removes secreted Wnts thus allowing an assessment of the effects of secreted wnts on cochlear development. Wnts have a well-known role in planar cell polarity and in convergent extension and both these processes are essential for cochlear development. In this report the consequence of blocking secretion of Wnts in the cochlear duct is examined. In addition, specific Wnts expressed in developing cochlea are identified and localized using RNAscope. The authors find that loss of Wnt secretion leads to shortening of the cochlear duct and a perturbation in hair cell orientation. They further examined the localization of planar cell polarity proteins and found that some, but not all, fail to localize. The use of 2 different cKOs in the wnt secretion pathway, the description of Wnt gene expression and finally the finding that haploinsufficiency in the PCP gene, Vangl2, leads to an accentuation of the phenotype makes for a very complete body of work. The experiments are well described and supported with images and quantitation. This report adds significantly to our understanding of the signals required for the development of the cochlea.

Comments for the author

What is lacking from this manuscript is a substantive discussion of the role of Wnt and β−catenin in cochlear development that would set previous Wnt data into the context of this study. A more complete discussion that includes references to other work by this group and theories of the role of Wnts taken from recent reviews would be very helpful (e.g. Groves and Fekete, 2012 and Munnamlai and Fekete, 2020). Especially since this study actually looks at expression of Wnts now perhaps some of the existing theories for roles of particular wnts can be supported or refuted. Of particular note the authors fail to cite or discuss one of their own previous studies that “reveals specific functions of β-catenin in governing cell identity and patterning mediated through cell adhesion in the developing cochlea”. The same group having studied the effects of removal of secreted Wnts or removal of β−catenin is in the perfect position to comment on Wnt dependent β−catenin actions and those that are Wnt independent.

The description of hair cell orientation in the Wls cKO cochlea implies that there is a significant deviation from center in the KO but in the figure legend for this data (Figure 2) there is a statement that significant difference between cKO and control was only detected in the IHCs. In looking at the Rose plots the p values would indicate a significant difference in all rows. Could the authors clarify this point in the text so there is not this apparent contradiction?

On page 7 beginning on line 14 the authors state that “Wls expression remained robust” at E 16.5 and E18.5 but looking at both ages in fact the expression is decreased relative to E14.5 and this is also supported by their PCR data in Fig1b. I suggest changing “robust” to “evident” to match the data presented.

Where numbers of cells are given for control and mutants (e.g. in Figures 2 and 4) specific numbers of mice of each genotype should also be specified.

Reviewer 2

Advance summary and potential significance to field

If substantiated, this work would transform our understanding of the mechanisms that establish and guide PCP.

Comments for the author

The manuscript by Najarro and colleagues evaluates the contribution of Wnt-signaling towards the establishment of Planar Cell Polarity (PCP) in the developing mouse cochlea. While consensus in the

field is that Wnt ligands should function upstream of hair cell PCP, the mild phenotypes of available mutant mouse lines belies this presumption. Furthermore, the relevant in vitro studies by Dabdoub et al 2003 suggested that Wnt-signaling was more likely to contribute to the refinement of hair cell PCP rather than the initial establishment. Najarro and colleagues address these discrepancies using Wntless and Porcupine conditional knockouts in which the secretion of all Wnt ligands is abolished. Remarkably these mutants have similarly mild PCP phenotypes. In addition, core PCP proteins are differentially impacted by the loss of Wnt secretion with clear changes in the sub-cellular distribution of Frizzled and Dishevelled while Vangl2 and CELSR are not affected. Based upon these results, the authors conclude that PCP is guided by two independent pathways, one that is WNT dependent and another functioning through Vangl2. The approach is logical and well presented, with good figures in a well written document. However, while I am also keen to the prospect that Wnt signaling is not sufficient to guide hair cell PCP, a significant caveat of this study is its dependence upon the efficacy of cre-mediated gene deletion in these CKO lines. As outlined in the comments below, there is reasonable concern that Wnt signaling is not completely disrupted which tempers enthusiasm for the work. Thus, while the overall results are intriguing, it is not clear that the proposed model is correct.

1) It is not clear that Wnt signaling is sufficiently depleted in Emx2-Cre Wntless CKO mice. Work from Albert Edge and colleagues would argue that the complete loss of (canonical) Wnt signaling would prevent sensory domain proliferation. Consistent with this, no cochlear structures develop in the Pax2-Cre Wntless CKO (page9, lines1-2). As a result, it seems likely that canonical, and likely non-canonical, Wnt signaling persists in Emx2-Cre driven CKOs. The authors cannot rule out the possibility that this is sufficient to initiate PCP.
2) It is not clear why IHC phenotypes resembling that found in the Fzd3/6 KO cochlea do not occur in in Wntlss CKOs where Fzd3 and Fzd6 localization is lost. Is it possible that residual Wnt signaling occurring earlier in development is sufficient to rescue IHC PCP?
3) It is not clear that the same region of the cochlea is being compared between mutants and CKOs. Differences comparable to those reported in Figure 2 can be seen when comparing OHCs between the basal and apical turn of wild type mice due to the gradient of differentiation that occurs along the length of the cochlea. Since the mutant cochlea consists of just ¾ of a turn, it is not clear whether mutant hair cells should be compared to apical or basal cells in the control.
4) It is not clear why the shortened cochlea in the Wntless CKO is attributed to convergent extension deficits rather than the simple consequence of having fewer hair cells. An additional 4th row of OHCs is relatively common in wild type mice and does not signify convergent extension deficits which are typically more severe as seen in Looptail mutants where there are 6-8 extra rows in the most apical turn (Montcouquiol 2003).
5) The distribution of PCP proteins presented in schematic diagrams in multiple figures does not match the description of protein distributions provided in the introduction. Specifically, Vangl2 should be depicted in the adjacent supporting cell and CELSR should be located on both sides of the junction. In schematics of Figures 3,6,7 these proteins are only shown in hair cells.
6) There is no discussion of stereociliary bundle reorientation or refinement despite the striking similarity in phenotypes between Wntless/Vangl2 compound mutants and the SFRP treated cochlea of Dabdoub et al. 2003. In both experiments the orientation of bundles are not random (as they are in Vangl2 KOs) and instead tend to point in a single direction. One explanation is that these bundles are oriented towards the cochlear apex because they have not reoriented.

Reviewer 3

Advance summary and potential significance to field

The manuscript by Najarro et al., investigates the potential role of secreted Wnt proteins in establishing planar cell polarity in the mouse cochlea. The authors bypass the issue of variability and redundancy in the various Wnts and Frizzled receptors previously reported and use conditional deletion of Wntless and Porcupine which are necessary for Wnt protein secretion.
They report that conditional deletion of either Wntless or Porcupine resulted in shortened cochleae and sensory cell stereociliary bundle misorientation; both are clear indicators of planar cell polarity defects.
They demonstrate that in the Wntless conditional knockout mice, Dishevelled 1 and 2 as well as Frizzled 3 and 6 were no longer polarized (absent) in the sensory hair cells while Vangl 1 and 2,

Celsr1 and Dishevelled 3 were polarized. Furthermore, they demonstrate that in the Wntless conditional knockout cochleae on a Vangl2 mutant (haploinsufficiency) background, there is a clear increase in planar cell polarity defects as measured by bundle orientation.

Comments for the author

The major issue here is in the interpretation of the data. The main finding is that when Wntless is conditionally deleted, some planar cell polarity proteins are disrupted while others are not. Is this because secreted Wnts only regulate some of the planar cell polarity proteins? Or is this because the Wntless conditional knockout is basically a hypomorph with about 35-40% expression remaining as demonstrated by the most sensitive test used quantifying mRNA (Figure 1 I). As this is an incomplete deletion, does a reduced level of Wnt secretion continue in the Wntless conditional knockout?
Are these lower levels sufficient to maintain the polarization of Vangl2 but not Frizzled 3 for example? The same applies for the Porcupine conditional knockout and should provide rt-qPCR quantification.

I appreciate all the efforts that went into this study, the images and quantification are excellent and so is the writing; however, revisiting the interpretation of the data is necessary.

Minor points
The majority of reviews referenced in this manuscript are old. To give readers more updated information, provide reviews that are 3-5 years old or less.

The first reference in the Results section line 5/6 Wang et al., 2012 should be Wang et al., 2005.

First revision

Author response to reviewers' comments

Reviewer #1:
What is lacking from this manuscript is a substantive discussion of the role of Wnt and β–catenin in cochlear development that would set previous Wnt data into the context of this study. A more complete discussion that includes references to other work by this group and theories of the role of Wnts taken from recent reviews would be very helpful (e.g. Groves and Fekete, 2012 and Munnamalai and Fekete, 2020). Especially since this study actually looks at expression of Wnts now perhaps some of the existing theories for roles of particular wnts can be supported or refuted. Of particular note the authors fail to cite or discuss one of their own previous studies that "reveals specific functions of β-catenin in governing cell identity and patterning mediated through cell adhesion in the developing cochlea". The same group having studied the effects of removal of secreted Wnts or removal of β–catenin is in the perfect position to comment on Wnt dependent β–catenin actions and those that are Wnt independent.
This is an excellent point. We have added a section in the discussion portion (p.18-19, section titled: Disruption of Wnt secretion and Wnt/β-catenin signaling in the embryonic cochlea) of the manuscript to discuss the roles of Wnts and β–catenin in cochlear development. First, we stated the potential role(s) for Wnt ligands in the embryonic cochlea as described in previous publications (Groves and Fekete, Development 2012 and Munnamalai and Fekete, Dev Dyn 2020). The previous data describing the potential role of Wnts on stereocilia bundle reorientation and how they relate to the present study is also discussed (Dabdoub et al., Development 2003). Second, we discussed the relationships between Wnt secretion and the Wnt/β–catenin pathway, specifically by contrasting the phenotypes between mutant mice deficient in Wnt secretion versus those with ablated β–catenin. For example, hair cell differentiation appears unaffected in mice deficient in Wnt secretion (current study), whereas early ablation of β–catenin prevented hair cell specification and differentiation (Shi et al., J Neurosci 2014). Third, how timing and extent of gene deletion affects phenotype is discussed. For instance, early ablation of β–catenin prevents hair cell formation (Shi et al., J Neurosci 2014) whereas late ablation perturbs primarily cell patterning in

the cochlea (Jansson et al., PNAS 2019). Lastly, we discussed how timing and extent of gene deletion may affect the phenotype of Wnt secretion mutants in the current study.

The description of hair cell orientation in the Wls cKO cochlea implies that there is a significant deviation from center in the KO but in the figure legend for this data (Figure 2) there is a statement that significant difference between cKO and control was only detected in the IHCs. In looking at the Rose plots the p values would indicate a significant difference in all rows. Could the authors clarify this point in the text so there is not this apparent contradiction?
We clarified this point in the manuscript. The reviewer is correct that orientation of both outer and inner hair cells are affected.

On page 7 beginning on line 14 the authors state that "Wls expression remained robust" at E 16.5 and E18.5 but looking at both ages in fact the expression is decreased relative to E14.5 and this is also supported by their PCR data in Fig1b. I suggest changing "robust" to "evident" to match the data presented.
We edited the description as suggested.

Where numbers of cells are given for control and mutants (e.g. in Figures 2 and 4) specific numbers of mice of each genotype should also be specified.
We have included this information in Fig. 2 and 4 legends.

Reviewer #2:
1) It is not clear that Wnt signaling is sufficiently depleted in Emx2-Cre Wntless CKO mice. Work from Albert Edge and colleagues would argue that the complete loss of (canonical) Wnt signaling would prevent sensory domain proliferation. Consistent with this, no cochlear structures develop in the Pax2-Cre Wntless CKO (page9, lines1-2). As a result, it seems likely that canonical, and likely non-canonical, Wnt signaling persists in Emx2-Cre driven CKOs. The authors cannot rule out the possibility that this is sufficient to initiate PCP.
We thank this reviewer for the comments. We added to the discussion (p.18-19) and results sections how the timing and extent of *Wntless* deletion may affect the phenotypes observed in this study, and contrast it with β-catenin deficient models (also see point #1 for review #1).

To overcome the redundancy of multiple Wnts, several groups have also taken this same approach of ablating *Wntless* (Snowball et al., Dev Biol 2015; Carpenter et al., Development 2015; Jiang et al., Development 2013). *Emx2-Cre* has been used to effectively delete genes in the developing cochlea by several groups because of its high efficiency and selectivity (Campbell et al., Sci Reports 2016; Tateya et al., Development 2013; Stoller et al., Dev Biol 2018). Its expression precedes hair cell specification by 5-6 days. Our data indicate that both *Wntless* mRNA and proteins are effectively deleted in the cochlear duct prior to hair cell specification.

We have clarified that our approach is to ablate Wnt secretion from the cochlear duct without disrupting Wnt secretion from the periotic mesenchyme, which is another potential source of Wnts in the cochlea. **Therefore, we do not expect canonical or non-canonical Wnt signaling to be absent.** As stated in point #1 for reviewer #1, ubiquitous and early deletion of β-catenin causes different defects in cochlear development from late and spatially restricted deletion. Similarly the finding that cochlear development is prevented when *Wntless* is deleted earlier using the *Pax2-Cre* line indicates that early deletion also causes a different phenotype from late deletion using the Emx2-Cre line. Since we cannot rule out the possibility of residual Wnt PCP signaling in the cochlear duct as a result of Wnts originating from the periotic mesenchymal, we revised the results and discussion sections accordingly (p.11 and p.19-20). First, we stated that *Wntless* and *Porcn* models likely represent a partial loss of Wnt signaling in the cochlea (e.g. a hypomorph). Second, we stated that it is likely that Wnts from the periotic mesenchyme are not affected by our manipulation. Third, we stated that an earlier and broader ablation of *Wntless* may be required to more severely disrupt PCP in the cochlea.

p.11: "It is likely that redundant mechanisms regulate planar polarization of HCs in the cochlear duct. For example, it is possible that Wnts originating from the periotic mesenchyme outside the cochlear duct also play a role in governing hair cell polarity." p.18-19: Section titled: Disruption of Wnt secretion and Wnt/β-catenin signaling in the embryonic cochlea

2) It is not clear why IHC phenotypes resembling that found in the Fzd3/6 KO cochlea do not occur in in Wntless CKOs where Fzd3 and Fzd6 localization is lost. Is it possible that residual Wnt signaling occurring earlier in development is sufficient to rescue IHC PCP?
This is an excellent point. We clarified this in the discussion sections (p.21) in the following way:

The phenotypes of *Wntless* cKO are milder, but still resemble Fzd3/6 KOs in that polarity defects are more prominent among inner hair cells than outer hair cells (Wang et al., J Neuro 2006). There are three main possibilities for this difference. First, Fzd3/6 KO represent a loss of function model where neither of these two proteins are translated, whereas in *Wntless* cKOs, we detected a mislocalization of Fz3/6 proteins, which appear to remain detectable. Second, in *Wntless* cKOs (as stated in point #1), *Wntless* is ablated only in the cochlear duct after around E12.5 when Emx2 Cre activity is detected. Fzd3/6 KOs display no proteins at the germline level and throughout the embryos, thus the deletion is not spatiotemporally restricted. It is likely that both longer and broader deletion of Fz3 and Fz6 contribute to this difference. Lastly, there are other Fzds expressed in the embryonic cochlea (Geng and Noda et al., PloS One 2016), which could play a compensatory role in the *Wntless* model.

p.21: One possible explanation for this difference in severity is that mislocalized Fz3 and Fz6 still carried out partial function in the cochlea, or that mislocalized Fz proteins are more readily compensated for by other Fz members in the developing cochlea (Geng et al., 2016). Also, given the fact that membrane localization of PCP core proteins is dependent on each other, such as with Fz and Vangl2, the above arguments may also explain why some of the PCP core components remained polarized in the cochlea of *Wls* cKO animals.

3) It is not clear that the same region of the cochlea is being compared between mutants and CKOs. Differences comparable to those reported in Figure 2 can be seen when comparing OHCs between the basal and apical turn of wild type mice due to the gradient of differentiation that occurs along the length of the cochlea. Since the mutant cochlea consists of just ¾ of a turn, it is not clear whether mutant hair cells should be compared to apical or basal cells in the control.
As pointed out by this reviewer, the *Wls* KO cochlea is shorter than the control cochlea (about half). We have compared the base of *Wls* cKO to both the control middle and basal turns and found that stereocilia bundle orientations are both significantly different. We have stated this in the results section.

p.10: Since the *Wls* CKO cochleae were significantly shorter than control cochleae, we also compared the base of *Wls* cKO cochleae to the middle turn of control cochlea. Consistent with the above results, we found significantly greater deviation in stereocilia bundle orientation in *Wls* cKO compared to control cochleae (n=232 *Wls* cKO IHCs and 616 OHCs, and 166 control IHCs and 511 OHCs, $p<0.05$).

4) It is not clear why the shortened cochlea in the Wntless CKO is attributed to convergent extension deficits rather than the simple consequence of having fewer hair cells. An additional 4th row of OHCs is relatively common in wild type mice and does not signify convergent extension deficits which are typically more severe as seen in Looptail mutants where there are 6-8 extra rows in the most apical turn (Montcouquiol 2003).
We agree that a shortened cochlea and that a fourth row of OHCs by themselves may not always be attributed to convergent extension/PCP defects. Nonetheless, when both are present, they represent convergent extension (CE) and PCP deficits used to characterize many mutant mouse models (Montcouquiol et al., Nature 2003; Wang et al., Nat Genet 2005; Wang et al., Development 2006, Chacon-Heszele et al., Development, 2012). Moreover, we observed mislocalization of PCP core proteins including that of Fzd3 and Fzd6, with polarity defects observed of inner hair cells similar to those of Fzd3/Fzd6 KO cochlea. Based on this constellation of findings, we attribute the phenotype to convergent extension/PCP defects.

5) The distribution of PCP proteins presented in schematic diagrams in multiple figures does not match the description of protein distributions provided in the introduction. Specifically, Vangl2 should be depicted in the adjacent supporting cell and CELSR should be located on both sides of the junction. In schematics of Figures 3,6,7 these proteins are only shown in hair cells.
We have changed the diagram so that Vangl2 is located in supporting cells and Celsr1 on both sides of the junction. We also changed the wording throughout the manuscript to reflect this modification.

6) There is no discussion of stereociliary bundle reorientation or refinement despite the striking similarity in phenotypes between Wntless/Vangl2 compound mutants and the SFRP treated cochlea of Dabdoub et al. 2003. In both experiments the orientation of bundles are not random (as they are in Vangl2 KOs) and instead tend to point in a single direction. One explanation is that these bundles are oriented towards the cochlear apex because they have not reoriented.
We have discussed the potential role(s) of Wnts in stereociliary bundle reorientation/refinement in the discussion section (also see point #1 for reviewer 1). We agree with reviewer#2 that SFRP treated cochlea has some resemblance with that of *Wntless*/*Vangl2* compound mutants in that bundles are oriented toward the apex of the cochlea. Also, a Vangl2 independent mechanism has been reported to reorient/refine hair bundles in the postembryonic cochlea (Copley et al., J Neurosci 2013), and Wnts may serve as candidates governing stereociliary bundle reorientation/refinement.

p. 22-23: Interestingly, addition of Wnt7a protein or secreted Wnt antagonists to cultured cochlear explants caused defects in stereocilia bundle orientation in more laterally located OHCs (Dabdoub et al., 2003), suggesting that secreted Wnts may function to refine the orientation of OHCs. This is consistent with the concept that a Vangl2- independent mechanism governs stereociliary bundle refinement (Copley et al., 2013).

Reviewer #3:
The major issue here is in the interpretation of the data. The main finding is that when Wntless is conditionally deleted, some planar cell polarity proteins are disrupted while others are not. Is this because secreted Wnts only regulate some of the planar cell polarity proteins? Or is this because the Wntless conditional knockout is basically a hypomorph with about 35-40% expression remaining as demonstrated by the most sensitive test used quantifying mRNA (Figure 1 I). As this is an incomplete deletion, does a reduced level of Wnt secretion continue in the Wntless conditional knockout?
We thank this reviewer for the comments. *Wntless* cKO can be viewed as a hypomorph (as described in #1 for Reviewers #1 and #2) because the deletion is spatiotemporally restricted. While we used *in situ* hybridization and *Wntless* immunostaining to show that *Wntless* was efficiently deleted from the cochlear duct, *Wntless* expression remained in the periotic mesenchyme. The qPCR experiment showed in Figure 1I measured *Wntless* mRNA expression of the whole cochlea (both duct and periotic mesenchyme), thus residual *Wntless* mRNA expression in the periotic mesenchyme cells is expected. We also addressed this in the discussion in point#1 for Reviewers #1 and 2.

p.11: It is likely that redundant mechanisms regulate planar polarization of HCs in the cochlear duct. For example, it is possible that Wnts originating from the periotic mesenchyme outside the cochlear duct also play a role in governing hair cell polarity.

p.19: In both the *Wls* and *Porcn* cKO models, ablation was spatially restricted to the cochlear duct (and spiral ganglia neurons for *Porcn*). Mice with earlier deletion of *Wls* (*Pax2-Cre; Wls*$^{fl/fl}$) failed to develop any cochlear structures, thereby preventing the analysis of hair cell specification. Considering that numerous Wnt members have been detected in the periotic mesenchyme, it is possible that more severe PCP phenotypes and HC specification defects could be observed by a broader and earlier deletion of *Porcn* and *Wls*. As we did not measure the levels of Wnt target genes, the *Wls* and *Porcn* cKO cochleae likely represent models with partial loss of Wnt signaling. Future experiments to more broadly ablate these two genes from the periotic mesenchyme should be considered.

Are these lower levels sufficient to maintain the polarization of Vangl2 but not Frizzled 3 for example?

This is an excellent point and we have included it in the discussion section. It is possible that Vangl2 and Frizzled 3 (Fzd3) have different sensitivities to the levels of secreted Wnts in the cochlea duct. This possibility would be more thoroughly tested by earlier and/or broader deletion of *Wntless*. We also stated that we did not detect a loss of asymmetric localization of Vangl2, Vangl1, Celsr1 and Dvl3 in the *Wntless* cKO cochlea, but this does not rule out more subtle changes that require more sensitive and quantitative assays than immunostaining to detect.

p. 12: Moreover, both Vang1 and Celsr1 remained asymmetrically localized in *Wls* cKO cochleae akin to control tissues (Fig. 3j-m, S7j-m), although subtle changes in their localization or level of expression cannot be ruled out.

p.15: In both control and *Wls* cKO cochleae, the polarized expression of Vangl2 was observed in the lateral side of supporting cells (Fig. 6a-c, S13a-c), suggesting that conditional ablation of *Wls* from the cochlear duct is not sufficient to affect the asymmetric localization of Vangl2. Alternatively, it is possible that Vangl2 localization does not depend on secreted Wnts in the cochlear duct.

The same applies for the Porcupine conditional knockout and should provide rt-qPCR quantification.
We have performed this experiment and have included the *Porcn* qPCR data. (Figure S4m).

Minor points
The majority of reviews referenced in this manuscript are old. To give readers more updated information, provide reviews that are 3-5 years old or less.
We have added more recent references (Groves and Fekete, Development 2012 and Munnamalai and Fekete, Dev Dyn 2020) to provide readers more updated information. (p. 18-19), section titled "Disruption of Wnt secretion and Wnt/β-catenin signaling in the embryonic cochlea".

The first reference in the Results section line 5/6 Wang et al., 2012 should be Wang et al., 2005.
This has been corrected in the manuscript.

Second decision letter

MS ID#: DEVELOP/2020/191981

MS TITLE: Dual regulation of planar polarization by secreted Wnts and Vangl2 in the developing mouse cochlea

AUTHORS: Alan Gi-Lun Cheng, Elvis Huarcaya Najarro, Nicolas Grillet, Adrian Jacobo, Jennifer Huang, and Lee Quiruz
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

In this manuscript the authors describe the effects of conditional deletion of two genes Wls and Porcn both of which are required for wnt secretion. Loss of either of these proteins removes secreted Wnts thus allowing an assessment of the effects of secreted wnts on cochlear development. Wnts have a well-known role in planar cell polarity and in convergent extension and both these processes are essential for cochlear development. In this report the consequence of blocking secretion of Wnts in the cochlear duct is examined. In addition specific Wnts expressed in developing cochlea are identified and localized using RNAscope. The authors find that loss of Wnt secretion leads to shortening of the cochlear duct and a perturbation in hair cell orientation. They further examined the localization of planar cell polarity proteins and found that some, but not all, fail to localize. The use of 2 different cKOs in the wnt secretion pathway, the description of Wnt gene expression and finally the finding that haploinsufficiency in the PCP gene, Vangl2, leads to an accentuation of the phenotype makes for a very complete body of work.

Comments for the author

In this revised version of the manuscript by Huarcaya Najarro et al. the authors have been responsive to my critiques. Including discussion of all points made by the reviewers has improved the manuscript and clarified the strengths and limitations of the study. It is clear that both the canonical and noncanonical wnt pathways are very complicated and active at several stages of

cochlea development in both the epithelial and mesenchymal compartments. Thus it is not easy to design simple experiments to tease out the particular contributions of all the players. This study adds to our appreciation of the complexities of the wnt pathways and placing the data in the context of other previous studies.

Reviewer 2

Advance summary and potential significance to field

An outstanding question in planar polarity research is the identity of the global cues that act upstream of PCP signaling and orient the PCP axes relative to the overall tissue, organ or body plan. While it has been broadly assumed that these cues are the Wnt ligands, the phenotypes of Wnt mutants are variable and do not account for all instance of PCP. This manuscript is an important advance because the findings strongly suggest that Wnts are not sufficient and that other signaling molecules act in conjuction with Wnts to establish PCP.
This manuscript will direct and influence future studies in the field.

Comments for the author

The authors adequately responded to reviewers' comments and have appropriately discussed the caveats and limitations of the study. I support publication of this paper in Development.

Reviewer 3

Advance summary and potential significance to field

The authors have addressed all my concerns.

Comments for the author

The authors have addressed all my concerns. Great work, looking forward to seeing it published.