New mouse models for high resolution and live imaging of planar cell polarity proteins in vivo

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MS TITLE: New mouse models for high resolution and live imaging of planar cell polarity proteins in vivo

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I apologise for the delay before beeing able to come back to you. 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 as a Techniques and Ressource Article (ie. not as a Research Article), provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them 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

The manuscript reads like a “Tools and Resources” manuscript. I’m not sure if it was submitted as a “Research Article” in error (or it’s a glitch in the editorial system?). Although the manuscript does report some new findings, they largely seem to be confirming or following-up previous observations

– many from the same research group e.g. compare use of super-resolution in Stahley et al 2021, or previous fixed-tissue studies on PCP protein localization during cell division. As a Research Article I'm not sure it really holds-up. If the intention was to publish it this way it would make sense for the authors to go further and deeper into a particular line of investigation. Hence I'm going to assess this as a "Tools and Resources" manuscript, as this better fits the contents. (Apologies if this isn't what the authors or editors want. Hopefully my comments will be helpful either way.)
In brief, the authors report generation of 3 transgenic mouse strains with fluorescent protein knock-ins to endogenous PCP loci, specifically, Fz6-3xGFP, Celsr1-3xGFP and tdTomato-Vangl2. They show that in the developing skin these three tagged protein localize and function normally by the criteria comparison to immunolabeling of untagged proteins and correct hair follicle orientation. (Although other data suggest tdTomato-Vangl2 is not full functional, for instance in neural tube closure).
They further demonstrate the utility of the reagents for tracking protein localizations, primarily in the mouse skin, but also for instance the adult trachea, the embryonic neural tube and a range of internal organs. These experiments provide useful "proof-of-principle" data, but in themselves do not add too much to our knowledge of PCP (and I don't think the authors intended otherwise). However, this survey of tissue expression does highlight the range of tissues in which these PCP protein fusions are expressed thus demonstrating that they will be useful as reagents to many workers in the mouse PCP field.
Overall, as a "Tools & Resources" manuscript I think this could be a valuable contribution.

Comments for the author

Major points:
• The STED imaging in Fig. 4 and Fig. S3 seems to paint a rather more complex picture than the description in the text. Depending where you look, GFP/tdTomato seem to almost exactly overlap in punctate structures, or they seem to be in separate but overlapping blobs (at the reported 89 nm spacing?), or they seem to be entirely separate. Also sometimes GFP and tdTomato look like they are on the "wrong" side of the junctions. The data answer the question of whether these proteins can be detected by STED, but in terms of describing their distributions and colocalizations the analysis is a bit preliminary.

– "Within individual puncta, there was consistent 'cross-junctional asymmetry' where Fz6-3xGFP localized to the anterior of the junction and tdTomato-Vangl2 localized to the posterior" – this statement does seem a bit subjective. Even in the chosen crops it only seems to be true some of the time?

– could a membrane marker be included to help distinguish between "junctional" puncta and e.g. endocytic populations? (And/or an endocytic marker?)

– what z-resolution is achieved? Could we be looking through a large z-range and seeing structures stacked on top of each other? Is STED similar to confocal in providing ~3x less resolution in z, so we might be resolving e.g. ~150 nm? (Is it possible to report on the resolution, even if just in xy?)

– "These results demonstrate how coupling super-resolution microscopy with endogenously tagged PCP protein reporters enables the clear and consistent resolution of Fz6 and Vangl2 unipolar" – given all we know from past studies this seems likely to be true, but I'm unsure this can be safely concluded without a third marker for e.g. cell membranes?

• It is nicely shown that Fz6-3xGFP, Celsr1-3xGFP and tdTomato-Vangl2 localize similarly to the endogenous proteins in the developing skin. However, if these reagents are going to be useful to groups looking in other tissues (as I would hope they would be), it would be good to provide the comparison elsewhere (e.g. adult trachea, neural tube?). This would be particularly useful as tdTomato-Vangl2 does not appear to retain full function in all tissues and this might be associated with localization defects.

• The survey of tissue expression in Fig.8 and S8 usefully highlights a range of tissues in which the fusion proteins could provide useful readouts of PCP protein localization. I wonder whether as a resource (assuming that is the authors' intention), a table could be provided summarizing known data on PCP protein function and localization in mouse tissues e.g. which tissues express Fz6,

Celsr1, Vangl2 (from publications and public databases?), which tissues show PCP defects in mutants for these genes, and summarizing the authors' own data. Then potential users could see at a glance what was already known.

Minor points:
p.2 "Vang Gogh" should be "Van Gogh" (after the painter)?

p.3 "Mosaic or clonal expression is achieved by overexpression or deletion of the PCP proteins" – is this specifically referring to vertebrate systems? I think even early studies in e.g. Drosophila used rescuing transgenes rather than overexpression. Also are examples in mouse skin such as Devenport et al 2011 actually relying on "overexpression" (that wasn't how I read the original paper)?
p.4 "The N-terminus of Vangl2 was chosen for tagging to avoid interfering with a highly conserved, C-terminal PDZ-binding motif" – I infer from this that Fz6 and Celsr1 do not have C-term PDZ-binding motifs?

I think some homologs of these proteins do?

Figure 1 – legend has (D) and (E) duplicated in place of (F) and (G)
p.8 "The generation of endogenous fluorescent PCP reporters provides for the first time in a mammalian system the ability to monitor the dynamics of PCP asymmetry through epithelial divisions and rearrangements" – I'm unsure if the authors mean to imply that lack of fluorescent PCP reporters in mammals was a barrier to live imaging, but they may be unaware that such reagents have been reported before? e.g. Wang et al 2006 doi:10.1242/dev.02347, Hashimoto et al 2010 doi:10.1038/ncb2020. Although maybe these are not bright enough to be useful? Arguably such studies could probably also be carried out in primary culture systems using transient transfection of reports, such as that reported by Vladar et al 2012 doi:10.1016/j.cub.2012.09.046.

Figure 5 and S5 – I'm trying to see the previously reported transcytosis of Fz6 during mitosis (Heck & Devenport 2017). Is there some reason why this isn't obvious?

Figure 6 – anti-GFP and anti-tdTomato are used for detection here due to weak overall signal? Or interference of MeOH fixation with the signal? This could be a helpful technical point for other groups that want to use these reagents.

p.11 "we suspect PCP asymmetries are highly dynamic and transient and may only be detectable in this tissue by live imaging" – would live-imaging make a difference? Surely looking over a large field of cells in fixed images would reveal if asymmetry was ever visible? I suspect more likely that scattered labeling of cells via some form of mosaic analysis would be most likely to reveal transient asymmetries.

Figure 7 legend – inserts of "(of how many microns)" possibly were not intended for the reviewers?
Figure S7 legend – not sure I can see the yellow arrowheads?

Figure 8 and S8 – there's probably a lot of valuable information here, but it's hard to appreciate with all the images shown as 3 channel overlays. Could grayscale channel separations be shown too (possibly in supplemental, or show some in main figure and some in supplemental)?

Reviewer 2

Advance summary and potential significance to field

The manuscript by Basta et.al describes the establishment of planar cell polarity during mouse development using three novel mouse lines they generated using CRISPR to label core PCP proteins with fluorescent proteins. They generated Celsr1-3XGFP, Fz6-3X GFP and tdTomato-Vangl2. The manuscript uses these tools to examine polarity using confocal microscopy and super-resolution STED imaging to confirm that localization of these proteins. Interestingly, they use these mice to examine live changes in Fz6-3xGFP during cell rearrangements and divisions. They finish the manuscript by very briefly documenting localization of the PCP proteins using the three lines. The authors make a convincing argument that overexpression and loss of function of PCP proteins can have defects. Tagging the endogenous proteins can also be useful in the case that high quality

antibodies are not available, and live imaging can allow visualization of PCP proteins dynamics that can be highly informative. The authors show that the animals are viable, and have bright PCP protein labeling in the expected places. They use super-resolution imaging with these to see the Fx3XGFP and tdTomato-Vangl2 go to the opposite sides of the cell. Importantly Celsr1-3X-GFP and Fz6-3XGFP mice are homozygous viable and fertile with no obvious phenotypes, however the tdTomato-Vangl2 mice show a broad range of PCP defects, indicating function is affected. This manuscript nicely describes new tools/reagent for PCP visualization with novel mouse lines, though not much novelty in terms of new insights into developmental biology mechanisms. This may be more appropriate for a resource/tools paper in Development.

Comments for the author

Comments/questions Is there a tdTomato-Vangl2 heterozygous phenotype? Similar to Vangl2 Looptail mutation?

Fig 2 was not well described for the naïve reader- it was not clear from an initial reading of the figure legend how the polarity was scored in D-F. Possibly a diagram of the approach, then referencing supplemental figure 1 should be included in Fig 1.

Fig 3 could be improved by including the pictures from Sup2. A key aspect of these tools is the degree of similarity of endogenously tagged protein vs antibody staining. If the authors feel there are too many figures I believe Figure 1 could be compressed in size and added to one of the others without detriment. Is the staining in the bottom of the hair germ that is so much stronger in tdTomato-Vangl2 present in antibody staining?

Fig4-6 use a variety of approaches to show that these proteins are polarized, consistent with previous studies using antibodies and fixed tissues. Fig 4 clearly shows the asymmetric distribution of Fz6-3XGFP and tdTomato-Vangl2, as well as puncta, consistent with previous studies on these proteins distribution. Previous work from these authors had shown that super-resolution microscopy can resolve asymmetries across junctional interfaces (Stahley et al, 2021 elife), using SIM , here now they use STED microscopy which can resolve ~ 50nm. Fig 5 nicely shows time lapse imaging of skin explants from Fzd6-3XGFP mice, showing the loss and gain of polarity during divisions. Especially nice is Fig 5c illustrating the polarity of mother and daughter cells. Fig 6 documents in lovely images that Celsr1-3X-GFP, tdTomator-Vangl2 and Fz6-3X-GFP are planar polarized in the adult trachea. This has been shown before with Fz6, but not Vangl2 or Celsr1.

Fig 7 and 8 are a rather cursory look at the expression patterns of the tagged proteins in other places. Fig 7A-E shows a broad staining of the neural tube at E8.5, F-H shows somites are brightly labeled with tdTomato-Vangl2, less so with Fzd6 or Celsr1. The legend of Fig 7 has typos (of how many microns?) Fig 8 continues with the broad survey, and shows that the tagged proteins have interesting expression patterns in stomach, intestine, kidney, liver and lung. But without co-staining, higher magnification or more explanations, this is not very illuminating. Given that Figures 1-6 largely show what has been previously shown with other methods, this is the most biological novelty of the manuscript. It is interesting that the vascular endothelium expresses Fz6-GFP, smooth muscle and stroma express PCP proteins Vang2. It is also interesting that tdTomato-Vangl2 seems to function normally in the epidermis, as opposed to other tissues.
The authors note that an long-isoform has been recognized—is there available RNAseq from epidermis to see if this isoform is not present there?

Reviewer 3

Advance summary and potential significance to field

This manuscript describes the generation of three new mouse models allowing analysis of the core planar cell polarity (PCP) pathway live in tissues, namely the generation of of endogenously-tagged Celsr1-3xGFP, Fz6-3xGFP and tdTomato-Vangl2 fusion proteins.
The authors show that these are functional and are localised asymmetrically.
In particular, they use their labs favourite model of PCP, the mouse epidermis, combined with live imaging to show that PCP in the basal progenitors is not fixed in time but that cell divisions and cell rearrangements lead to shifting patterns whilst still observing the overall tissue polarity.

Furthermore, they show that using these novel tools combined with super-resolution STED microscopy Frizzled6-3xGFP and tdTomato-Vangl2 can be resolved across cell-cell junctions with a resolution of 50nm, and this allowed the authors to address discrepancies in the literature, for instance the asymmetric localization of Fz6.
Finally, the authors show localisation of the endogenously tagged PCP components in a variety of embryonic tissues in the mouse where PCP is present, as well as several novel patterns of PCP but also expression of the proteins in possible non PCP roles.

Comments for the author

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Finally, the authors show localisation of the endogenously tagged PCP components in a variety of embryonic tissues in the mouse where PCP is present, as well as several novel patterns of PCP but also expression of the proteins in possible non PCP roles.

The tools generated and described in this study will be extremely useful to the PCP community and beyond allowing to tackle important open questions in the field that have remained despite the components themselves being known for years.
The authors elegantly demonstrate the localisation and functionality of the tagged proteins, and especially the functionality in super-res microscopy approaches is very impressive and will be very useful for further studies.

In summary, the tools described and characterised here will be of wide use and the paper widely cited once published. The authors have clearly demonstrated and identified novel unknowns aspects of these PCP components function and localisation, demonstrating how useful the tools described here will be.

First revision

Author response to reviewers' comments

We thank the reviewers for their close reading of the paper, their overall positive comments and thoughtful critiques. We were able to address all three reviewers' concerns in full and believe the revisions have greatly improved the manuscript. Below is a point-by-point response to the reviewers' comments, with our responses shown in blue text.

Reviewer 1 Advance Summary and Potential Significance to Field:
The manuscript reads like a "Tools and Resources" manuscript. I'm not sure if it was submitted as a "Research Article" in error (or it's a glitch in the editorial system?). Although the manuscript does report some new findings, they largely seem to be confirming or following-up previous observations – many from the same research group e.g. compare use of super-resolution in Stahley et al 2021, or previous fixed-tissue studies on PCP protein localization during cell division. As a Research Article I'm not sure it really holds-up. If the intention was to publish it this way it would make sense for the authors to go further and deeper into a particular line of investigation. Hence I'm going to assess this as a "Tools and Resources" manuscript, as this better fits the contents. (Apologies if this isn't what the authors or editors want. Hopefully my comments will be helpful

either way.)

In brief, the authors report generation of 3 transgenic mouse strains with fluorescent protein knock-ins to endogenous PCP loci, specifically, Fz6-3xGFP, Celsr1-3xGFP and tdTomato-Vangl2. They show that in the developing skin these three tagged protein localize and function normally by the criteria comparison to immunolabeling of untagged proteins and correct hair follicle orientation. (Although other data suggest tdTomato-Vangl2 is not fully functional, for instance in neural tube closure). They further demonstrate the utility of the reagents for tracking protein localizations, primarily in the mouse skin, but also for instance the adult trachea, the embryonic neural tube and a range of internal organs. These experiments provide useful "proof-of-principle" data, but in themselves do not add too much to our knowledge of PCP (and I don't think the authors intended otherwise). However, this survey of tissue expression does highlight the range of tissues in which these PCP protein fusions are expressed, thus demonstrating that they will be useful as reagents to many workers in the mouse PCP field. Overall, as a "Tools & Resources" manuscript I think this could be a valuable contribution.

The reviewer is correct that we intended to submit our paper as a "Tools & Resources" article. We also wanted the paper to be considered for the special issue on Imaging Development, Stem Cells and Regeneration, and there was not a way to select both criteria in the manuscript submission system. We apologize for any confusion and appreciate that the reviewer guessed correctly and evaluated the manuscript as a Tools paper, as we had intended it to be.

Reviewer 1 Comments for the Author:
Major points:

1. The STED imaging in Fig. 4 and Fig. S3 seems to paint a rather more complex picture than the description in the text. Depending where you look, GFP/tdTomato seem to almost exactly overlap in punctate structures, or they seem to be in separate but overlapping blobs (at the reported 89 nm spacing?), or they seem to be entirely separate. Also sometimes GFP and tdTomato look like they are on the "wrong" side of the junctions. The data answer the question of whether these proteins can be detected by STED, but in terms of describing their distributions and colocalizations the analysis is a bit preliminary. "Within individual puncta, there was consistent 'cross-junctional asymmetry' where Fz6- 3xGFP localized to the anterior of the junction and tdTomato-Vangl2 localized to the posterior" – this statement does seem a bit subjective. Even in the chosen crops it only seems to be true some of the time?

We agree that the distribution of puncta along the junction show a level of complexity that is not encapsulated solely by "cross-junctional asymmetry". To address this and provide a more complete and accurate representation of Fz6 and Vangl2 distribution by STED, we quantified the percentage of puncta pairs that had Fz6-3xGFP on the anterior and tdTomato-Vangl2 on the posterior ("F-V" orientation) and vice versa ("V-F" orientation). These data are now shown in Fig. 4E and represented as the fraction of each orientation per junction. Further, we added new imaging data of Fz6-3xGFP and tdTomato-Vangl2 organization at horizontal, non-PCP junctions (mediolateral junctions) as a comparison. Although the overall levels of Fz6-3xGFP and tdTomato-Vangl2 at horizontal junctions is much lower than at vertical junctions and their organization much more diffuse, puncta can still be resolved and segmented at horizontal junctions. To determine if there was any mediolateral bias in Fz6- 3xGFP and/or tdTomato-Vangl2 localization, we performed a similar analysis of puncta pairs and found F-V and V-F orientations in roughly equal proportions. New data analyzing PCP localization at horizontal junctions by STED are shown in Fig. 4F-I and Supplementary Figure D-I.

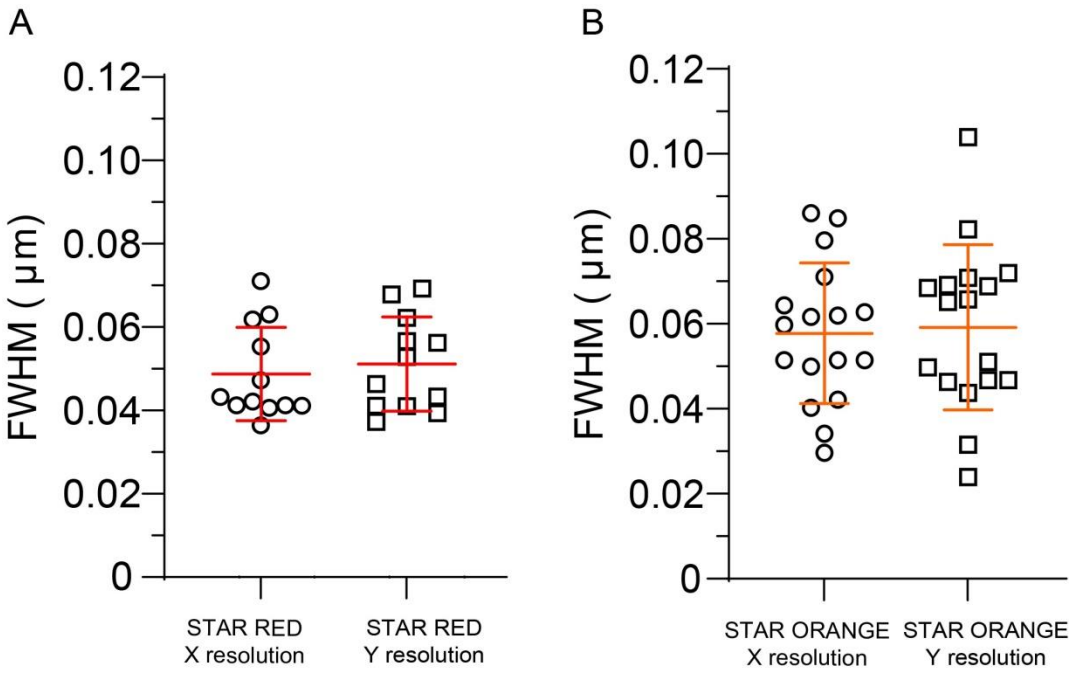
- Could a membrane marker be included to help distinguish between "junctional" puncta and e.g. endocytic populations? (And/or an endocytic marker?)

We would love to be able to do this, but unfortunately, our current STED system only allows for dual- labeling, which precludes us from staining for either membrane or endocytic markers in conjunction with Fz6-3xGFP and tdTomato-Vangl2. Based on our junctional segmentation, it's possible we're including some membrane-proximal endocytic vesicles in our analysis, but by making masks around the junctions (see Methods, Line 647) we tried to exclude as much cytoplasmic signal as possible.

- What z-resolution is achieved? Could we be looking through a large z-range and seeing structures stacked on top of each other? Is STED similar to confocal in providing ~3x less resolution in z, so we might be resolving e.g. ~150 nm? (Is it possible to report on the resolution, even if just in xy?)

We can report the XY resolution for both the STARRED and STARORANGE secondary antibodies. To do so we imaged smears of the STARORANGE and STARRED antibodies to determine the point spread function of signal coming from single molecules of antibody. The STARRED antibody showed an average FWHM (across 12 molecules) of 48.7 nm in the X axis and an average of 51.1 nm in the Y axis. The STARORANGE antibody showed a reduction in resolution, with an average of (across 17 molecules) 57.8 nm in the X axis and 59.1 nm in the Y (See reviewer Figure 1A-B below). We have added these data to the Materials and Methods.

We don't know the precise resolution in Z, but assume like confocal it is ~3x less and around 150nm. Therefore, it is possible some of the punctate structures we're visualizing are stacked in the Z. PCP proteins localize all along the lateral edges of basal cells (see localization in sagittal sections shown in Figure 2), and we're reporting their organization along a just subset of the junction along the apical- basal axis. We do observe Fz6-Vangl2 puncta pairs in multiple Z-planes along the lateral membrane surface, but haven't characterized whether there are differences in organization along the apical-basal axis, which would be interesting to do in the future.



Reviewer Figure 1. STED antibody XY resolution. (A) Single molecule signal point spread function of the STARRED secondary antibody across 12 molecules in X and Y. An average FWHM of 48.7 nm in the X axis and 51.1 nm in the Y axis was found. **(B)** Single molecule signal point spread function of the STARORANGE secondary antibody across 17 molecules in X and Y. An average FWHM of 57.8 nm in the X axis and 59.1 nm in the Y axis was found.

- "These results demonstrate how coupling super-resolution microscopy with endogenously tagged PCP protein reporters enables the clear and consistent resolution of Fz6 and Vangl2 unipolar" – given all we know from past studies this seems likely to be true, but I'm unsure this can be safely concluded without a third marker for e.g. cell membranes?

We have toned down these conclusions.

2. It is nicely shown that Fz6-3xGFP, Celsr1-3xGFP and tdTomato-Vangl2 localize similarly to the endogenous proteins in the developing skin. However, if these reagents are going to be useful to groups looking in other tissues (as I would hope they would be), it would be good to provide the

comparison elsewhere (e.g. adult trachea, neural tube?). This would be particularly useful as tdTomato- Vangl2 does not appear to retain full function in all tissues and this might be associated with localization defects.
We thank the reviewer for this suggestion. We now show in Supplemental Figure 5 staining of Celsr1 and Fz6 endogenous proteins in WT adult trachea by immunofluorescence. Similar to the localization of the tagged proteins shown in Figure 6, we observe proximal-distal enrichment of both Celsr1 and Fz6. By contrast, we were unable to achieve good labeling of adult tracheas with a commercially available Vangl2 antibody that works well in many other tissues. Yet we do observe strong P-D localization of tdTomato-Vangl2 in the trachea, so it appears this is another benefit of our tagged lines. Both Celsr1 and Vangl2 have been previously shown to localize asymmetrically in mouse trachea epithelial cells in culture (MTECs) with bipolar localization of Celsr1and unipolar localization of Vangl2 (Vladar et al, 2012). Therefore, the localization of the tagged proteins seems consistent with the endogenous proteins, both in our hands and with previously reported data.

We also now show in Supplemental Figure 7 staining of all three endogenous proteins in the neural tube, both at the anterior region of the embryo and along the midline and somites. We see similar distributions of the endogenous proteins compared to the tagged proteins, with Vangl2 displaying broader expression in the somites compared with Fz6 and Celsr1. We did not observe any obvious differences in the localization of untagged Vangl2 in WT embryos and tdTomato-Vangl2 in the neural tube. The reasons for the neural tube phenotypes in homozygous tdTomato-Vangl2 remain unclear.

3. The survey of tissue expression in Fig.8 and S8 usefully highlights a range of tissues in which the fusion proteins could provide useful readouts of PCP protein localization. I wonder whether as a resource (assuming that is the authors' intention), a table could be provided summarizing known data on PCP protein function and localization in mouse tissues e.g. which tissues express Fz6, Celsr1, Vangl2 (from publications and public databases?), which tissues show PCP defects in mutants for these genes, and summarizing the authors' own data. Then potential users could see at a glance what was already known.

The primary goal of Fig 8 and S8 was to highlight that these mice can be used as tools to study PCP across a variety of tissues in the mouse, rather than be a full resource for PCP protein expression across the mouse. Nevertheless, we did attempt to construct a table containing the information suggested by the reviewer, however it quickly became so large and unwieldy as to not be very helpful. Ultimately, we felt that although such a table would be useful, it would be more appropriate for separate review article given the breadth of information it would cover.

We hope the paper highlights that these tools are powerful to study PCP across a variety of contexts, and that new insights about PCP localization and dynamics can be gained across tissues and development using these tools. As a resource, we have many embryos embedded and stored in our freezer, ready to ship to any interested researcher to explore expression in their favorite tissue.

Minor points:
p.2 "Vang Gogh" should be "Van Gogh" (after the painter)?
Thank you. This has been fixed (Line 55).

p.3 "Mosaic or clonal expression is achieved by overexpression or deletion of the PCP proteins" – is this specifically referring to vertebrate systems? I think even early studies in e.g. Drosophila used rescuing transgenes rather than overexpression.

We apologize for the imprecise wording of this statement. It was not our intention to suggest that all prior studies on PCP localization involve overexpression. Rather we wanted to highlight that mosaic overexpression and mosaic deletion are known to cause PCP phenotypes, and therefore care must be taken in interpreting localization patterns of PCP proteins that are not endogenously expressed. Fz6 localization in the mouse is a case in point. A major conclusion of a 2018 paper published in Development by Dong et al, is that Fz6 is not asymmetrically localized in the epidermis - the same tissue at the same stages we investigate here. To quote from their abstract,

"we provide evidence that, contrary to the prevailing model, asymmetrical localization of the Fzd6 protein is not observed in skin epithelial cells". This conclusion was based on mosaic deletion of Fz6 and mosaic overexpression HA- Fz6. By contrast, in our Fz6-3xGFP line and in our wild-type controls, Fz6 is just as polarized as Vangl2 and Celsr1 in the skin. This kind of discrepancy was the major point we wanted to convey by the above statement. We have removed the above statement from the current version of the manuscript to avoid overstating the use of overexpression in the PCP field.

Also are examples in mouse skin such as Devenport et al 2011 actually relying on "overexpression" (that wasn't how I read the original paper)?
Mosaic PCP protein expression in Devenport et al 2011 was driven by the K14-promotor. So, while they were not necessarily overexpressed, they were not under endogenous regulation and different transgenic lines did have different levels of expression and mosaicism.

p.4 "The N-terminus of Vangl2 was chosen for tagging to avoid interfering with a highly conserved, C terminal PDZ-binding motif" – I infer from this that Fz6 and Celsr1 do not have C-term PDZ-binding motifs? I think some homologs of these proteins do?

Neither Fz6 nor Celsr1 have a C-terminal PDZ-binding motif (PDZBM), but Drosophila Fmi does. Our main reasoning for targeting the C-termini of Celsr1 and Fz6 was to avoid tagging their N-terminal extracellular domains. In Vangl2, both N- and C-termini are cytoplasmic, so in having to choose one or the other we decided to avoid tagging near the PDZBM (and in retrospect, this was probably the wrong choice). We have modified the text to make our rationale clearer.

Line 118: To generate mouse strains that express fluorescently-tagged PCP proteins from their endogenous loci, we used 2C-HR-CRISPR to insert 3xGFP at the intracellular, C-terminus of Celsr1 and Fz6 (Fig.1B,D), and tdTomato at the N-terminus of Vangl2 (Fig. 1F) (Gu et al., 2020b). All three core PCP components are multipass transmembrane proteins, but unlike Celsr1 and Fz6 which each have a single C-terminal cytoplasmic domain and extracellular N-terminal domains, Vangl2 is a four- pass transmembrane protein with cytosolic domains at both N- and C-termini. The N-terminus of Vangl2 was chosen for tagging to avoid interfering with a highly conserved, C terminal PDZ-binding motif…"

Figure 1 – legend has (D) and (E) duplicated in place of (F) and (G)
Thank you. This has been fixed.

p.8 "The generation of endogenous fluorescent PCP reporters provides for the first time in a mammalian system the ability to monitor the dynamics of PCP asymmetry through epithelial divisions and rearrangements" – I'm unsure if the authors mean to imply that lack of fluorescent PCP reporters in mammals was a barrier to live imaging, but they may be unaware that such reagents have been reported before? e.g. Wang et al 2006 doi:10.1242/dev.02347, Hashimoto et al 2010 doi:10.1038/ncb2020 Although maybe these are not bright enough to be useful? Arguably such studies could probably also be carried out in primary culture systems using transient transfection of reports, such as that reported by Vladar et al 2012 doi:10.1016/j.cub.2012.09.046.

We apologize for any unintended meaning in the wording of this statement. We intended to highlight that we report PCP dynamics during cell rearrangements and cell division in mouse for the first time. We have rephrased this statement to read:

Line 241: "Using the fusion PCP reporters generated here, we were able to monitor the dynamics of PCP asymmetry through epithelial divisions and rearrangements".

Figure 5 and S5 – I'm trying to see the previously reported transcytosis of Fz6 during mitosis (Heck & Devenport 2017). Is there some reason why this isn't obvious?
Yes, the conditions that allow us to live image the skin for long time periods (>4 hours) require growing the skin at an air-liquid interface and imaging with an air objective. Thus, we must use a 20X air objective on a spinning disc confocal, and this does not provide the magnification or resolution to observe all but the largest endosomes (which can be seen in the movies shown in Supplemental Figure 5 and Supplemental Video 5,6). All of our prior work investigating PCP trafficking and endosome dynamics used scanning confocal microscopes and 100X objectives, or

was performed in cultured keratinocytes, which are much larger and flatter than basal cells *in vivo*. We do observe endogenously-tagged PCP proteins localized to endosomes in dividing cells when imaged at higher magnification with immersion objectives. We are working on optimizing live-imaging conditions to capture dynamics of PCP-containing endosomes, and are optimistic about these early efforts, so stay tuned.

To address this point in the manuscript, the Discussion reads:
Line 449: Although the conditions required to perform long-term live imaging of the epidermis were insufficient to track endosome movements over the course of mitosis (explants are grown at an air-liquid interface, and images are captured using air objectives at 10-20 minute intervals), we believe high speed imaging of the tagged lines with immersion objectives will be possible for shorter time periods and enable high resolution imaging of PCP trafficking during mitosis.

Figure 6 – anti-GFP and anti-tdTomato are used for detection here due to weak overall signal? Or interference of MeOH fixation with the signal? This could be a helpful technical point for other groups that want to use these reagents.

Both PFA and especially MeOH fixation weaken the GFP and mTomato signals, so antibodies were used to boost the signal. To clarify, we have added the following to the Materials and Methods.

Line 565: Samples were incubated in primary antibody in PBT2 overnight at 4˚C, including antibodies against GFP and tdTomato as PFA fixation weakens endogenous signals.

Line 783: GFP and tdTomato were stained against as trachea fixation in methanol and PFA weakens the endogenous signal of both GFP and tdTomato.

p.11 “we suspect PCP asymmetries are highly dynamic and transient and may only be detectable in this tissue by live imaging” – would live-imaging make a difference? Surely looking over a large field of cells in fixed images would reveal if asymmetry was ever visible? I suspect more likely that scattered labeling of cells via some form of mosaic analysis would be most likely to reveal transient asymmetries
Live imaging could make a difference. In Butler and Wallingford, 2018 they live image PCP protein localization during neural tube closure and show that PCP enrichment correlates better with junction shrinkage than with junction angle. But yes, we agree that mosaic analysis could reveal asymmetries that we are not able to appreciate in large fields of cells. We have added this as a potential explanation to the paper.

Line 346: Alternatively, mosaic expression of the fusion proteins may be required to reveal asymmetries of PCP proteins during neural tube development. This could be achieved by generating embryo chimeras between endogenously-tagged PCP reporter and wild-type embryos to produce mosaic expression of the tagged PCP proteins.

Figure 7 legend – inserts of “(of how many microns)” possibly were not intended for the reviewers?
This has been corrected.

Figure S7 legend – not sure I can see the yellow arrowheads?
This has been fixed.

Figure 8 and S8 – there’s probably a lot of valuable information here, but it’s hard to appreciate with all the images shown as 3 channel overlays. Could grayscale channel separations be shown too (possibly in supplemental, or show some in main figure and some in supplemental)?

We now show high magnification regions of each tissue with the channels separated and displayed in grayscale, allowing a more direct comparison between E-Cadherin and PCP protein expression. See revised Figure 8 and Supplementary Figure 8.

Reviewer 2 Advance Summary and Potential Significance to Field:
The manuscript by Basta et.al describes the establishment of planar cell polarity during mouse development, using three novel mouse lines they generated using CRISPR to label core PCP proteins with fluorescent proteins. They generated Celsr1-3XGFP, Fz6-3X GFP and tdTomato-

Vangl2. The manuscript uses these tools to examine polarity using confocal microscopy and super-resolution STED imaging to confirm that localization of these proteins. Interestingly, they use these mice to examine live changes in Fz6-3xGFP during cell rearrangements and divisions. They finish the manuscript by very briefly documenting localization of the PCP proteins using the three lines. The authors make a convincing argument that overexpression and loss of function of PCP proteins can have defects.
Tagging the endogenous proteins can also be useful in the case that high quality antibodies are not available, and live imaging can allow visualization of PCP proteins dynamics that can be highly informative. The authors show that the animals are viable, and have bright PCP protein labeling in the expected places. They use super-resolution imaging with these to see the Fx3XGFP and tdTomato- Vangl2 go to the opposite sides of the cell. Importantly Celsr1-3X-GFP and Fz6-3XGFP mice are homozygous viable and fertile with no obvious phenotypes, however the tdTomato-Vangl2 mice show a broad range of PCP defects, indicating function is affected. This manuscript nicely describes new tools/reagent for PCP visualization with novel mouse lines, though not much novelty in terms of new insights into developmental biology mechanisms. This may be more appropriate for a resource/tools paper in Development.

We thank the reviewer for the overall positive summary. We did intend for the paper to be considered as a Tools and Resources paper, as the reviewer suggested.

Reviewer 2 Comments for the Author:

1. Is there a tdTomato-Vangl2 heterozygous phenotype? Similar to Vangl2 Looptail mutation?
In a Bl6 background, heterozygotes occasionally display curly tails, but not in a CD1 background. We have also seen hermaphroditism in heterozygotes in the CD1 background (a common phenotype in Looptail heterozygotes), but for the most part heterozygotes are viable and fertile. Looptail heterozygous phenotypes are much more penetrant. We have added these additional details to the description of the mouse lines in the Materials and Methods.

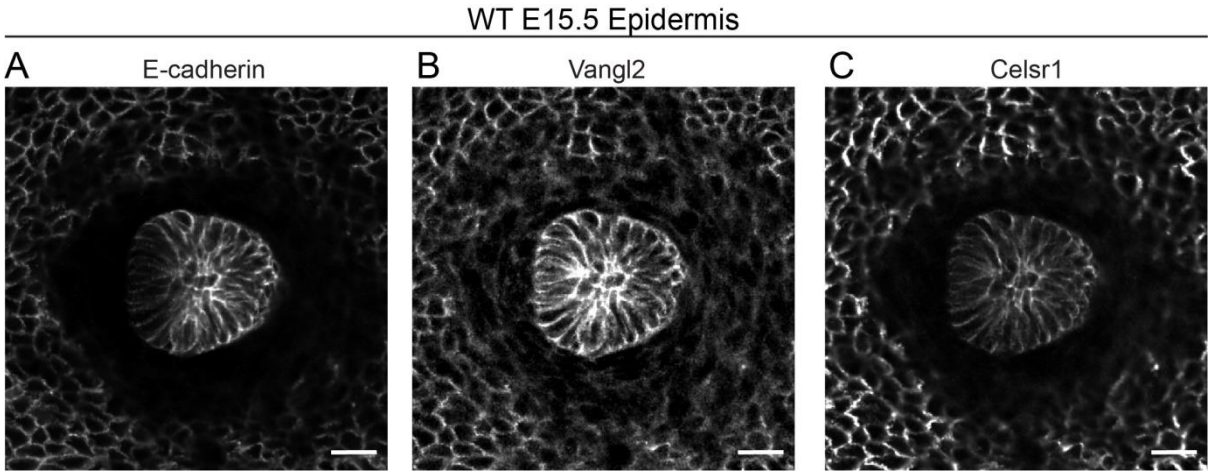
Line 542: Although heterozygous tdTomato-Vangl2 mice were viable and fertile, phenotypes were observed with variable penetrance when backcrossed into different backgrounds. In BL6, heterozygous mice displayed curly tails, whereas in the CD1 background, heterozygous mice displayed hermaphroditism. Homozygous animals at postnatal stages were viable and fertile, but displayed curly tails, hermaphroditism, and head shaking behaviors.

2. Fig 2 was not well described for the naïve reader- it was not clear from an initial reading of the figure legend how the polarity was scored in D-F. Possibly a diagram of the approach, then referencing supplemental figure 1 should be included in Fig 1.
We use the overall morphology and expression of E-Cadherin to measure the polarity of embryonic hair follicles. E-Cad expression is downregulated in the anterior of the hair follicle, but when there is a defect in hair follicle polarity, as in a PCP mutant, the position of the E-Cad low zone is randomized or located in the center of the follicle (Devenport and Fuchs, 2008). We have revised Figure 2 to include a zoomed-in image of a representative follicle labeled with E-Cadherin, and the polarity vector resulting from drawing an arrow through the center of the E-Cad low zone. We also updated Supplemental Figure 2 to include single follicle images together with larger areas of skin that include several follicles labeled with E-Cadherin. Magenta lines overlay each follicle to indicate its polarity (Supplemental Figure 1).

3. Fig 3 could be improved by including the pictures from Sup2. A key aspect of these tools is the degree of similarity of endogenously tagged protein vs antibody staining. If the authors feel there are too many figures, I believe Figure 1 could be compressed in size and added to one of the others without detriment.
Figure 3 is now revised to include the localization of endogenous, untagged tagged PCP proteins in WT epidermis. This allows for direct comparison of WT and tagged PCP protein localizations.

4. Is the staining in the bottom of the hair germ that is so much stronger in tdTomato-Vangl2 present in antibody staining?
Yes, we observe high Vangl2 expression in embryonic hair follicles when staining for the endogenous protein in wild type skin (Devenport and Fuchs, 2008), similar to the tdTomato-Vangl2. Please see Reviewer Figure 2 below showing an example of an embryonic hair follicle

from wild type skin stained for Vangl2, Celsr1 and E-cadherin. Shown is a planar view of a flat mounted skin explant.



Reviewer Figure 2. Representative planar view of a hair follicle in E15.5 wild-type epidermis. Anterior is to the left. **(A)** E-cadherin. Note the low E-cadherin expression in the anterior of the follicle. **(B)** Vangl2. Note high expression throughout the hair follicle. **(C)** Celsr1. Note low overall expression in the hair follicle compared to the surrounding interfollicular cells and Vangl2.

5. Fig 8 continues with the broad survey, and shows that the tagged proteins have interesting expression patterns in stomach, intestine, kidney, liver and lung. But without co-staining, higher magnification or more explanations, this is not very illuminating. Given that Figures 1-6 largely show what has been previously shown with other methods, this is the most biological novelty of the manuscript. It is interesting that the vascular endothelium expresses Fz6-GFP, smooth muscle and stroma express PCP proteins Vang2. It is also interesting that tdTomato-Vangl2 seems to function normally in the epidermis, as opposed to other tissues.
To improve this figure and make it easier for the reader to decipher localization patterns across different tissues, we now show high magnification regions of each tissue with the channels separated and displayed in grayscale. This allows for a more direct comparison between E-Cadherin and PCP protein expression. See revised Figure 8 and Supplementary Figure 8.

6. The authors note that an long-isoform has been recognized—is there available RNAseq from epidermis to see if this isoform is not present there?
A blast search for the long isoform matched with 100% identity to Vangl2 cDNAs isolated from mouse tail tissue, brain, embryonic limb bud, embryonic testis, and embryonic spinal cord (Okazaki et al., 2003; Strausberg et al., 2002; Shibata et al., 2000). Vangl2-long does appear to be an isoform that exists in mouse and is expressed in a variety of tissues.

To determine whether Vangl2-long is expressed in skin, we searched for sequences matching the 5'- extended region of Vangl2-long in our own unpublished RNAseq datasets, and found a match that indicates the alternative splice mRNA is present. We were unable to mine published RNAseq and ssRNAseq of the skin as raw sequence files were unavailable and we are unsure whether the 5' extended region is annotated as Vangl2. Although this information doesn't clarify why tdTomato-Vangl2 mice display defects in the neural tube and not the epidermis, it serves as a useful starting point for future investigations.

Okazaki, N. et al. (2003). Prediction of the coding sequences of mouse homologues of KIAA gene: III. The complete necleotide sequences of 500 mouse KIAA-homologous cDNAs identified by screening of terminal sequences of cDNA clones randomly samples from size-fractionation libraries. *DNA Res* **10**, 167-80.

Shibata, K. et al. (2000) RIKEN integrated sequence analysis (RISA) system--384-format sequencing pipeline with 384 multicapillary sequencer. *Genome Res* 10, 1757-71.

Strausbery, R.L., et al. (2002). Generation and initial analysis of more than 15,000 full length human and mouse cDNA sequences. *PNAS* **99**, 16899-903.

Reviewer 3 Advance Summary and Potential Significance to Field:
This manuscript describes the generation of three new mouse models allowing analysis of the core planar cell polarity (PCP) pathway live in tissues, namely the generation of of endogenously-tagged Celsr1-3xGFP, Fz6-3xGFP and tdTomato-Vangl2 fusion proteins. The authors show that these are functional and are localised asymmetrically. In particular, they use their lab's favourite model of PCP, the mouse epidermis, combined with live imaging to show that PCP in the basal progenitors is not fixed in time but that cell divisions and cell rearrangements lead to shifting patterns whilst still observing the overall tissue polarity. Furthermore, they show that using these novel tools combined with super- resolution STED microscopy Frizzled6-3xGFP and tdTomato-Vangl2 can be resolved across cell-cell junctions with a resolution of 50nm, and this allowed the authors to address discrepancies in the literature, for instance the asymmetric localization of Fz6.Finally, the authors show localisation of the endogenously tagged PCP components in a variety of embryonic tissues in the mouse where PCP is present, as well as several novel patterns of PCP but also expression of the proteins in possible non PCP roles.

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The tools generated and described in this study will be extremely useful to the PCP community and beyond, allowing to tackle important open questions in the field that have remained despite the components themselves being known for years. The authors elegantly demonstrate the localisation and functionality of the tagged proteins, and especially the functionality in super-res microscopy approaches is very impressive and will be very useful for further studies.

In summary, the tools described and characterised here will be of wide use and the paper widely cited once published. The authors have clearly demonstrated and identified novel unknowns aspects of these PCP components function and localisation, demonstrating how useful the tools described here will be.

We thank the reviewer for their positive evaluation of the paper and hope our tools will indeed be useful to the developmental biology community.

Second decision letter

MS ID#: DEVELOP/2021/199695

MS TITLE: New mouse models for high resolution and live imaging of planar cell polarity proteins in vivo

AUTHORS: Lena P Basta, Michael Hill-Oliva, Sarah V Paramore, Rishabh Sharan, Audrey Goh, Abhishek Biswas, Marvin Cortez, Katherine A Little, Eszter Posfai, and Danelle Devenport
ARTICLE TYPE: Research Article

I have looked carefully at your revision and in lighht of this I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.