Planar cell polarity-dependent asymmetric organization of microtubules for polarized positioning of the basal body in node cells

Xiaorei Sai, Yayoi Ikawa, Hiromi Nishimura, Katsutoshi Mizuno, Eriko Kajikawa, Takanobu A Katoh, Toshiya Kimura, Hidetaka Shiratori, Katsuyoshi Takaoka, Hiroshi Hamada and Katsura Minegishi
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MS TITLE: Planar cell polarity dependent asymmetric organization of microtubules for polarized positioning of the basal body in node cells

AUTHORS: Xiaorei Sai, Yayoi Ikawa, Hiromi Nishimura, Katsutoshi Mizuno, Eriko Kajikawa, Hidetaka Shiratori, Katsuyoshi Takaoka, Hiroshi Hamada, and Katsura Minegishi

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 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

In this manuscript, the authors generated Dchs1 and Dchs2 double mutants mouse embryos, which showed partial failure of asymmetrical localization of basal bodies in the nodal pit cells as well as

altered nodal flow, consistent with previous findings that PCP is required for posterior localization of basal bodies in the pit cells. The authors then turned to investigate whether microtubules (MTs) and actomyosin are involved in basal body positioning. By treating in vitro cultured embryos with drug that alter the dynamic MT and actin assembly, the authors found that asymmetrical basal body positioning was disrupted. These data led the authors to conclude that asymmetry in baMT organization may play a role in correct positioning of the basal body for establishment of left-right asymmetry. The data shown are of high quality and it is important to ask how core PCP proteins like Vangl, Dchs, Prickle regulate asymmetrical basal body positioning in the node pit cells.

Comments for the author

However, the current studies are mostly descriptive and did not provide a strong causal connection between baMT organinization and the basal body localization.
Specific comments:
1. It is quite skeptical that the mouse Dchs1/2 regulate PCP. If the mouse Dchs1/2 truly regulate PCP, the authors should show PCP-specific phenotypes including left-right asymmetry of the Dchs1/2 mutant embryos.
2. The authors need to explain how proteins without asymmetrical localization could result in defects in asymmetrical localization of other PCP proteins such as Vangl1. Is Vangl2 localization also altered? If so, why the phenotypes were milder?
3. The authors should quantify immunohistochemistry results to justify many of the claims (for example, in Figure 5, 6 and 8).
4. Does Rac1 inhibition abolish Vangl1 localization and basal body posterior localization?
5. It is not surprising that dynamic assembly of MT and actin is required for basal body positioning, as has been indicated in spindle body assembly. However there is no definitive evidence that PCP is required for such dynamics.
Therefore, the manuscript appears to contain two not so well-developed stories: phenotypical description of Dchs1-/-;Dchs2-/- embryos and the connection between PCP and dynamic assembly of MT or actin. It is likely that disrupted baMT and actinmyosin organization are defects downstream of core PCP genes associated with mispositioning of the basal bodies. The observed basal body localization defect in the in vitro experiments may simply reflect the requirement for dynamic MT and actin remodeling, which is likely not regulated by PCP.
Minor comments.
The manuscript should benefit from careful proof reading. In P6, Mouse embryos at the early{late?} headfold…

Reviewer 2

Advance summary and potential significance to field

The manuscript by Sai et al addresses the development of basal body (bb) asymmetry in the mouse embryonic node. It is well known that asymmetric positioning of the node cilia is an essential aspect of left-right determination, yet the molecular mechanism driving asymmetry remain largely unknown. In this manuscript the authors address the contribution of PCP signaling and both microtubule and acto-myosin on bb asymmetry. First they show that the PCP component Vangl1s asymmetry in node cells is disrupted in Dchs1/2 mutant mice as is the polarized flow and bb distribution. Additionally, they perform a detailed drug analysis on explanted tissue to address the function of cytoskeletal regulators on bb movements and find that both MTs and actin/myosin function is critical for bb asymmetry establishment and maintenance. They show that pMLC is asymmetric and downstream of PCP signaling and they propose that this is an important feature of driving bb asymmetry based on the timing of various asymmetric features (e.g. PCP—pMLC—bb). Finally they identify a shift in the angle and attachment of MTs that accompanies the establishment of bb asymmetry and they propose that differential MT functions (either nucleation or stabilization) could underlie the movement of bbs during establishment of asymmetry. The authors do a nice job of performing quantification of important aspects of their data and in particular the novel characterization of MT angle in developing cells is very important and novel. In general this is a beautiful paper that adds a new layer of insight into the establishment of left-right patterning and will be of interest to a wide range of developmental biologists.

Comments for the author

Comments:
"The plus ends of anterior baMTs appeared to reach the adherens junctions given that the termini of the MTs were located at the region positive for E-cadherin (Fig. 8A)."…..It is certainly reasonable and likely that the minus ends are at the bb, but there are numerous examples of MTs nucleating from other places including the cell cortex. Given that the actual plus and minus ends are not being assessed here I would recommend using a more conservative terminology or actually performing + and - end staining (which would probably be very helpful).
"However, our observations at the resolution level of microscopy applied indicated that the plus ends of both anterior and posterior baMTs are localized to adherens junctions.". ….I am not sure that I entirely agree with this statement. The MTs appear to run along both the apical cell cortex as well as the lateral cortex (adherens junction). What seems to change is that in the EHF and 3ss the posterior MTs no longer associate with apical cortex but are more restricted to the lateral cortex. The terminology of saying the MTs localize with the adherins junctions is a bit misleading. It implies a specific localization but in fact it is quite broad to the overall cell cortex. There is no specific localization that indicates strong co-localization with specific foci of cadherin. I would urge a more vague terminology.

Minor In FigS7 LB the inset box is not surrounding the correct cell but the one just above.

First revision

Author response to reviewers' comments

Response to the reviewers' comments
We thank the reviewers for their constructive comments. We have addressed these comments by performing additional experiments and analyses wherever necessary. Please note that abstract has been shortened to 176 words according to the journal's guidline.

Reviewer 1 *Advance Summary and Potential Significance to Field:*
In this manuscript, the authors generated Dchs1 and Dchs2 double mutants mouse embryos, which showed partial failure of asymmetrical localization of basal bodies in the nodal pit cells as well as altered nodal flow, consistent with previous findings that PCP is required for posterior localization of basal bodies in the pit cells. The authors then turned to investigate whether microtubules (MTs) and actomyosin are involved in basal body positioning. By treating in vitro cultured embryos with drug that alter the dynamic MT and actin assembly, the authors found that asymmetrical basal body positioning was disrupted. These data led the authors to conclude that asymmetry in baMT organization may play a role in correct positioning of the basal body for establishment of left-right asymmetry. The data shown are of high quality and it is important to ask how core PCP proteins like Vangl, Dchs, Prickle regulate asymmetrical basal body positioning in the node pit cells.

Reviewer 1 Comments for the Author:
However, the current studies are mostly descriptive and did not provide a strong causal connection between baMT organinization and the basal body localization.

Response:
Unfortunately, it is not feasible to directly disrupt A-P asymmetry of baMT organization alone. However, there is a very close connection between baMT organization and the basal body localization, since the latter was impaired whenever the former was disrupted (stage-dependence, Pk and Dchs mutants, treatment with inhibitors).

Specific comments:
1. It is quite skeptical that the mouse Dchs1/2 regulate PCP. If the mouse Dchs1/2 truly regulate PCP, the authors should show PCP-specific phenotypes including left-right asymmetry of the Dchs1/2 mutant embryos.

Response: We have examined Nodal *expression in the Dchs1/2 mutant embryos. Among eight Dchs1/2 mutant embryos examined, left-sided expression of* Nodal *in the lateral plate was lost in 4/8 embryos, down-regulated in 2/8 embryos and remained normal in 2/8 embryos (now shown in Fig. 1F, and mentioned on page 5).. This is consistent with the impaired nodal flow in the Dchs1/2 mutant embryos (Fig. 1E).*

2. The authors need to explain how proteins without asymmetrical localization could result in defects in asymmetrical localization of other PCP proteins such as Vangl1. Is Vangl2 localization also altered? If so, why the phenotypes were milder?

Response: We believe that Dchs1, 2 have a permissive role. Thus, they are required for polarized localization of other PCP proteins such as Vangl1 &2, but Dchs 1,2 proteins themselves do not need to be localized asymmetrically. The exact function of Dchs proteins remains unknown but they may be required to anchor Vangl proteins at the cell membrane, since Vangl1 protein was markedly detected in the cytoplasm in Dchs1/2 mutant embryos (Fig. 1A, C). As requested by the reviewer, we have examined Vangl2 localization in Dchs1/2 mutant embryos. Vangl2 was localized at the anterior side of node cells in the WT embryos (as previously reported by Minegishi et al, 2018), while its localization was not polarized in the Dchs1/2 mutant embryos (shown in Supplementary Fig. S2).

3. The authors should quantify immunohistochemistry results to justify many of the claims (for example, in Figure 5, 6 and 8).

Response: as requested by the reviewer, we have quantified immunohistochemistry and other results by multiple ways.
1) The frequency of embryos showing a particular staining pattern is now described as xx/xx embryos, in Fig. 1, while the number of embryos examined is indicated in Fig. 5 and Fig.6. An embryo showing the representative staining pattern is shown in each panel of Fig. 5 and Fig. 6 (all the embryos examined showed similar staining pattern).
2) We have quantified the level of pMLC staining in Fig. 5 and Fig. 6. The staining level increased from the LB stage to the 3ss stage (Fig. 5G), and was reduced in Dchs1,2 double KO, Pk1,2 double KO, Sfrp1,2,5 triple KO embryos (Fig. 6F).
3) We have quantified localization pattern of Valgl1 in drug-treated embryos (shown in a new figure, Fig. 7) and that of Vangle2 (Fig. S2) and results are shown as rose diagrams. These new data further confirmed that localization of Vangl1/2 is polarized along the A-P side of node cells in WT embryos while its asymmetric localization is disrupted in Dchs1/2 and PK1/2 mutant embryos. We also provide the frequency of node cells showing asymmetric staining pattern of pMLC at different stages in Fig. 5F.
4) baMT pattern shgwn in Fig.9 was quantitatively analyzed and shown as rose diagrams in Fig. 10. In Fig. S9C and the legend to Fig. 9, we now provide the frequency of node cells that had asymmetric baMTs:2/20 cells at LB stage, 24/24 at the 3ss stage, 2/23 in Pk1,2 DKO embryos etc.
5) We have performed statistical analysis wherever possible, and now provide statistical significance in these figures.

4. Does Rac1 inhibition abolish Vangl1 localization and basal body posterior localization?
Response: We thank the reviewer for this constructive comment. We have addressed this important question by performing rotation culture with the Rac1 inhibitor, NSC23766. Vangl1 localization was not affected by Rac1 inhibition, which is shown in a new figure (Fig. 7). The basal body position was affected by the Rac1 inhibitor (shown in Fig. x), as we reported previously (Hashimoto et al., NCB 2010). In addition, we have examined the effects of ML-7 on Vangl1 localization. A-P polarized localization of Vangl1 was maintained by ML-7 treatment (Fig. 7). These results collectively suggest that Rac1-mediated activation of myosin II is required for posterior shift of the basal body, but it is an event downstream of polarization of Vangle1 localization.

5. It is not surprising that dynamic assembly of MT and actin is required for basal body positioning, as has been indicated in spindle body assembly. However, there is no definitive evidence that PCP is required for such dynamics.
Therefore, the manuscript appears to contain two not so well-developed stories: phenotypical description of Dchs1-/-;Dchs2-/- embryos and the connection between PCP and dynamic assembly of MT or actin. It is likely that disrupted baMT and actinmyosin organization are defects

downstream of core PCP genes associated with mispositioning of the basal bodies. The observed basal body localization defect in the in vitro experiments may simply reflect the requirement for dynamic MT and actin remodeling, which is likely not regulated by PCP.

Response: We agree with the reviewer's suggestion that disrupted baMT and actinmyosin organization are defects downstream of core PCP genes, since A-P asymmetric baMT organization was lost in Dchs1/2 and PK mutants (Fig. 8, 9-→Fig.9 and Fig. 10 in the revised manuscript). However, A-P asymmetric organization of baMT is unlikely the consequence of the posterior shift of basal body, because the asymmetric MT organization takes place before the posterior shift of the basal body (Fig. 9). Although it is not feasible to test directly, our current data support an idea that asymmetric MT organization is required for the posterior shift of the basal body.

As suggested by the reviewer, MTs and actomyosin are required at multiple steps during the posterior shift of the basal body in node cells. Their presence (amount) would be required for (asymmetric) localization of PCP protein at the cell membrane. Indeed, upon treatment with nocotazole, Vangle1 level at the cell membrane was reduced with its increase in the cytoplasm, and A-P polarized localization was affected (Fig. 7). Distribution pattern of MTs also regulates the position of the basal body.

Minor comments.
The manuscript should benefit from careful proof reading. In P6, Mouse embryos at the early{late?} headfold…

Response: Thank you. It was supposed to be late headfold, and we have corrected it.

Reviewer 2 Advance Summary and Potential Significance to Field:
The manuscript by Sai et al addresses the development of basal body (bb) asymmetry in the mouse embryonic node. It is well known that asymmetric positioning of the node cilia is an essential aspect of left- right determination, yet the molecular mechanism driving asymmetry remain largely unknown. In this manuscript the authors address the contribution of PCP signaling and both microtubule and acto-myosin on bb asymmetry. First they show that the PCP component Vangl1s asymmetry in node cells is disrupted in Dchs1/2 mutant mice as is the polarized flow and bb distribution. Additionally, they perform a detailed drug analysis on explanted tissue to address the function of cytoskeletal regulators on bb movements and find that both MTs and actin/myosin function is critical for bb asymmetry establishment and maintenance. They show that pMLC is asymmetric and downstream of PCP signaling and they propose that this is an important feature of driving bb asymmetry based on the timing of various asymmetric features (e.g. PCP— pMLC—bb). Finally they identify a shift in the angle and attachment of MTs that accompanies the establishment of bb asymmetry and they propose that differential MT functions (either nucleation or stabilization) could underlie the movement of bbs during establishment of asymmetry. The authors do a nice job of performing quantification of important aspects of their data and in particular the novel characterization of MT angle in developing cells is very important and novel. In general this is a beautiful paper that adds a new layer of insight into the establishment of left-right patterning and will be of interest to a wide range of developmental biologists.

Reviewer 2 Comments for the Author:

Comments:

"The plus ends of anterior baMTs appeared to reach the adherens junctions given that the termini of the MTs were located at the region positive for E-cadherin (Fig. 8A)."…..It is certainly reasonable and likely that the minus ends are at the bb, but there are numerous examples of MTs nucleating from other places including the cell cortex. Given that the actual plus and minus ends are not being assessed here I would recommend using a more conservative terminology or actually performing + and - end staining (which would probably be very helpful).

Response: We attempted to distinguish plus and minus ends of baMTs with specific markers such as CLIP-170 and EB1. Unfortunately, however, neither antibodies worked for node cells of mouse embryos although these antibodies work for cultured cells. Transgenic embryos expressing

EB1::EGFP protein did not give rise to sufficient signals in embryos either. In the absence of such data, we have deleted “plus or minus-end” and simply describe as “termini.”

“However, our observations at the resolution level of microscopy applied indicated that the plus ends of both anterior and posterior baMTs are localized to adherens junctions.”. ….I am not sure that I entirely agree with this statement. The MTs appear to run along both the apical cell cortex as well as the lateral cortex (adherens junction). What seems to change is that in the EHF and 3ss the posterior MTs no longer associate with apical cortex but are more restricted to the lateral cortex. The terminology of saying the MTs localize with the adherins junctions is a bit misleading. It implies a specific localization but in fact it is quite broad to the overall cell cortex. There is no specific localization that indicates strong co-localization with specific foci of cadherin. I would urge a more vague terminology.

Response: We agree with the reviewer. Node cells were co-stained for MTs and E-cadherin, and both saining regions are closely located, but not at a high enough resolution that would warrant precise co-localization. As suggested by the reviewer, we now use a more vague terminology, the cell cortex, instead of adherens junction throughout the text.

In FigS7 LB the inset box is not surrounding the correct cell but the one just above.
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Response: This was corrected

Second decision letter

MS ID#: DEVELOP/2021/200315

MS TITLE: Planar cell polarity dependent asymmetric organization of microtubules for polarized positioning of the basal body in node cells

AUTHORS: Xiaorei Sai, Yayoi Ikawa, Hiromi Nishimura, Katsutoshi Mizuno, Eriko Kajikawa, Takanobu A Katoh, Toshiya Kimura, Hidetaka Shiratori, Katsuyoshi Takaoka, Hiroshi Hamada, and Katsura Minegishi
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

The authors have thoroughly revised the manuscript with addition experiments and data. The previous concerns have been adequately addressed. The study provides good evidence how PCP signaling regulates basal body positioning.

Comments for the author

The previous concerns have been adequately addressed. The manuscript is suitable for publication in Development.

Reviewer 2

Advance summary and potential significance to field

Same as previous version.

Comments for the author

None. I think this is a really beautiful paper. The changes have made a very good paper even better.