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Phosphorylation and dephosphorylation of Ser852 and Ser889 control the clustering, localization and function of PAR3

Kazunari Yamashita, Keiko Mizuno, Kana Furukawa, Hiroko Hirose, Natsuki Sakurai, Maki Masuda-Hirata, Yoshiko Amano, Tomonori Hirose, Atsushi Suzuki and Shigeo Ohno
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MS TITLE: Phosphorylation and dephosphorylation of Ser852 and Ser889 control clustering, localization, and function of PAR-3

AUTHORS: Kazunari Yamashita, Keiko Mizuno, Kana Furukawa, Hiroko Hirose, Natsuki Sakurai, Maki Masuda-Hirata, Yoshiko Amano, Tomonori Hirose, Atsushi Suzuki, and Shigeo Ohno
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

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 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. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

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Reviewer 1

Advance summary and potential significance to field

In this manuscript, Yamashita et al. report that Par3 localization and clustering is subject to regulation through ASPP1 and protein phosphatases. The study is overall very thoroughly conducted and includes comprehensive biochemical and cell biological analyses of Par3 phosphorylation. The authors report a novel phosphorylation site in Par3 (Ser852), demonstrate a principle role for its modification in junction biogenesis, and made efforts to identify the kinase responsible for phosphorylation at this site. While they could not pinpoint the kinase(s) modifying Par3 at physiologic conditions (Par1b inhibition only suppressed ectopic phosphorylation but not phosphorylation of endogenous Par3), they focused on the process of dephosphorylation of Par3, which involves ASPP2 and its interplay with PP1 or PP2A. The concept that Par3-Ser852 and Ser889 (de)phosphorylation determines where and how long Par3 localized to distinct membrane domains is certainly interesting.

Comments for the author

Major Points:
Given the importance of the protein clustering aspect in this study, the manuscript would benefit from an improved and more detailed presentation of the FRAP data. This would substantiate the conclusion that Par3 clustering depends on phosphorylation at specific residues. Although suggestive that the cytoplasmic pool of Par3 is the one responsible for signal recovery, the appearing Par3 molecules could also derived from more apical or more basal membrane domains (as the case for certain cadherin sub-pools). More details on the FRAP calculations and micrographs to clarify which fraction is actually measured would be of help.

The authors use a single drug targeting PP1A (tautomycetin/TMC), and calyculin A, which inhibits PP1 and PP2A. The results from these experiments do not exclude that also PP2A dephosphorylates Ser852. PP2A has been implicated in dephosphorylation of Bazooka (e.g. Nam et al 2007; Krahn et al., 2009), and of Par3-binding partners in mammals (like JAM-A, Iden et al. 2012), thus it would not be too surprising if PP1 and PP2A both modify Ser852. In fact, it seems that Calyculin A has a stronger effect on Ser852-P than TMC alone. Adding a quantification of P-Par3 in Calyculin A treated cells vs. controls vs. TMC-treated cells in Fig. 3H would thus be helpful. Did the authors test additional drugs like okadaic acid (PP1/PP2A inhibition) or a PP1A activator, which could further serve to dissect the phosphatase specificity?
At least, a more careful discussion on this point should be provided.

The summary scheme in Figure 8 is nice, but should be restricted to information that has been shown in the presented study or previous studies. Alternatively, suggestive molecular connections should be unambiguously identified by specific drawings, question marks etc., to avoid overstating.

Minor Points:
The statistical analysis requires at least validation. Using a Student’s t-test for each and every data set (also for multiple comparisons, and without stating the outcome of normality tests and type of raw data) is questionable.

The authors used and developed tools that are important for the field. Congratulations on this. Adding a table that summarizes the antibodies used with dilutions, source etc. would be very helpful for the reader.

The citations should be revisited - integrating some more work in the area relevant for this manuscripts would increase objectivity.

The running title is not very informative and is slightly misleading.

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Reviewer 2

Advance summary and potential significance to field

This paper provides important new insights into the regulation of the polarity protein PAR3. The authors identify a previously unknown phosphorylation site in PAR3. A mechanism of direct protein interactions is shown to link a phosphatase to PAR3, and this phosphatase is shown to control the phosphorylation status of the site. Par-1 and additional unknown kinases are also implicated. In cell culture, phosphorylation of the newly identified site together with additional known sites is shown to prevent ectopic clustering of Par-3 and to allow normal rates of recruitment to cell-cell junctions. Inhibition of kinases also leads to abnormal clustering. Indicating the importance of the phosphorylation events for polarized epithelial structure, the phosphorylation sites are also needed for efficient tight junction assembly. Overall, the paper combines high quality biochemical and cell biological studies to reveal a molecular mechanism for controlling the localized clustering of a cell polarity scaffold protein. The study should be of interest to the readers of JCS. A number of points should be addressed.

Comments for the author

1. On lines 30 and 281, the unphosphorylatable forms of PAR3 are described as “static”. This is an overstatement. They displayed reduced dynamics but are not fully static.

2. It is suggested several times (and indicated in the final model) that the ectopic PAR3 clustering might reduce PAR3 recruitment to cell-cell junctions because of the “low diffusion rate of clustered PAR3”. It also seems possible that the ectopic clusters could trap/sequester PAR3 molecules that would otherwise diffuse individually to the junctions. Furthermore, although the authors provide evidence for the ectopic clusters not associating with VACs, it remains possible that they associate with other intracellular membranes, and may not freely diffuse through the cytosol. The authors should acknowledge these alternate possibilities in the text (and model), or provide more data to support the cluster diffusion idea.

3. On line 270, it is stated that “The ASPP2–PP1 complex is efficiently recruited to the PAR3 cluster”. Are the authors saying that ASPP2–PP1 complex recruitment to PAR3 clusters is more efficient than to PAR3 monomers? If so then the data supporting this should be referenced. If not, then re-wording is warranted.

4. The PAR-1 phosphorylation of Drosophila PAR3 can also have a positive relationship with PAR3 clusters associated with centrosomes (shown normally in Drosophila male germline stem cells by the Yamashita lab [PMID: 25793442] and with mis-regulation in the Drosophila embryo ectoderm by the Harris lab [26455305]). These studies should be briefly mentioned to highlight some of the complexity of PAR3 cluster regulation.

5. In Fig 2B, the left asterisk may be mis-positioned, and positions of Mw markers should be indicated.

6. In Fig 3D, statistically significant differences should be indicated.

Reviewer 3

Advance summary and potential significance to field

PAR3 is an evolutionary conserved polarity protein that acts in epithelial cells to regulate apical basal polarity and the formation of apical junctions. In vertebrate epithelia, PAR3 is enriched at tight junctions (TJs) and is important for TJ assembly. Phosphorylation at multiple sides is an essential component of PAR3 regulation as has been documented through numerous studies, including work from the Ohno laboratory. This is an important area of research that clarifies the molecular mechanisms of cell polarization. The present paper identifies an additional phosphorylation side within PAR3, Ser852, and shows that dynamic phosphorylation of Ser852 as well as Ser889 plays a key role in PAR3 clustering and localization at the TJ domain. Phospho-

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Ser852 binds 14-3-3, which presumably interferes with molecular interactions that would enhance PAR3 clustering. The authors further show that an ASPP2-PP1 complex can dephosphorylate Ser852 and Ser889. These data suggest that the non-phosphorylated forms of PAR3 act at TJs to promote polarity and TJ formation. However, it is also shown that non-phosphorylatable forms of PAR3 have reduced turnover and their ability to promote normal TJs formation at new cell contacts is compromised, suggesting that phosphorylation – dephosphorylation cycles are crucial for normal PAR3 activity.

The authors present a detailed and well-documented study that is very appropriate for JCS. I have listed a number of small points. While I realize and appreciate that the authors are not native English speakers, the paper would benefit from a revision to improve the English grammar.

Line 2: PAR-3 or PAR3 – settle on one version. Same goes for PAR1.
Line 24: …..we demonstrate that ASPP2, which controls PAR3 localization…..
Line 45: PAR proteins were first identified in the context of asymmetric cell division of the C.elegans zygote Line 52 etc: two key papers that establish negative feedback between apical and basolateral polarity factors are Tanentzapf and Tepass, NCB 2003 and BIlder et al., NCB 2003.
Line 67: a key paper showing that PAR3 is upstream of AJ assembly is Harris and Peifer, JCB 2004.
Line 73: Par3 does not have a transmembrane …..
Line 106 ….This mutant was severely compromised in its interaction…..
Line 109…. ASPP2 mutant lacking the C-terminus () failed to interact….
Line 111…This suggests that the REVD…..
Line 113…. REVD mutant in previously….
Line 115…REVD mutant did not (Fig.1E,F….
Line 128 ….Among them, the 1-269aa fragment ….. whereas the 710-936 aa fragment showed a strong affinity…. This result suggests that ….
Line 133… short isoform of mouse PAR3, which lacks….
Line 136… Ser point mutants and identified Ser852 as a novel 14-3-3 binding sitte in PAR3 Line 137: the alignments seems to show conservation of Ser852; explain why this statement is qualified ('may be conserved')
Line 198: delete 'In addition'
Line 199:…triple mutant were used to evaluate the function….
Line 204: if a molecule is observed in puncta (assuming multiple molecules need to be present to make it visible) does that not imply that the molecule is 'clustered'. So this statement would be tautological.
Line 217 etc: the authors may want to consider how there diffusion model could be affected be the fact that their constructs a 10x overexpressed.
Line 224: …a low turnover at a developing ….
Line 233 ….prolonged in clone #6 but not in clone #3…
Line 249….cells organized unique intercellular…
Line 259…can be dephosphorylated by…
Line 260 …In comparison to normal PAR3… (wild-type would be the endogenous unmodified PAR3, a GFP-tagged exogenous PAR3 is not wild-type. This should be corrected throughout.)
Line 263….localized to cell-cell contact sites….
Line 267…
Line 268 etc: delete 'as depicted in Figure 4'….these data suggest that the normal localization of PAR3 …….clustering of PAR3 at a specific….
Line 270: …PAR3 clusters…
Line 271:…sites, which would promote ASPP2-PP1-mediated dephosphorylation of PAR3 and , consequently, clustering. …
Line 273…at a specific….
Line 288…candidates…
Line 317….PAR3 clusters anchor centrosomes…

I did not edit M+M or Figure legends; please check

Figure 1A: Is the increase of PAR3, aPKC, and PAR6 in the ASPP2 lane a reproducible finding?
Figure 2B: replace 'deletion mutants' with 'fragments'
Figure 5: the control here is overexpressed PAR3-GFP. How does this control compare to endogenous PAR3 in these type of experiment. Are they the so same?

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Comments for the author

see above

First revision

Author response to reviewers' comments

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AUTHORS: Kazunari Yamashita, Keiko Mizuno, Kana Furukawa, Hiroko Hirose, Natsuki Sakurai, Maki Masuda-Hirata, Yoshiko Amano, Tomonori Hirose, Atsushi Suzuki, and Shigeo Ohno

September 17, 2020
Our response to the Reviewers' comments:

We thank all the reviewers for reading our manuscript and giving constructive advices. We performed reanalysis of our data and rewriting the manuscript to address all the issues raised by the reviewers.

In main text, alterations relating to suggestions of reviewer 1, reviewer 2, and reviewer 3 were marked in yellow, green, and turquoise, respectively. Pink markers indicate our corrections. Please see original document file 'main_text_rev_YamashitaK.docx', especially for confirmation of line numbers. Our point by point response to the reviewers' comments follows.

Reviewer 1 Advance Summary and Potential Significance to Field:
In this manuscript, Yamashita et al. report that Par3 localization and clustering is subject to regulation through ASPP1 and protein phosphatases. The study is overall very thoroughly conducted and includes comprehensive biochemical and cell biological analyses of Par3 phosphorylation. The authors report a novel phosphorylation site in Par3 (Ser852), demonstrate a principle role for its modification in junction biogenesis, and made efforts to identify the kinase responsible for phosphorylation at this site. While they could not pinpoint the kinase(s) modifying Par3 at physiologic conditions (Par1b inhibition only suppressed ectopic phosphorylation but not phosphorylation of endogenous Par3), they focused on the process of dephosphorylation of Par3, which involves ASPP2 and its interplay with PP1 or PP2A. The concept that Par3-Ser852 and Ser889 (de)phosphorylation determines where and how long Par3 localized to distinct membrane domains is certainly interesting.

Reviewer 1 Comments for the Author:
Major Points:

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This standpoint is potentially interesting. However, our experimental system doesn't have enough resolution along Z-axis to analyze influxes of PAR3-EGFP from the apical or basal membrane, because high power bleaching beam bleached not only the focal plane but also upper and lower sides (please see attached data, 'Figures only for reviewers, Yamashita'). In addition to this, we

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used plastic dishes for FRAP experiment instead of transwell because of compatibility with the live-imaging chamber. The MDCK cells which is cultured on dishes poorly develops lateral membrane domain even when they reached at confluence. This further makes it difficult to achieve this experiment. From these reasons, we can argue the exchange of membrane-associated PAR3 only along the X-Y direction. In revised version, we precisely analyzed recovery of PAR3-EGFP along the plasma membrane in X-Y plane (supplementary Fig S6A). This data shows that the exchange within membrane-associated PAR3 looks enough slower than the exchange between the membrane and the cytoplasm. From these data, we speculate that the exchange of PAR3 along the apical-basal axis might also be inefficient.

The authors use a single drug targeting PP1A (tautomycetin/TMC), and calyculin A, which inhibits PP1 and PP2A. The results from these experiments do not exclude that also PP2A dephosphorylates Ser852. PP2A has been implicated in dephosphorylation of Bazooka (e.g. Nam et al 2007; Krahn et al., 2009), and of Par3-binding partners in mammals (like JAM-A, Iden et al. 2012), thus it would not be too surprising if PP1 and PP2A both modify Ser852. In fact, it seems that Calyculin A has a stronger effect on Ser852-P than TMC alone. Adding a quantification of P-Par3 in Calyculin A treated cells vs. controls vs. TMC-treated cells in Fig. 3H would thus be helpful. Did the authors test additional drugs like okadaic acid (PP1/PP2A inhibition) or a PP1A activator, which could further serve to dissect the phosphatase specificity?
At least, a more careful discussion on this point should be provided.

As reviewer1 suggests, we performed quantification of Calyculin A-treatment (Fig. 3H) and added following sentences (line 184).
This result would suggest the specific function of PP1 on Ser852 dephosphorylation. However, we do not exclude the possibility of the involvement of other phosphatases including PP2A on Ser852 dephosphorylation, because calyculin A treatment was relatively more effective than tautomycetin treatment, although no significant difference was found between them (Fig. 3G,H).

We consider that Calyculin A can not specify the functional phosphatase species because they also inhibit PP4, PP5, and PP6, besides PP1 and PP2A (Zhang M et.al., FEBS J, 2013). In MDCK cells, we could not clearly detect the interaction between PAR3 and PP2A, which has been reported in *Drosophila* (Krahn et al., 2009) (Fig1A and Fig3C). Thus, we didn't focus on PP2A in this study. We discuss about the functional difference between PP1 and PP2A in the discussion section (from line 305).

The summary scheme in Figure 8 is nice, but should be restricted to information that has been shown in the presented study or previous studies. Alternatively, suggestive molecular connections should be unambiguously identified by specific drawings, question marks etc., to avoid overstating.

We agree with this opinion. As reviewer1 suggested, S852/889 kinase has not been identified yet, and its localization was based on our speculation. We improved Fig8, making in mind with another suggestion from reviewer2.

Minor Points:

The statistical analysis requires at least validation. Using a Student's t-test for each and every data set (also for multiple comparisons, and without stating the outcome of normality tests and type of raw data) is questionable.

We adopted unpaired t-test for statistical analyses. As reviewer1 suggested, our previous representations would give readers the misleading impression that multiple comparison tests were used in Fig3B, Fig4B, Fig5E, Fig6EF, Fig7B, and Fig S6BC. So, in revised manuscript, we improved graphs (P value was represented even if there is no significance) and legends (the name of tests and compared pairs were declared) to prevent misunderstanding in such figures.
We know about "Multiple comparisons problem". However, we did not adopt multiple comparison procedures in this study because our experiments were basically designed to test the difference between two groups (e.g. WT PAR3-expressing cells and S852A mutant PAR3-expressing cells) even

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if multiple samples were experimented. In this study area (molecular cell biology), t-test has generally been used for such purpose (e.g. Huo Y and Macara IG, Nature Cell Biology, 16(6), 2014 or Zihni C et.al., J Cell Biology, 204(1) 111–127, 2013 or Inaba et al., eLife;4:e04960, 2015).

The authors used and developed tools that are important for the field. Congratulations on this. Adding a table that summarizes the antibodies used with dilutions, source etc. would be very helpful for the reader.

We agree with this suggestion. We made Table S1 in supplementary information.

The citations should be revisited - integrating some more work in the area relevant for this manuscripts would increase objectivity.

In revised manuscript, we added following references:

Benton, R. and St Johnston, D. (2003a). A conserved oligomerization domain in drosophila Bazooka/PAR-3 is important for apical localization and epithelial polarity. Curr Biol 13, 1330-4.

Bilder, D., Schober, M. and Perrimon, N. (2003). Integrated activity of PDZ protein complexes regulates epithelial polarity. Nat Cell Biol 5, 53-8.

Tanentzapf, G. and Tepass, U. (2003). Interactions between the crumbs, lethal giant larvae and bazooka pathways in epithelial polarization. Nat Cell Biol 5, 46-52.

Harris, T. J. and Peifer, M. (2004). Adherens junction-dependent and -independent steps in the establishment of epithelial cell polarity in Drosophila. J Cell Biol 167, 135-47.

Chen, S., Chen, J., Shi, H., Wei, M., Castaneda-Castellanos, D. R., Bultje, R. S., Pei, X., Kriegstein, A. R., Zhang, M. and Shi, S. H. (2013). Regulation of microtubule stability and organization by mammalian Par3 in specifying neuronal polarity. Dev Cell 24, 26-40.

Zhang, M., Yogesha, S. D., Mayfield, J. E., Gill, G. N. and Zhang, Y. (2013a). Viewing serine/threonine protein phosphatases through the eyes of drug designers. FEBS J 280, 4739-60.

Jiang, T., McKinley, R. F., McGill, M. A., Angers, S. and Harris, T. J. (2015). A Par-1-Par-3-Centrosome Cell Polarity Pathway and Its Tuning for Isotropic Cell Adhesion. Curr Biol 25, 2701-8.

Roman-Fernandez, A. and Bryant, D. M. (2016). Complex Polarity: Building Multicellular Tissues Through Apical Membrane Traffic. Traffic 17, 1244-1261.

Choy, M. S., Swingle, M., D'Arcy, B., Abney, K., Rusin, S. F., Kettenbach, A. N., Page, R., Honkanen, R. E. and Peti, W. (2017). PP1:Tautomycetin Complex Reveals a Path toward the Development of PP1-Specific Inhibitors. J Am Chem Soc 139, 17703-17706.

Jouette, J., Guichet, A. and Claret, S. B. (2019). Dynein-mediated transport and membrane trafficking control PAR3 polarised distribution. Elife 8.

The running title is not very informative and is slightly misleading.

We agree with this suggestion. Although 32 characters is too limited to express our point, we changed the running tile to “Regulations of PAR3 clusters” (28 characters).

Reviewer 2 Advance Summary and Potential Significance to Field:
This paper provides important new insights into the regulation of the polarity protein PAR3. The authors identify a previously unknown phosphorylation site in PAR3. A mechanism of direct protein interactions is shown to link a phosphatase to PAR3, and this phosphatase is shown to control the phosphorylation status of the site. Par-1 and additional unknown kinases are also implicated. In cell culture, phosphorylation of the newly identified site together with additional known sites is shown to prevent ectopic clustering of Par-3 and to allow normal rates of recruitment to cell-cell junctions. Inhibition of kinases also leads to abnormal clustering. Indicating the importance of the

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phosphorylation events for polarized epithelial structure, the phosphorylation sites are also needed for efficient tight junction assembly. Overall, the paper combines high quality biochemical and cell biological studies to reveal a molecular mechanism for controlling the localized clustering of a cell polarity scaffold protein. The study should be of interest to the readers of JCS. A number of points should be addressed.

Reviewer 2 Comments for the Author:
1. On lines 30 and 281, the unphosphorylatable forms of PAR3 are described as “static”. This is an overstatement. They displayed reduced dynamics but are not fully static.

We agree with this suggestion. We changed the expression as follows:
Line 29
We also demonstrate that unphosphorylatable forms of PAR3 ~~are static in~~ exhibited a low molecular turnover and fail to coordinate rapid reconstruction of the tight junction, supporting that both phosphorylated and dephosphorylated states are essential for the functional integrity of PAR3.

Line 287
Dephosphorylated PAR3 molecules can concentrate and exert a strong activity that promotes the formation of the tight junction and the apical domain. However, since dephosphorylated form is ~~static in molecular turnover~~ less diffusive and can be trapped by some cellular structures, it fails to coordinate the rapid reconstruction of the tight junction. Therefore, both phosphorylated and dephosphorylated states are essential for the rapid recruitment and the functional integrity of PAR3 (Fig. 8).

2. It is suggested several times (and indicated in the final model) that the ectopic PAR3 clustering might reduce PAR3 recruitment to cell-cell junctions because of the “low diffusion rate of clustered PAR3”. It also seems possible that the ectopic clusters could trap/sequester PAR3 molecules that would otherwise diffuse individually to the junctions. Furthermore, although the authors provide evidence for the ectopic clusters not associating with VACs, it remains possible that they associate with other intracellular membranes, and may not freely diffuse through the cytosol. The authors should acknowledge these alternate possibilities in the text (and model), or provide more data to support the cluster diffusion idea.

We agree with this suggestion. We have to discuss this possibility besides molecular size effect by clustering. We also added this notion to our model (Fig.8).

We added following sentences:
To line 229
In addition to this mechanism, it would be possible that the interaction between PAR3 and cellular structures, such as microtubules and vesicles, can trap PAR3 clusters and prevent diffusion of dephosphorylated PAR3 (Chen et al., 2013; Jouette et al., 2019).

To line 288
However, since dephosphorylated form is less diffusive and can be trapped by some cellular structures, it fails to coordinate the rapid reconstruction of the tight junction.

To legend for figure 8 (line 595)
In addition, the association with some cell structures may prevent diffusion of clustered PAR3.

3. On line 270, it is stated that “The ASPP2–PP1 complex is efficiently recruited to the PAR3 cluster”. Are the authors saying that ASPP2–PP1 complex recruitment to PAR3 clusters is more efficient than to PAR3 monomers? If so, then the data supporting this should be referenced. If not, then re-wording is warranted.

We agree with this suggestion. Some speculations are included in this statement/hypothesis. Because numerous ASPP2-binding sites are included in the PAR3 clusters, we expected that the recruitment of the ASPP2-PP1 complex to the PAR3 clusters would be efficient. However, we should not use the word “efficiently” to avoid overstatement. We altered the sentence and sentences

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around that as below. Green markers indicate the corrected words responding to this suggestion, while blue one responding to reviewer3's suggestion.

Revised version (line 277):
Altogether, these data suggest that the normal localization of PAR3 is the consequence of local clustering of PAR3 on the specific plasma membrane domain. ASPP2-PP1 complexes are probably concentrated in the PAR3 clusters, which harbors numerous ASPP2-binding sites, which would promote ASPP2-PP1-mediated dephosphorylation of PAR3 and, consequently, clustering. Thus, this positive feedback loop would accumulate PAR3 at a specific membrane domain.

Previous version:
Altogether, these data suggest that the so-called "localization" of PAR3 is the consequence of local clustering of PAR3 on the specific plasma membrane domain. The ASPP2-PP1 complex is efficiently recruited to the PAR3 cluster, which harbors several ASPP2-binding sites, and this would further promote ASPP2-PP1-mediated dephosphorylation of PAR3. Our results suggest that this positive feedback loop accumulates PAR3 at the specific membrane domain.

4. The PAR-1 phosphorylation of Drosophila PAR3 can also have a positive relationship with PAR3 clusters associated with centrosomes (shown normally in Drosophila male germline stem cells by the Yamashita lab [PMID: 25793442] and with mis-regulation in the Drosophila embryo ectoderm by the Harris lab [26455305]). These studies should be briefly mentioned to highlight some of the complexity of PAR3 cluster regulation.

We agree with reviewer's suggestion. We added following discussion to the discussion section (line 333).

However, it has been shown that Bazooka/PAR3-accumulated patches contain Bazooka/PAR3 molecules phosphorylated on Ser151 and/or Ser1085 in both epithelial cells and male germline stem cells of Drosophila (Inaba et al., 2015; Jiang et al., 2015). In addition, the data shown by Jiang et al. suggests that Bazooka/PAR3-S151A/S1085A mutant molecules poorly accumulated to the Bazooka/PAR3 clusters. Together with our results, these observations suggest that proper expression and phosphorylation level and/or the phosphorylation-dephosphorylation cycle would be important for the organization of PAR3 clusters in some contexts. Alternatively, yet unidentified mechanisms may underlie cluster formation of PAR3 in those contexts.

5. In Fig 2B, the left asterisk may be mis-positioned, and positions of Mw markers should be indicated.

We deleted left asterisk, and added the information of Mw markers.

6. In Fig 3D, statistically significant differences should be indicated.

We added results of t-test. We improved quantifications of these data.

Reviewer 3 Advance Summary and Potential Significance to Field:
PAR3 is an evolutionary conserved polarity protein that acts in epithelial cells to regulate apical basal polarity and the formation of apical junctions. In vertebrate epithelia, PAR3 is enriched at tight junctions (TJs) and is important for TJ assembly. Phosphorylation at multiple sides is an essential component of PAR3 regulation as has been documented through numerous studies, including work from the Ohno laboratory. This is an important area of research that clarifies the molecular mechanisms of cell polarization. The present paper identifies an additional phosphorylation side within PAR3, Ser852, and shows that dynamic phosphorylation of Ser852 as well as Ser889 plays a key role in PAR3 clustering and localization at the TJ domain. Phospho-Ser852 binds 14-3-3, which presumably interferes with molecular interactions that would enhance PAR3 clustering. The authors further show that an ASPP2-PP1 complex can dephosphorylate Ser852 and Ser889. These data suggest that the non-phosphorylated forms of PAR3 act at TJs to promote polarity and TJ formation. However, it is also shown that non-phosphorylatable forms of PAR3 have reduced turnover and their ability to promote normal TJs formation at new cell contacts is compromised, suggesting that phosphorylation – dephosphorylation cycles are crucial for normal

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PAR3 activity.

The authors present a detailed and well-documented study that is very appropriate for JCS. I have listed a number of small points. While I realize and appreciate that the authors are not native English speakers, the paper would benefit from a revision to improve the English grammar.

Your suggestions improved our manuscript. We are deeply grateful to you for your kindness.

Line 2: PAR-3 or PAR3 – settle on one version. Same goes for PAR1.
We agree with this suggestion. We settled on ‘PAR3’ and ‘PAR1’ throughout the manuscript.

Line 24: …..we demonstrate that ASPP2, which controls PAR3 localization…..
We agree with this correction (line 24).

Line 45: PAR proteins were first identified in the context of asymmetric cell division of the C.elegans zygote
We agree with this correction (line 44).

Line 52 etc: two key papers that establish negative feedback between apical and basolateral polarity factors are Tanentzapf and Tepass, NCB 2003 and BIlder et al., NCB 2003.

We agree with this suggestion. We referenced these two papers.

Revised version (line 58):
The antagonistic relationship between apical determinants and basolateral determinants were demonstrated using Drosophila genetics (Bilder et al., 2003; Tanentzapf and Tepass, 2003).

Line 67: a key paper showing that PAR3 is upstream of AJ assembly is Harris and Peifer, JCB 2004.

We agree with this suggestion. We referenced suggested paper and added information around the sentence.

Revised version (line 66):
PAR3 localizes to primordial adherens junctions prior to other PAR complex components in mammalian epithelial cells (Suzuki et al., 2002), whereas Bazooka/PAR3 is positioned by cytoskeletal cues and acts upstream of adherens junctions to position and develop cadherin-catenin clusters in embryonic epithelia of Drosophila (Harris and Peifer, 2004; Harris and Peifer, 2005). In both cases, PAR3 plays a unique role in the PAR complex in determining the initial formation of the PAR complex at the cell-cell contact region that becomes the subapical region as a molecular landmark after polarity establishment (Roman-Fernandez and Bryant, 2016; Suzuki and Ohno, 2006).

Line 73: Par3 does not have a transmembrane …..
We agree with this correction (line 77).

Line 106 ….This mutant was severely compromised in its interaction…..
We agree with this correction (line 111).

Line 109…. ASPP2 mutant lacking the C-terminus () failed to interact….
We agree with this correction (line 114).

Line 111…This suggests that the REVD…..
We agree with this correction (line 116).

Line 113…. REVD mutant in previously….
We agree with this correction (line 117).

Line 115…REVD mutant did not (Fig.1E,F….
We agree with this correction (line 120).

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Line 128 ….Among them, the 1-269aa fragment ….. whereas the 710-936 aa fragment showed a strong affinity…. This result suggests that ….
We agree with this correction (line 132).

Line 133… short isoform of mouse PAR3, which lacks….
We agree with this correction (line 136).

Line 136… Ser point mutants and identified Ser852 as a novel 14-3-3 binding sitte in PAR3
We agree with this correction (line 140).

Line 137: the alignments seems to show conservation of Ser852; explain why this statement is qualified ('may be conserved')
The alignments just showed that S852 is conserved among these specific animal species. Although we surveyed several vertebrate and chordate species, we cannot completely deny the possibility that some species lack Ser852.

Line 198: delete 'In addition'
We agree with this correction (line 206).

Line 199:…triple mutant were used to evaluate the function….
We agree with this correction (line 207).

Line 204: if a molecule is observed in puncta (assuming multiple molecules need to be present to make it visible) does that not imply that the molecule is 'clustered'. So this statement would be tautological.

We agree with your opinion. We changed the sentence as follows:

Revised version (line 211):
In several animal species, PAR3 has been observed as puncta, and this structure was thought to be organized by oligomerization-mediated clustering of PAR3

Previous version: In several animal species, PAR3 has been observed as puncta, and this structure was believed to be clustered PAR3

Line 217 etc: the authors may want to consider how there diffusion model could be affected be the fact that their constructs a 10x overexpressed.

We reconsidered about this. As reviewer2 suggested, PAR3 clusters can associate some intracellular structures, and this interaction may interfere diffusion of clustered PAR3 molecules. This model could explain the dysfunction of mutant PAR3 even when exogenous proteins were overexpressed. Thus, we discussed about cellular structure-trapping model besides molecular size effect by clustering.

We added following sentences:
To line 229
In addition to this mechanism, it would be possible that the interaction between PAR3 and cellular structures, such as microtubules and vesicles, can trap PAR3 clusters and prevent diffusion of dephosphorylated PAR3 (Chen et al., 2013; Jouette et al., 2019).

To line 288
However, since dephosphorylated form is less diffusive and can be trapped by some cellular structures, it fails to coordinate the rapid reconstruction of the tight junction.

To legend for figure 8 (line 595)
In addition, the association with some cellular structures may prevent diffusion of clustered PAR3.

Line 224: …a low turnover at a developing ….
We agree with this correction (line 235).

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Line 233 ….prolonged in clone #6 but not in clone #3…
We intended to emphasize the lack of reproducibility. So, we think we should keep the phrase 'not reproduced'.

Line 242
The half time of recovery of PAR3-2SA (852/889) appeared to be prolonged in #6 clone (Fig. 6A,C,E) but was not reproduced in #3 clone (Fig. S6B).

Line 249….cells organized unique intercellular…
We agree with this correction (line 258).

Line 259…can be dephosphorylated by…
We agree with this correction (line 268).

Line 260 …In comparison to normal PAR3… (wild-type would be the endogenous unmodified PAR3, a GFP-tagged exogenous PAR3 is not wild-type. This should be corrected throughout.)

We basically agree with this opinion. The word 'wild-type' originally means the organisms which possess original form of endogenous genes. However, many researchers have practically used the word 'wild-type' as the word meaning not mutated genes or proteins (e.g. Benton R and St Johnston D, Cell, 115, 691–704, 2003, Figure3A-C: Rodriguez J et.al. Developmental Cell 42, 400-415, 2017, Figure 4D-H and Figure 5F-K: Inaba M et.al. eLIFE 4:e04960, 2015, Figure 5F: Jiang T et.al. Current Biology 25, 2701–2708, 2015, Figure S4 D-F). Although we continue to use 'wild-type' in this manuscript, we changed the expression that 'intact ASPP2' to 'normal ASPP2 (ASPP2-WT)' in Line 117, where the first place 'WT' emerges.

Line 263….localized to cell-cell contact sites….
We agree with this correction (line 272).

Line 267…
Line 268 etc: delete 'as depicted in Figure 4'….these data suggest that the normal localization of PAR3 …….clustering of PAR3 at a specific….

We basically agree with this correction. We added some information to specify the data which we argue.

Revised version (line 275):
i.e., GFP-fused unphosphorylatable mutants of Bazooka/PAR3 tended to form clusters compared with the wild-type Bazooka/PAR3 in the follicle cells~~, as depicted in Figure 4~~. Altogether, these data suggest that the ~~so-called "localization"~~ normal localization of PAR3 is the consequence of local clustering of PAR3 ~~on the~~ at a specific plasma membrane domain.

Line 270: …PAR3 clusters…

Line 271:…sites, which would promote ASPP2-PP1-mediated dephosphorylation of PAR3 and , consequently, clustering. …

We agree with these corrections.
Corrected sentences are shown as below. Blue markers indicate the corrected words responding to reviewer2's suggestion, while green one responding to reviewer2's suggestion.

Revised version (line 278):
ASPP2-PP1 complexes are probably concentrated in the PAR3 clusters, which harbors numerous ASPP2-binding sites, which would promote ASPP2-PP1-mediated dephosphorylation of PAR3 and, consequently, clustering. Thus, this positive feedback loop would accumulate PAR3 at a specific membrane domain.

Previous version:

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The ASPP2-PP1 complex is efficiently recruited to the PAR3 cluster, which harbors several ASPP2-binding sites, and this would further promote ASPP2-PP1-mediated dephosphorylation of PAR3. Our results suggest that this positive feedback loop accumulates PAR3 at the specific membrane domain.

Line 273…at a specific….
We agree with this correction (line 278).

Line 288…candidates…
We agree with this correction (line 297).

Line 317….PAR3 clusters anchor centrosomes…
We agree with this correction (line 325).

I did not edit M+M or Figure legends; please check

Figure 1A: Is the increase of PAR3, aPKC, and PAR6 in the ASPP2 lane a reproducible finding?

This is not reproducible. Fig. 3A and 3C are the examples.

Figure 2B: replace 'deletion mutants' with 'fragments'

We agree with this suggestion. We changed the figure label and the figure legend.

Figure 5: the control here is overexpressed PAR3-GFP. How does this control compare to endogenous PAR3 in these type of experiment. Are they the so same?

We have shown the data using endogenous PAR3-expressing cell (NS_EGFP #22, which expresses non-silencing shRNA and EGFP) (Fig.5E), although we omitted the photo data for this line due to space limitation. Basically, they are the same. However, PAR3-EGFP-WT-rescued cells showed a slight delay in junction formation (Fig.5E). This suggests that excess expression of PAR3 has a slightly negative effect on junction formation. We added the information about #22 and #21 clones to the figure legend for Fig5E.

Reviewer 3 Comments for the Author:
see above

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Figures only for reviewers

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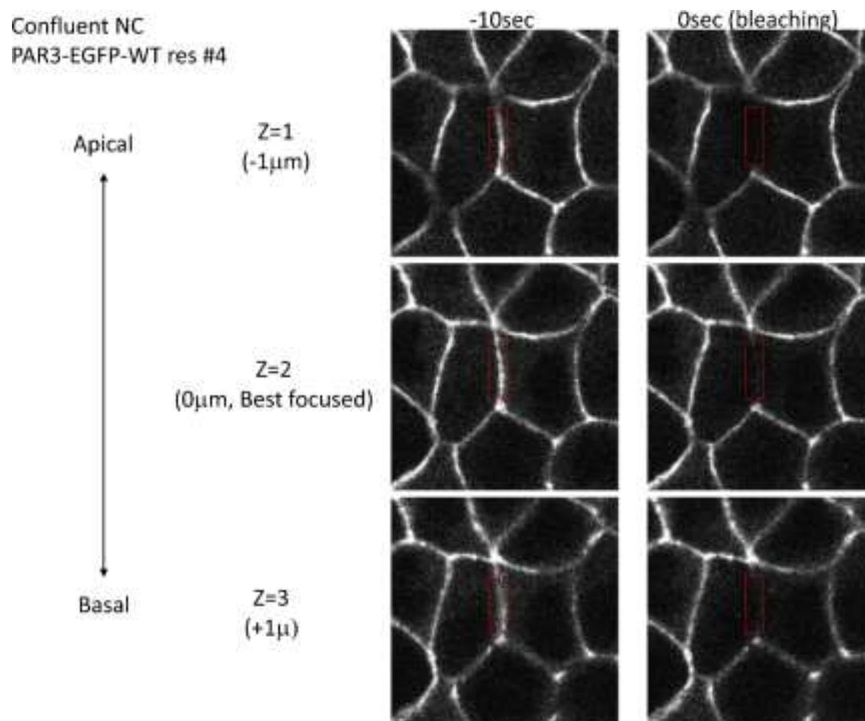

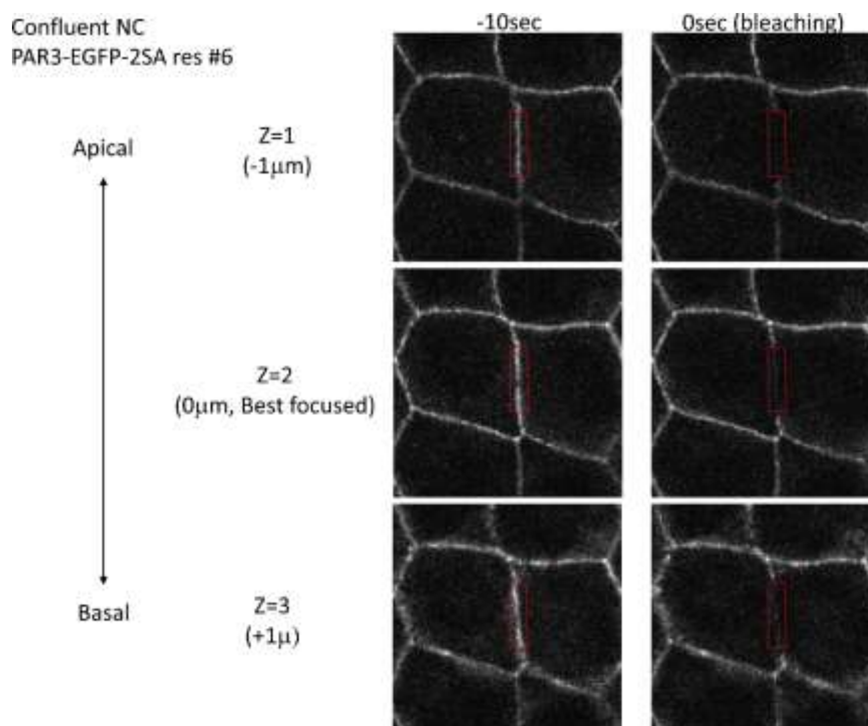

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Second decision letter

MS ID#: JOCES/2020/244830

MS TITLE: Phosphorylation and dephosphorylation of Ser852 and Ser889 control clustering, localization, and function of PAR3

AUTHORS: Kazunari Yamashita, Keiko Mizuno, Kana Furukawa, Hiroko Hirose, Natsuki Sakurai, Maki Masuda-Hirata, Yoshiko Amano, Tomonori Hirose, Atsushi Suzuki, and Shigeo Ohno
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

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