Plakophilin 3 and Par3 Facilitate Desmosomes' association with the Apical Junctional Complex

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)
Dear Sergey,

as you can see, both reviewers are supportive of your manuscript but also see the need to back up some conclusions experimentally. Reviewer 1 makes the strong point that no data are shown how Par3 mediates desmosome distribution via PKP3. Other issues raised by this reviewer can be addressed while preparing the ms for resubmission. Rev 2 raises the question whether Par3-dependent desmosome reorganization may simply result from the reorganization of E-cad which may depend on Par3. Again, this issue requires some experimental support. I am convinced that including a model outlining your hypothesis how the Par3-PKP3 axis might work will strengthen the manuscript and contribute to it being cited. Provided these topics are addressed, I can't see anything that precludes acceptance of a revised manuscript.

Sincerely,

Thomas Magin
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Troyanovsky,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

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Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
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Reviewer #1 (Remarks to the Author):

system they use DLD1 simple epithelial cells where they knockout the major desmosomal plaque proteins to generate a series of DLD-1-KO cell lines. Using these cells, they show that PKP3 is essential for the apical localization of desmosomes. Moreover, they identify an extra-desmosomal pool of PKP3 at tricellular junctions. They then proceed to analyze the role of the Lgl, the scribble/erbin/Lano and the par polarity complexes in regulating the apical localization of desmosomes and the tricellular localization of PKP3. Whereas the knockout of Lgl- or scribble complexes have little effect on desmosomes, knockout of Par3 abolishes sorting of desmosomal proteins to the apical junctional complex and leads to the loss of PKP3 and tricellulin from tricellular contacts.

While a tricellular localization of PKP3 as well as association with E-cadherin and the requirement of at least one PKP for desmosome formation has been shown before, the role of the polarity protein par3 described here is novel. This is an important finding showing that not only TJ and AJ localization depend on the apical polarity complex but that this applies to desmosomes as well. However, the interplay between the junctions and the par complex has not been characterized in more detail. I suggest to cut down the first part and elaborate on the new findings.

Most data and conclusions rely on immunofluorescence data. Although these data are of high quality, only a few experiments have been quantitatively evaluated. Some biochemical experiments should be performed to complement and support the conclusions.

P5 last paragraph of introduction:

"While the basolateral polarity Scrib module is required for proper localization of the entire AJC, genetic interference with Scrib proteins fails to uncouple DSMs from AJC or abolish the extra-DSM pool of Pkp3." This refers to the tC pool only whereas the cytoplasmic pool which is also "extra-DSM" has not been studied.
Fig. 1:
p. 5: „all DSM arrays were located at the apical end of the lateral membrane”. z-slices should be added for DSG2 + ZO-1 in Fig.1A as shown for DSG2 and E-Cad in Fig 1B.

Fig. 2:
Abbreviation for plakoglobin is usually either pg or JUP.
The statement „Further, no changes were detected in expression of other tested DSM proteins” (p.7) is not supported by the Western Blot data: Pg appears reduced in the PKP3 knockout and vice versa. DSP is clearly reduced at least in pg and PKP3 KO cells. Expression levels of the desmosomal proteins must be quantified from at least three independent experiments and changes should be described.

Fig. 3:
WT cells should be shown as reference/control.
3A,B: E-cadherin localization is also altered in the PKP2 and PKP3 KO cells. This should be either mentioned or the staining should be omitted. Since TJs are the generally accepted marker for the „Apical Junctional Complex”, co-staining of DSG2 and ZO-1 would be a better indicator of desmosome localization in the apical region.
„In Pkp3-KO cells the number of rDSMs was dramatically decreased” (p6, last paragraph). This conclusion should be validated by quantification.
3C: To compare the localization of desmosomal proteins in the apical versus basal region, the scale should be the same in all samples. The number of optical slices varies between 13 and 19. Does this reflect different height of the cells? It is not apparent what the different colors mean. What does „relative fluorescence” indicate? What is the reference?
For a quantification, the apical region could be defined by ZO-1 (or occludin) localization. Every fluorescence signal underneath would be defined as basal. The ratio of mean apical fluorescence intensity to mean basal fluorescence intensity of DSG2 in all cell lines (WT and KO-cells) would allow to compare the effects including a statistical evaluation.
Since all cells are DLD1-cells the unmodified cells should be designated „wild type" instead of „DLD1”

Fig. 4:
Quantification in Pkp2/3-KO cells is missing.

Fig. 5:
A control is required to show that the Pearson's correlation coefficient is suitable to conclude that a major portion of membrane associated PKP3 is extra-desmosomal. A positive (e.g. Dsg2+Dsp) as well as a negative control should be included. The number of independent experiments and images used for quantification must be described in the figure legend. The Pearson's correlation for tricellulin + PKP3 (compared to tricellulin + DSG2 and tricellulin +DSP) would support the conclusion that PKP3 co-localizes with tricellulin in contrast to other desmosomal proteins. The Pearson’s values rely on "six randomly taken images". This is a very low number of images that are obviously taken from a single experiment.
Fig 5G shows a “tC/bC index” for PLA signals. According to the text, it is the ratio of tricellular PLA signals to total PLA signals outside tCs and not to bicellular signals. „Quantification of the tC/bC PLA index, determined as a ratio of the PLA signal detected within 5μm tC circles centered at tC junctions (positions as indicated above) to the total signal outside of these circles,...”
Co-immunoprecipitation of PKP3 with proteins from tricellular junctions should be performed to support the imaging data.
Fig. 6:
6A: PKP2 staining is of low quality. Pearson's correlation should be shown for PKP2 + Dsg2 and PKP2 + tricellulin to assess the extent of co-localization.
6A, B: "This Pkp2 staining in tC contacts was specific since it also roughly corresponded to MARVELD2-marked junctions and was undetectable in Pkp2-KO cells (Fig. 6A, B)" (p9). 6B does not show MARVELD2/tricellulin staining but instead ZO-1. The co-staining should be the same to allow a comparison.
6C: "Further, no difference in Pkp2 distribution was detected in Pkp3-KO cells" (p9). PKP2 membrane association appears considerably improved compared to wt cells and no tricellular accumulation is detected. Without a quantification, this conclusion cannot be drawn.
6D: Quantification of the ratio of tricellular versus bicellular PKP3 is required in PKP2 KO and wt cells.

Fig. 7:
The switch from DSG2 staining as a marker for desmosomes (all previous figures) to DSP impedes a comparison with previous experiments.
PKP3 localization differs strongly between cells co-stained for tricellulin (strong tC PKP3, weak bC PKP3) and those co-stained for DSP and ZO1 (strong bC PKP3). Tricellular localization is even partially lost in cells co-stained for DSP (7C). Since processing of cells for immunofluorescence was done following the same protocol, it is not clear why this is so.
Again, different numbers of optical slices are compared.
The ratio of tC versus bC PKP3 is a much better indication of PKP3 sorting to tCs.

Abb. S4A:
Shows PLA signals for PKP3+par3. This is much too small to spot the PLA signals in relation to par3 staining. An enlargement is required as well as a quantification as shown in Fig. 5. Use of the same negative control (PKP3-KO cells) would facilitate the comparison of experiments.

Abb. 8:
The PKP3 level should be determined in Par3-KO cells. Immunofluorescence suggests that it might be reduced. Quantification of PKP3 localization in tC is missing.
Since loss of Par3 abolishes polarity completely, one would not expect desmosomal proteins or any other protein to reveal a polar localization in these cells. Thus, the effect on PKP3 and desmosomes could be indirect. Since PKP3 associates with E-cadherin before it localizes at desmosomes e.g. the loss of E-cadherin apical localization could be responsible for loss of desmosomal polarity. Therefore, it is mandatory to show an association between PKP3 and the polarity complex by immunoprecipitation to develop the hypothesis of an interplay between the polarity complex and desmosomes.

Discussion:
"It is possible that Pkp3 recruitment in the cortex of tC-contacts is partially mediated by direct interactions of Pkp3 with the plasma membrane due to Pkp3 palmitoylation". This is rather unlikely in view of the fact that other desmosomal proteins/PKPs are also palmitoylated and the palmitoyl moiety can also be integrated into lateral membranes.
"Dsg2 in the Par3-KO DLD cells disperses into numerous small clusters.... It seems plausible that this phenotype reflects an uncontrolled formation of rDSMs, possibly resulting from hyperactivity of Pkp3". What is "hyperactive" Pkp3? This should be explained.
"A possible explanation for such an interrelationship is that Par3-based signaling maintains Pkp3 in
an inactive pool concentrated with other TJ proteins in tC contact areas. In such a model, only a fraction of Pkp3 is permitted to be released from this pool and functions to maintain DSM assembly. What means inactive PKP3? How are these fractions regulated? What might control the release? What are "Par3-dependent modifications"? This requires further explanation.

The authors should present a hypothetical model how par3 might interact with PKP3 and affect desmosomal localization.

Reviewer #2 (Remarks to the Author):

This work by Indra et al. addressed the localization of desmosomal proteins along epithelial lateral cell-cell contacts, using an epithelial colon carcinoma cell lines, DLD1, and asked how desmosomes are integrated into the apical junctional complex (AJC). By gene inactivation of various desmosomal components, the authors delineate individual desmosomal gene functions in assembly and apical localization of desmosomes in this cell line (in part confirming previous reports by other laboratories). Among these, Pkp3 inactivation resulted in reduced apical enrichment of desmosomes while not affecting desmosome assembly. Moreover, inactivation of basolateral and of apical polarity proteins was used to determine the extent to which desmosomal protein localization and desmosome assembly depend on polarity proteins, which have previously been connected to organization of the AJC. The authors report an interesting dependency of desmosomal pools on the polarity protein Par3 in this cell line.

The experiments are thoroughly performed, and the results properly described and discussed. In general, the findings seem of interest for the readership of MBoC. However, in the current version some conclusions are not fully supported by the actual data shown, and data presentation could be improved.

General points
• Desmosome formation in keratinocytes depends on Adherens Junction proteins (e.g. PMID 19262605, 29999492). How is the hierarchy between AJs and DSM in DLD1 cells? How does abrogation of all AJ-based cadherins (E- and P-cadherin, others in DLD1 cells?) affect DSM? I feel more insight or at least discussion on this is required to understand whether the effect of impaired apical polarity machinery is direct or indirect regarding desmosomal assembly and localization (see later comments).
• A limitation of the study is that only one (cancerous) cell line has been investigated. Desmosome distribution varies among different cell types and systems. Is the extra-DSM pool at tricellular junctions specific for DLD1 cells? Could the authors state anything about the generality of these findings? Moreover, what are the dynamics of these pools during junction maturation, i.e. is the extra-DMS pool of desmosomal proteins larger (or smaller) in recently formed vs. mature cell-cell contacts (e.g. after calcium depletion or in freshly formed junctions after scratch-wounding)? This could be easily tested in Wild-type" DLD1 cells.
• In mammary epithelial cells, Par3 has been reported to serve as exocyst receptor, among others targeting E-cadherin to the plasma membrane (PMID 28358000). How does E-cadherin localize in the Par3-KO DLD1 cells and in the Par3-GFP rescue cells? Are the effects on DMS proteins perhaps simply an indirect consequence of altered AJs following Par3 inactivation?
• Figure 8 and related: The authors confirm that Par3 deletion results in fragmented TJs and in impaired tricellular contacts. They seem to further interpret there data as such that Pkp3 tC localization is Par3-regulated. The fact that the formation and/or maintenance of tricellular contacts
itself is Par3-dependent actually implies that any tC protein is mislocalized following Par3 inactivation (as the case for tricellulin itself as the authors showed). Hence the reduced tC localization of Pkp3 is likely not a specific effect but a systemic consequence of the lack of tCs. Similarly, it therefore seems counterintuitive to quantify the extent of PLA at tCs in Par3-KO cells when the authors state those structures are (almost) absent in these cells. I suggest rephrasing the authors conclusions in this respect.

- Page 16 "...And from our work here, specific Par3-dependent modifications may create a Pkp3 pool that is concentrated in tC contacts and unable to assemble into this framework." Could the authors be more explicit what they refer to here? Which data shown imply what type of protein modifications?
- Manuscript title: To me, the evidence that Pkp3 and Par3"JOINTLY" dictate AJC association of desmosomes is insufficient. I recommend rephrasing the title accordingly.

Minor points:
- Evaluation of the manuscript was somewhat difficult because in several figures the information on the respective genotype was not provided. I recommend consistent integration of genotypes in figure panels (beyond figure legends) to enhance readability.
RESPONSE TO THE REVIEWERS

Reviewer #1

General concern:

While the reviewer commented that we made “an important finding showing that not only TJ and AJ localization depend on the apical polarity complex but that this applies to desmosomes as well”, the reviewer identified numerous technical and minor issues, most of which we addressed in the revised version of the manuscript and outlined below.

I suggest to cut down the first part and elaborate on the new findings.

We believe that the first part of our work is important to include since it compares the contribution of major desmosomal proteins to desmosome formation using the same cell model and under the same experimental conditions. This part shows that Pkps, in contrast to other DSM proteins, are absolutely essential for normal DSM formation and distribution in our experimental cell model.

“... a tricellular localization of PKP3 as well as association with E-cadherin and the requirement of at least one PKP for desmosome formation has been shown before...”.

We of course agree with this and we quoted the corresponding papers in Discussion.

Most data and conclusions rely on immunofluorescence data. Although these data are of high quality, only a few experiments have been quantitatively evaluated.

In the revised version, we quantified all experiments where, in our opinion, quantitative validation of the visual observations adds value to the analysis. This includes Figures 3, 4, 5, 6, 7, 8, S4. In some instances (e.g. E-cadherin distribution in Fig. 3 and 4), where the observed phenomenon is obvious from visual inspection of the presented figure, we did not include quantification, but in each case we made it clear that these images are representative of at least 3 independent experiments.

Some biochemical experiments should be performed to complement and support the conclusions.

We agree with the referee that biochemical analysis would provide a valuable complementary approach, and toward this end we determined the extent to which we could identify Par3 and Pkp3 interactions using standard co-immunoprecipitation experiments. We were unable to detect interactions using this approach in our several pilot Co-IP experiments, which could indicate that the interactions are indirect, weak, transient, or that the sensitivity of the assay is compromised by having insufficient protein in the detergent-soluble pool. So we turned to the PLA approach, which has the advantage of providing spatial information and gives confidence that proteins are actually...
in proximity in situ. We briefly discuss the potential mechanisms of these interactions in the Discussion and, furthermore, as requested, we incorporated a corresponding hypothetical model of these interactions (Fig. 9). Note that this model does not claim that Par3 and Pkp3 are in the same complex. The detailed biochemical characterization of Par3-Pkp3 interplay requires more in depth analysis that will be the subject of a separate study.

Other concerns:

P5 last paragraph of introduction:

„While the basolateral polarity Scrib module is required for proper localization of the entire AJC, genetic interference with Scrib proteins fails to uncouple DSMs from AJC or abolish the extra-DSM pool of Pkp3.” This refers to the tC pool only whereas the cytoplasmic pool which is also „extra-DSM” has not been studied.

Throughout the paper, “extra-DSM pool of Pkp3” refers to the cortex-located pool proximal to TJs. To clarify this point we modified the statement: “… genetic interference with Scrib proteins fails to uncouple DSMs from AJC or abolish this cortical extra-DSM pool of Pkp3.”

Fig. 1: p. 5: „all DSM arrays were located at the apical end of the lateral membrane”. z-slices should be added for DSG2 + ZO-1 in Fig.1A as shown for DSG2 and E-Cad in Fig 1B.

Fig. 1A is made using widefield microscope, using a low magnification lens, as indicated in the legend, so no z-slices were available for this figure. This figure intends to show a general pattern of DSMs in DLD1 cell culture. We also added in the Fig. 1 legend that quantifications of DSMs in DLD1 cells are presented in Fig. 3C, D.

Fig. 2: Abbreviation for plakoglobin is usually either pg or JUP. The statement „Further, no changes were detected in expression of other tested DSM proteins” (p.7) is not supported by the Western Blot data: Pg appears reduced in the PKP3 knockout and vice versa. DSP is clearly reduced at least in pg and PKP3 KO cells. Expression levels of the desmosomal proteins must be quantified from at least three independent experiments and changes should be described.

We changed the abbreviation for plakoglobin to “Pg” for convenience. We prefer to use lowercase letter in the protein name abbreviations since the use of only capital letters suggests the gene name and could be confusing.

As requested, we performed three more independent Western blots for DSM proteins and quantified protein levels. They are not included (but will be, if the reviewer considers it necessary) since no changes in expression levels of these proteins in any KO lines were detected. The apparent differences in expression levels noted by the reviewer were not
reproducible; therefore, we replaced the original blot with one that is more representative. We indicated that these results are representative of three independent experiments in the figure legend.

Fig. 3:
WT cells should be shown as reference/control.

The Dsg2-E-cadherin staining of the WT DLD1 cells is shown in Fig. 1B and C and in Fig. 4C. We added this point to the legend of Figure 3. We also added to the legend of Fig. 1 that DSM quantification of WT DLD1 cells is presented in Fig. 3C, D.

3A, B: E-cadherin localization is also altered in the PKP2 and PKP3 KO cells. This should be either mentioned or the staining should be omitted. Since TJs are the generally accepted marker for the „Apical Junctional Complex”, co-staining of DSG2 and ZO-1 would be a better indicator of desmosome localization in the apical region.

E-cadherin was select as a marker of AJC because it also visualizes the lateral membrane. We marked location of AJC in Figs. C and D using a red line.

Based on the referees’ concern we looked more carefully at E-cadherin localization throughout cell populations. While highly variable in both controls and KO lines, we did not detect any reproducible differences in AJs (or E-cadherin localization) between cell lines shown in Figs 3 and 4. We ensured that more representative images were used and also highlighted this point for clarity in the text (p. 7): “It is also important to note that E-cadherin staining was not detectably changed in these cells, showing that apically located Zonula Adherens (ZA) and numerous so-called spot-like adherens junctions (sAJs) were still abundant along the lateral membrane. “

In Pkp3-KO cells the number of rDSMs was dramatically decreased” (p6, last paragraph). This conclusion should be validated by quantification.

Quantification is now added (Fig. 3D).

3C: To compare the localization of desmosomal proteins in the apical versus basal region, the scale should be the same in all samples. The number of optical slices varies between 13 and 19. Does this reflect different height of the cells? It is not apparent what the different colors mean. What does „relative fluorescence" indicate? What is the reference?
For a quantification, the apical region could be defined by ZO-1 (or occludin) localization. Every fluorescence signal underneath would be defined as basal. The ratio of mean apical fluorescence intensity to mean basal fluorescence intensity of DSG2 in all cell lines (WT and KO-cells) would allow to compare the effects including a statistical evaluation.
Since all cells are DLD1-cells the unmodified cells should be designated „wild type" instead of „DLD1"
The scale is the same in all samples present in Fig 3 as well as the Z step size (0.5 μm, indicated in the Method section). Indeed, in 48-64h cell cultures the height of the cells varies from 7 to 10 μm and this variation results in differences in optical slice numbers. We added this point into the legend. Furthermore, the cell height not only differs from sample to sample but also between the cells in one image. That is the reason why we need at least 3 optical slices (1.5 μm) to present the AJC region of the cells in Fig. 3B. We also added several other minor corrections suggested by the reviewer. Finally, the differences in fluorescence intensities of rDSMs along the lateral membrane are provided in the apicobasal intensities charts (Fig. 3C). The revised version emphasizes (p. 6) that the intensities (in C) are determined for the entire image (50x50 μm²) that includes 8-10 cells. It clearly shows that the apically located rDSM are significantly brighter for all cells except in the Pkp3-KO. While we appreciate the suggestion of the reviewer to report the data as a ratio of mean apical to basal fluorescence intensity, we felt that this would mask important data regarding individual intensities along the membrane.

**Fig. 4:**
Quantification in Pkp2/3-KO cells is missing.

To address the reviewer’s concern, we added quantification of apical membrane Dsg2 fluorescence in WT DLD1 cells versus Pkp2/3-KO cells (Fig 4. D) to show the increase in apical Dsg2 at the expense of DSMs. We also added a representative optical cross-section of a control DLD1 cell for comparison (Fig. 4C).

**Fig. 5:**
A control is required to show that the Pearson's correlation coefficient is suitable to conclude that a major portion of membrane associated PKP3 is extra-desmosomal. A positive (e.g. Dsg2+Dsp) as well as a negative control should be included. The number of independent experiments and images used for quantification must be described in the figure legend. The Pearson's correlation for tricellulin + PKP3 (compared to tricellulin + DSG2 and tricellulin +DSP) would support the conclusion that PKP3 co-localizes with tricellulin in contrast to other desmosomal proteins. The Pearson's values rely on "six randomly taken images". This is a very low number of images that are obviously taken from a single experiment.

PCC values support the visual observation that a significant pool of Pkp3 is present outside of DSMs and that this pool is increased in tC contacts. We added PCC for both Dsg2-Dsp and Dsp/Pkp3-MARVELD as requested (Fig. 5D). From the data presented, is clear that PKP3 is localized in the vicinity of tC contacts (5E), and that its localization with desmosome molecules in these regions, in particular Dsp, is very low (5D). We also quantified additional images (each image presents about 8-10 cells) and added these data to the final graph. We specified in the Methods that PCC was determined for 4 independent images from each of 3 independent experiments, and indicated the number of bC and tC contacts evaluated for each antibody pair.
Fig 5G shows a "tC/bC index" for PLA signals. According to the text, it is the ratio of tricellular PLA signals to total PLA signals outside tCs and not to bicellular signals. "Quantification of the tC/bC PLA index, determined as a ratio of the PLA signal detected within 5μm tC circles centered at tC junctions (positions as indicated above) to the total signal outside of these circles,..." Co-immunoprecipitation of PKP3 with proteins from tricellular junctions should be performed to support the imaging data.

To be precise, we changed the name of the index from tC/bC to tC/outside tC. As it has been noted above, we did not include the co-immunoprecipitation experiments since currently it is not clear what proteins interact with extra-DSM pool Pkp3. It is also important that we do not claim that any of TJ proteins directly interact with Pkp3.

Fig. 6:
6A: PKP2 staining is of low quality. Pearson's correlation should be shown for PKP2 + Dsg2 and PKP2 + tricellulin to assess the extent of co-localization.
6A,B: "This Pkp2 staining in tC contacts was specific since it also roughly corresponded to MARVELD2-marked junctions and was undetectable in Pkp2-KO cells (Fig. 6A, B)" (p9). 6B does not show MARVELD2/tricellulin staining but instead ZO-1. The co-staining should be the same to allow a comparison.
6C: „Further, no difference in Pkp2 distribution was detected in Pkp3-KO cells"(p9). PKP2 membrane association appears considerably improved compared to wt cells and no tricellular accumulation is detected. Without a quantification, this conclusion cannot be drawn.

We included a new Pkp2-Dsg2 image as requested (Fig. 6A). We also included the Pkp2 and Pkp3 staining of methanol-fixed cells (Fig. S3) to show that the staining is independent of the staining protocols.
We did not include PCC for Pkp2 localization as our focus was on Pkp3 and no significant alterations in Pkp2 were detectable by visual inspection Pkp3-KO cells. We highlighted this point in the results. We also incorporated several changes into the text, so now the text corresponds to the shown figures more precisely.

6D: Quantification of the ratio of tricellular versus bicellular PKP3 is required in PKP2 KO and wt cells.

We added Pkp3/Dsg2-PCC quantification for Pkp2-KO cells (Fig. 6E). Again, we did not added PCC for Pkp2 since this protein was not intended to be the focus of the analysis. So as not to overstate the conclusions, we specified: “No obvious differences in Pkp2 distribution was detected in Pkp3-KO cells by visual inspection (Fig. 6C)".

Fig. 7:
The switch from DSG2 staining as a marker for desmosomes (all previous figures) to DSP impedes a comparison with previous experiments.
PKP3 localization differs strongly between cells co-stained for tricellulin (strong tC PKP3, weak bC PKP3) and those co-stained for DSP and ZO1 (strong bC PKP3). Tricellular localization is even partially lost in cells co-stained for DSP (7C). Since processing of cells for immunofluorescence was done following the same protocol, it is not clear why this is so. Again, different numbers of optical slices are compared. The ratio of tC versus bC PKP3 is a much better indication of PKP3 sorting to tCs.

We incorporated Dsg2 staining of Scrib/Erbin/Lano-KO cells into Fig. S3B. Dsg2 staining is also provided for Par3-KO cells (Fig. 8A). We switched to Dsp staining since it allows us to perform triple staining (using guinea pig anti-Dsp antibody). Both proteins, Dsg and Dsp, are reliable markers of desmosomes. While some variation in intensity of Pkp3 staining between cells is apparent, importantly, in all cases the DSM and extra-DSM localization of this protein is clear. Variations could be also due the fact that the triple-stained image was taken using a confocal whereas the double-stained imaged was taken using a widefield microscope. Figure 7C shows LAP protein-deficient cells that exhibit extremely irregular morphology and, as we specify in the Results, tC area in these cells does not coincide with the geometrical intersections of cell borders. It could be identified only using MARVELD-2 staining.

Abb. S4A: Shows PLA signals for PKP3+par3. This is much too small to spot the PLA signals in relation to par3 staining. An enlargement is required as well as a quantification as shown in Fig. 5. Use of the same negative control (PKP3-KO cells) would facilitate the comparison of experiments.

High magnification of Pkp3-Par3 is added along with the negative control and quantification.

Abb. 8: The PKP3 level should be determined in Par3-KO cells. Immuno fluorescence suggests that it might be reduced. Quantification of PKP3 localization in tC is missing. Since loss of Par3 abolishes polarity completely, one would not expect desmosomal proteins or any other protein to reveal a polar localization in these cells. Thus, the effect on PKP3 and desmosomes could be indirect. Since PKP3 associates with E-cadherin before it localizes at desmosomes e.g. the loss of E-cadherin apical localization could be responsible for loss of desmosomal polarity. Therefore, it is mandatory to show an association between PKP3 and the polarity complex by immunoprecipitation to develop the hypothesis of an interplay between the polarity complex and desmosomes.

The requested Western blot showing Pkp3 level in control and Par3-KO cells is added (Fig. S4D), showing that Pkp3 levels are comparable among the cell lines. Pkp3-Dsp PCC quantification is presented in Fig. 8F.
It is true that depolarization of DSMs in Par3-KO cells could be indirect and could proceed through some kind of depolarization of other structures. In fact the underlying mechanisms for TJ and AJ depolarization in Par3-KO cells also remained to be elucidated. However, as we noted in respond to the second reviewer (and highlighted in the paper, see below), the E-cadherin apical ZA is also lost in the LAP protein-deficient cells, without a dramatic DSM phenotype. It is also important to note that the most striking phenotype of the Par3-KO cells is a disappearance of the extra-DSM pool of Pkp3.

**Discussion:**

"It is possible that Pkp3 recruitment in the cortex of tC-contacts is partially mediated by direct interactions of Pkp3 with the plasma membrane due to Pkp3 palmitoylation". This is rather unlikely in view of the fact that other desmosomal proteins/PKPs are also palmitoylated and the palmitoyl moiety can also be integrated into lateral membranes. "Dsg2 in the Par3-KO DLD cells disperses into numerous small clusters.... It seems plausible that this phenotype reflects an uncontrolled formation of rDSMs, possibly resulting from hyperactivity of Pkp3". What is "hyperactive" PKP3? This should be explained.

"A possible explanation for such an interrelationship is that Par3-based signaling maintains Pkp3 in an inactive pool concentrated with other TJ proteins in tC contact areas. In such a model, only a fraction of Pkp3 is permitted to be released from this pool and functions to maintain DSM assembly" What means inactive PKP3? How are these fractions regulated? What might control the release? What are "Par3-dependent modifications"? This requires further explanation.

It is true that palmitoylation alone is unlikely to target Pkp3 into tC contacts. But, it is plausible that it could maintain the protein in the cortex. To highlight this point we changed the sentence to state: “It is possible that the cortical localization of extra-DSM pool of Pkp3 is partially mediated …”

“Hyperactive” and “inactive” forms of Pkp3: We agree that the exact role of Pkp3 in DSM assembly is not clear. However, some of its features are clearly essential for DSM formation. One may propose, therefore, that Pkp3 molecules, which expose these features, are active with respect to DSM assembly. Any modifications that remove these features inactivate the molecules. To minimize speculation, we replaced the term “hyperactivity”. Now the sentence reads: “It seems plausible that this phenotype reflects an uncontrolled formation of rDSMs, possibly resulting from deregulation of Pkp3.”

As requested, we presented the crude hypothetical model of the mechanism illustrating how these two pools (DSM-bound and extra-DSM) may be regulated and may control DSMs (Fig. 9). While it would be too preliminary to discuss the specific points of this model, we indicated that phosphorylation could play some role in regulation of Pkp3.

*The authors should present a hypothetical model how par3 might interact with PKP3 and affect desmosomal localization.*
The requested model is added (see Fig. 9).

**Reviewer # 2**

*Desmosome formation in keratinocytes depends on Adherens Junction proteins (e.g. PMID 19262605, 29999492). How is the hierarchy between AJs and DSM in DLD1 cells? How does abrogation of all AJ-based cadherins (E- and P-cadherin, others in DLD1 cells?) affect DSM? I feel more insight or at least discussion on this is required to understand whether the effect of impaired apical polarity machinery is direct or indirect regarding desmosomal assembly and localization (see later comments).*

The reviewer’s point is well-taken. We cannot directly rule out a role for altered AJs in spite of the fact that the lateral AJs still assemble in Par3-KO. To avoid unnecessary speculation, we simply state: “Since formation of DSMs is reported to depend on AJs, it is important to emphasize that both types of cells (LAP protein-deficient and Par3-deficient) formed very prominent E-cadherin-based AJs, whereas the DSM phenotype of these two types of cells appeared very different. Nevertheless, we cannot rule out a possible contribution of local changes in AJs to the observed DSM alterations” (p. 16).

*A limitation of the study is that only one (cancerous) cell line has been investigated. Desmosome distribution varies among different cell types and systems. Is the extra-DSM pool at tricellular junctions specific for DLD1 cells? Could the authors state anything about the generality of these findings? Moreover, what are the dynamics of these pools during junction maturation, i.e. is the extra-DMS pool of desmosomal proteins larger (or smaller) in recently formed vs. mature cell-cell contacts (e.g. after calcium depletion or in freshly formed junctions after scratch-wounding)? This could be easily tested in Wild-type” DLD1 cells.*

We agree that it is very important to show whether extra-DSM pool of Pkp3 is a general phenomenon. To study this, we stained three additional epithelial cell lines: MDCK, HBE, and Caco2. All three show extra-DSM Pkp3 staining, which was especially prominent in the vicinity of the tC contacts (Fig. S3A). The dynamics of the extra-DSM Pkp3 pool is also an important area for investigation, which will be facilitated once we have made some headway into determining mechanisms retaining this pool in the proximity to tC-TJs.

*In mammary epithelial cells, Par3 has been reported to serve as exocyst receptor, among others targeting E-cadherin to the plasma membrane (PMID 28358000). How does E-cadherin localize in the Par3-KO DLD1 cells and in the Par3-GFP rescue cells? Are the effects on DSM proteins perhaps simply an indirect consequence of altered AJs following Par3 inactivation?*
Figure S4 shows that E-cadherin in the Par3-KO cells, though incapable of forming restricted belt-like apical AJs, still produces very prominent AJs. DSM impairment was more dramatic, in contrast, with Dsg2 being broadly dispersed along the apical and lateral cell membranes. To clarify, we added: (Par3-KO cells showed) “… the resulting Par3-KO cells showed gross abnormalities in their general organization characterized by fragmentation of TJs (Fig. 8) and disintegration of the belt-like apical AJs. AJs were retained at the lateral cell membranes, however (Fig. S4).” As mentioned above, we also added a statement that we cannot rule out local changes in AJs making a contribution to the observed phenotype.

Figure 8 and related: The authors confirm that Par3 deletion results in fragmented TJs and in impaired tricellular contacts. They seem to further interpret there data as such that Pkp3 tC localization is Par3-regulated. The fact that the formation and/or maintenance of tricellular contacts itself is Par3-dependent actually implies that any tC protein is mislocalized following Par3 inactivation (as the case for tricellulin itself as the authors showed). Hence the reduced tC localization of Pkp3 is likely not a specific effect but a systemic consequence of the lack of tCs. Similarly, it therefore seems counterintuitive to quantify the extent of PLA at tCs in Par3-KO cells when the authors state those structures are (almost) absent in these cells. I suggest rephrasing the authors conclusions in this respect.

We specified in the text (p. 12) that Par3-KO cells were used as a negative control in our PLA staining. We also highlight in the Discussion (p. 16) that “Whether such mutual (tC-TJs and extra-DSM Pkp3) disappearance occurred because one of these structures depends on the other, or they both are independent downstream targets of Par3 is a subject for future research.”

Page 16 “...And from our work here, specific Par3-dependent modifications may create a Pkp3 pool that is concentrated in tC contacts and unable to assemble into this framework.” Could the authors be more explicit what they refer to here? Which data shown imply what type of protein modifications?

We added a sentence: “One of the most plausible candidates for such modifications is specific phosphorylation of Pkp3 that abolishes its binding with other DSM proteins.”

Manuscript title: To me, the evidence that Pkp3 and Par3"JOINTLY" dictate AJC association of desmosomes is insufficient. I recommend rephrasing the title accordingly.

We changed the title “Plakophilin 3 and Par3 Facilitate Desmosomes’ association with the Apical Junctional Complex “. We hope it more precisely reflects results of our paper.
2nd Editorial Decision

June 7, 2021

RE: Manuscript #E21-01-0001R
TITLE: "Plakophilin 3 and Par3 Facilitate Desmosomes' association with the Apical Junctional Complex"

Dear Sergey,

Both reviewers acknowledge your team’s effort to strengthen the manuscript and suggest that it merits acceptance by the MBoC, a view that I share. While reviewer 2 accepts your manuscript as it stands, reviewer 1 recommends to discuss findings by the Lechler lab. Further, this reviewer suggests to perform a Ca switch experiment to test your hypothesis that PKP3 first appears at tricellular junctions before accumulating at the desmosome. I agree with this suggestion and leave it up to you whether you want to strengthen your story by performing this straightforward experiment. Clearly, the manuscript would gain impact by including the proposed experiment. Reviewer 1 also suggests to improve your model, a suggestion that I strongly support.

Sincerely,
Thomas Magin
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Troyanovksy,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor’s decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

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Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

The authors have addressed most of my previous concerns:
1. Quantifications as requested are now included.
2. The Western Blot showing expression of the desmosomal proteins in the KO-cell lines has been exchanged. The authors state that the results from the original Western Blot indicating differential expression of several of the desmosomal proteins could not be reproduced.
3. An IP experiment to support the PLA data has failed. The authors did not try a GFP-trap approach with their par3-GFP cell line or Pkp3-GFP expressing cells. Supportive data from the literature should be discussed in more detail, including for example a BioID-IP experiment of desmoplakin which identified ZO1, ZO2 and Par3 among the co-precipitated proteins (PMID: 32238101).

The model in Fig. 9 is incomplete and partially misleading and has to be revised:
(1) The model suggests that only tC Pkp3 can exchange with desmosomal Pkp3 but not vice versa. In this context, the authors speculate that "Par3-based signaling maintains Pkp3 in a state, which is unable to interact with DSM targets, and which is concentrated in tC contact areas" (p17 top). Is there any evidence for this? An obvious candidate for a Par3 associated modification would be phosphorylation by aPKC, which is however not discussed.
(2) "Only a fraction of Pkp3 is permitted to be released from this pool and functions to maintain DSM assembly." This statement implies that upon contact formation Pkp3 would first appear at the tC contacts before it could be redistributed to the desmosomes. This could be easily tested in a Ca-shift assay.
(3) Why does the figure indicate an inhibitory effect of par3 on tC Pkp3 when extra-desmosomal (= tC) Pkp3 is lost in Par3 KO cells?
Reviewer #2 (Remarks to the Author):

In this revised version of the manuscript, the authors have fully addressed my previous concerns, clarified the points raised, or added necessary discussion.
RESPONSE TO THE REVIEWER # 1

We are pleased that the reviewer concluded that we have addressed most of his/her previous concerns.

Additional recommendations:

Supportive data from the literature should be discussed in more detail, including for example a BioID-IP experiment of desmoplakin which identified ZO1, ZO2 and Par3 among the co-precipitated proteins (PMID: 32238101).

We added a brief note about the indicated paper in our Discussion: “The role of Dsp in this process has been suggested by recent BioID proximity proteomics that shows a juxtapositioning of this DSM protein with some polarity proteins, such as Par3 or Scribble (Badu-Nkansah & Lechler, 2020).”

The model in Fig. 9 is incomplete and partially misleading and has to be revised:
(1) The model suggests that only tC Pkp3 can exchange with desmosomal Pkp3 but not vice versa. In this context, the authors speculate that "Par3-based signaling maintains Pkp3 in a state, which is unable to interact with DSM targets, and which is concentrated in tC contact areas" (p17 top). Is there any evidence for this? An obvious candidate for a Par3 associated modification would be phosphorylation by aPKC, which is however not discussed.

We completely agree with the reviewer that the model in Fig. 9 is hypothetical and incomplete. It only highlights our major observation that Pkp3 exists in two cell-cell contact-located pools. The fact that no other DSM proteins were detected in association with Pkp3 in the extra-DSM pool suggests that this pool does not interact with core DSM proteins. We emphasize this point in the new version: “…this pool of Pkp3 is concentrated in tC contact areas, where no other core DSM proteins are detected.” We hypothesize, based on disappearance of the extra-DSM pool in Par3-KO cells, that specific modifications of Pkp3 in this pool prevent this protein from binding to DSM targets. As suggested, we have added aPKC into Discussion: “One of the most attractive modifications that should be tested in future experiments is phosphorylation of Pkp3 by aPKC. This kinase is activated upon association with Par3 specifically in TJs (Harris, 2017; Humbert et al., 2006)” (p. 14).

(2) "Only a fraction of Pkp3 is permitted to be released from this pool and functions to maintain DSM assembly." This statement implies that upon contact formation Pkp3 would first appear at the tC contacts before it could be redistributed to the desmosomes. This could be easily tested in a Ca-shift assay.

The reviewer is right that it would be interesting to analyze formation of tC-contact-associated pool of Pkp3 in a Ca-shift assay. However, we respectfully disagree that this
experiment may directly test our idea that in an established polarized epithelial sheet, the DSM pool of Pkp3 derives from the extra-DSM pool. The Ca-shift may suggest how this pool is forming during the cell-cell contact formation. Indeed, published data showed that formation of the polarized cell-cell contact upon a Ca-shift is a complex process that proceeds through numerous transient and mobile contacts, which gradually, over several hours, produce a fully developed polarized cell-cell contacts. Many aspects of this process are not clear and, apparently, specific to the Ca-shift assay.

(3) Why does the figure indicate an inhibitory effect of par3 on tC Pkp3 when extra-desmosomal (= tC) Pkp3 is lost in Par3 KO cells?

To be more precise, we incorporated several corrections to our drawing.
Dear Sergey,

thank you for your swift revision. I am happy to accept your team's manuscript for MBoC.

Very best,
Thomas

Thomas Magin
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Troyanovksy:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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Sincerely,

Eric Baker
Journal Production Manager
