A specific isoform of Pyd/ZO-1 mediates junctional remodelling and formation of slit diaphragms

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December 6, 2018

Re: JCB manuscript #201810171

Dr. Mar Ruiz-Gómez
Centro de Biología Molecular Severo Ochoa, CSIC
Nicolás Cabrera, 1
Madrid 28049
Spain

Dear Dr. Ruiz-Gómez,

Thank you for submitting your manuscript entitled "A specific isoform of Pyd/ZO-1 mediates junctional remodelling and formation of slit diaphragms". Your manuscript has been assessed by expert reviewers, whose comments are appended below. Although the reviewers express potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB.

You will see that the reviewers felt that while the initial data set reporting the pyd phenotype was important as a foundation for the paper, it was not surprising. The excitements rests on the idea that Pyd controls junctional remodeling via affects on SD protein trafficking. However, all three Reviewers feel this aspect of the manuscript has to be strengthened, and I agree. They have solid suggestions for how to more completely analyze the putative trafficking of SD proteins in vesicles, and all would be valuable, though the live imaging requested by Reviewer 2 may be beyond the scope of the current work.

Please let me know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. Our typical timeframe for revisions is three to four months; if submitted within this timeframe, novelty will not be reassessed. I would be open to resubmission at a later date; however, please note that priority and novelty would be reassessed.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:
Text limits: Character count for Articles is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. To avoid delays in production, figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

***IMPORTANT: It is JCB policy that if requested, original data images must be made available.

Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.***

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Mark Peifer, PhD
Monitoring Editor
JCB

Tim Spencer, PhD
Deputy Editor
Journal of Cell Biology
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Reviewer #1 (Comments to the Authors (Required)):

Slit diaphragms (SDs) are a specialized cell junction in kidney podocytes, and are required for proper blood filtration. The manuscript from Carrasco-Rando et al., uses the fly nephrocyte to investigate the role of ZO-1 (fly Pyd) in SD formation. The experiments are well-designed and the imaging is excellent. The major new findings include how loss of Pyd leads to adherens-like junctions, the identification of the Pyd isoform regulating nephrocyte SD formation, and a description of the trafficking of pre-SD complexes to form SDs. From these observations, the authors propose a model of junctional remodeling that occurs when Pyd is reintroduced to Pyd-deficient cells. It is an intriguing model with implications for understanding how podocytes manage SD disruption, however, additional experiments and image analysis would help support some of the conclusions and the model.

Major comments:

1. It would be helpful to better define the junctions present between cells in the pyd mutants. Are these junctions possibly some version of SDs between neighboring cells-does Nephrin/Sns also

accumulate at these contacts? Or, if they are an adherens-like junction, do classic junctional proteins localize at these structures-perhaps E-Cadherin, which is presumably absent from normal nephrocyte SDs?

2. Reintroducing Pyd restores SD formation (Fig 6D), but does it lead to loss of the adherens junctions? The confocal images suggests this, but some images indicate Duf remains at cell-cell contacts (Fig6D'), implying there may still be adherens junctions present. Even when Pyd has been reintroduced for sufficient time to allow SD formation (i.e., 24hrs, Fig 6D), significant agglutination remains, suggesting the adherens junctions may still be present. This conversion of junctions is an important aspect of the proposed model. TEM with quantification of adherens-junction number and size at a couple different time points would be more convincing.

3. The authors interpret the presence of subcortical puncta/vesicles containing Duf and Pyd near cell-cell contacts as evidence they are "emanating" from those contacts. However, it also seems plausible that those vesicles could be on their way to the membrane, perhaps from the Golgi or other vesicle sorting bodies. To better define these vesicles, they could co-stain for different markers of the endocytic and exocytic pathways.

4. The authors infer a process of junctional transition based on the location and prevalence of Duf+Pyd containing vesicles near sites of cell-cell contact, and later moving to regions where SD form. Because the images indicate the number and location of these puncta can be quite variable, this progression needs to be quantified to be convincing.

5. The authors conclude the SD proteins are trafficked in a clathrin-independent manner. However, the evidence for this seems limited. Lack of a clathrin coat may be due to normal shedding of clathrin after internalization. Zip colocalization is presented as a marker of clathrin-independent trafficking, but no reference is provided that actin and myosins are uniquely involved in clathrin-independent endocytosis. Both actin and non-muscle Myosins have been implicated in clathrin mediated endocytosis (CME):
https://www.ncbi.nlm.nih.gov/pubmed/22663081
https://www.ncbi.nlm.nih.gov/pubmed/24443954
The larger size and irregular shape of the vesicles might reflect fusion of smaller vesicles to one another or with larger vesicles present in nephrocytes. More convincing evidence for the claim of clathrin-independence would require knockdown of CME-specific components (e.g., Chc or AP-1), or showing that Clathrin never colocalizes with these puncta (IF of anti-Chc in Drosophila is published).

Minor comments:

1. Does loss of Pyd after initial SD establishment lead to adherens-like junction formation? It is not clear from the confocal images if this is the case (Fig S4 E and F, for example). TEM images would be informative on this point.

2. pyd mutants have increased nephrocyte agglutination, and Duf accumulates at these cell-cell contacts in adherens-like junctions. Wildtype larval nephrocytes can sometimes also make cell-cell contacts (Fig1C shows at least one), presumably this is where FasIII accumulates. Do those wildtype cell-cell contacts also contain similar junctions-what do these contacts look like by TEM?

3. More citations should be added throughout the manuscript, particularly where others have made the same or related observations, for example, Duf localization pattern by IF and EM (Weavers et al., Nature 2009; Zhuang et al., Development 2009), Duf perturbation following Pyd knockdown (Na et

al., Cell Reports 2015), BM thickening when nephrocyte SDs are disrupted (Weavers et al., Nature, 2009), etc…

4. The study finds that the coiled-coil domain in fly Pyd is important in nephrocyte SD formation. Do any vertebrate ZO-1 isoforms possess a similar coiled-coil domain?

5. It would be helpful to include more detailed information on the CRISPR-induced mutations for the new pyd alleles. It is also important to show protein expression data (i.e., Western) to demonstrate the expressed truncated proteins are of the predicted size.

6. Figure 3 shows the presence of a non-SD type junction in the fish zo-1 mutant podocyte, but there is only one image showing a single junction. This should be quantified in control and mutants to determine if this is a common feature of ZO-1 loss.

7. Figure 3 F+G-red boxes don't appear to match the inset images

8. Figure 6, some of the ROI boxes in B,C,D don't seem to match to the images below them. Please clarify in the images which box matches which panel below.

9. Please show genotypes in Figures 6,7,S4.

Reviewer #2 (Comments to the Authors (Required)):

Carrasco-Rando et al. demonstrate that a specific isoform of Polychaetoid/ZO-1 (Pyd-P) is required for slit diaphragm formation and stability within Drosophila nephrocytes (a podocyte-like cell type). The authors convincingly show that without Pyd, another slit diaphragm component (Dumbfounded) accumulates ectopically at intercellular junctions but this phenotype can be rescued by re-introduction of Pyd-P. The authors also show that zebrafish ZO-1 (termed Tjp-1a) is similarly required for slit diaphragm (SD) formation within the zebrafish pronephros, confirming a role for vertebrate ZO-1 as previously shown for mouse podocytes. The authors go on to use elegant genetic manipulations to investigate the stability of SDs in the absence of Pyd-P and demonstrate that although SDs are very stable structures (lasting up to 5 days) in the absence of Pyd-P, new Pyd-P is required to maintain SDs after this time-point. Finally, the authors use this system for re-introducing Pyd to explore the subcellular dynamics/localisation of Duf and Pyd during SD formation.

The discovery of a specific isoform of Pyd/ZO-1 required for SD formation in Drosophila is an exciting step forward in understanding the cell biology of this conserved cell type, although it remains unclear whether a specific Pyd isoform (similar to Drosophila Pyd-P) is present and required in vertebrates. The observation of Duf and Pyd on nephrocyte intracellular vesicles might be an important clue to the mechanism of SD formation - perhaps in the future it might be possible to live-image trafficking within in vivo or ex vivo nephrocytes? This is a well written paper, however given the level of rigor expected for a JCB paper, I would only recommend this manuscript for publication as an Article in JCB if the following points are addressed.

Major comments:

1. In the first section of the Results, the authors state that it is the delayed expression of Pyd in

nephrocytes, that drives relocalisation of Duf from intercellular junctions to SDs. To bolster these conclusions, please provide an image to show that Duf initially localises to intercellular junctions in stage 11/12 nephrocytes before the onset of Pyd expression. Furthermore, if delayed Pyd expression is responsible for late SD formation in wild-type nephrocytes, does ectopic Pyd expression driven earlier in development lead to faster SD formation? A minor point - the authors describe the onset of Pyd expression in nephrocytes as 'considerably later' (at stage 12) than Duf/Sns at stage 11 - please adjust text to be more modest.

2. In the zebrafish experiments, were the control images taken from 'wild-type' fish or a suitable injected control? Ideally, the control images should be taken from fish injected with a control or 'scrambled morpholino', to confirm that the observed defects in the tjp1a morphants are due to knock-down of Tjp1a and not a side-effect of the MO injection. If possible, it should also be demonstrated the MOs successfully reduce Tjp1a expression.

3. In the experiments using the TARGET system to assess continued requirement for Pyd, Pyd and Duf were observed in vesicles within the nephrocytes. Please comment on (or ideally provide additional images) whether similar vesicle localisation is observed during normal embryonic development in wild-type nephrocytes.

4. Although I agree that the observations of vesicles with Duf/Pyd adjacent to different nephrocyte membrane zones are striking, care needs to be taken when making bold statements regarding the directionality with which these vesicles are trafficking. To fully support these claims, the authors would need to provide live-imaging data to show vesicles moving in specific directions - although I realise this might be technically challenging. Such imaging data would also be informative to strengthen the claims that Duf relocalises from intercellular junctional complexes to SDs. If such experiments are outside the scope of this manuscript, please tone down the language used to be more speculative about the directionality of vesicles observed.

Minor points:

1. Please include references to previous work in the Introduction to Drosophila nephrocytes (such as Zhuang et al. 2009 and Weavers et al., 2009) earlier in paragraph 2, page 3. Please also include references to support your statement on page 4 that "these discoveries allowed the use of nephrocytes to model kidney diseases" such as Na et al., 2015.

2. Please comment on how duf null mutant nephrocytes might aggregate in the absence of Duf or Pyd-mediated adhesion, do they require Fas3?

3. Please provide a higher magnification inset in Figure 3F' to show the intact 'wild-type' SDs in control pronephros samples.

4. Please comment on which domain of Drosophila or vertebrate ZO-1 is known to mediate the interaction with Duf and whether this domain is present in all isoforms (particularly Pyd-P) of Drosophila Pyd. Please also comment on what is known about ZO-1 isoforms of vertebrates and whether Pyd-P is likely to be conserved.

5. Please correct the first sentence of the section "Pre-SD complexed are sorted to non-clathrin....." as I think some words are missing.

Reviewer #3 (Comments to the Authors (Required)):

The manuscript from Carrasco-Rando et al describes a study of the role of Pyd, the Drosophila homolog of the conserved ZO-1/Neph1 protein family, in mediating formation and structural integrity of slit diaphragms (SDs), key nephrocyte cell-junctions, that serve as essential and conserved functional components of the kidney blood filtration apparatus. As elaborated below, I found the paper to be an uneven presentation, in which the latter part puts forward an interesting and thought-provoking cellular model, backed by limited but significant data, while the bulk of the reported results are rather mundane and of minor/questionable significance. This is not to fault the genetic and cytological (light and electron microscopy) experimental work, which is of high quality throughout, but an assessment of the significance of the results. My strong recommendation, therefore, is that the authors restructure and shorten the manuscript so as to focus on the data directly relevant and supportive of their model, and consider resubmission to JCB as a Report.

The data presented in the latter part of the manuscript (Figs 6-7 and accompanying text), stemming from gradual restoration of pyd function to pyd mutants during larval stages, leads the authors to propose a model, whereby Pyd contributes to SD structure by mediating vesicular trafficking of the adhesion protein Duf/Kirre (and other SD components) from nephrocyte cell-cell contacts to the external membrane of these cells, where SDs form and perform their filtration-related functions. This apparently novel view of Pyd function in nephrocytes suggests an intriguing mechanism by which cells acquire functional features, a notion that is potentially of broad implications, given the conserved nature of nephrocyte filtration mechanisms and the molecular identity of the relevant components.
As might be expected, the model raises many questions, among them:
 What is the nature of the "vesicles" trafficking Duf, Pyd and other components towards the external membrane?
 How do these vesicles move? The reported presence of myosin II is suggestive of a microfilament-based transport system, but much further elaboration is clearly required.
 What makes Pyd such a critical contributor to this process? Do the vesicles fail to form in the absence of Pyd or is there another explanation?
 Is this mode of trafficking conserved during vertebrate/mammalian SD formation?
 What is the functional significance of utilizing the distinct isoform of Pyd, Pyd-P, in this setting? Are any unique structural characteristics of this isoform conserved in other species?

These questions and others set the stage for future research, and are generally beyond the scope of the current study. However, there are a couple of issues which the authors should address, experimentally or via discussion, in the context of the submitted work:
1. The model suggests that the nephrocyte "agglutination" phenotype observed in pyd mutants/knockdown results in part from abnormal accumulation of Duf at nephrocyte cell-cell contacts. However, the same phenotype is observed in duf null mutants (i.e., in the absence of Duf- Fig 2C)- how can this be explained?
2. The data suggests that the described mode of action of Pyd, which functions in a variety of tissues, is performed primarily (exclusively?) in nephrocytes, and is based on the use of the "P" isoform. This could imply that the nephrocyte-specific activity would be detrimental in other settings. Indeed, ectopic expression of the P isoform in thoracic sensory organs results in apparently dominant-negative effects (Fig. S3G), but the analysis does not explore the cellular basis for this phenotype. The authors should examine whether expression of Pyd-P is detrimental in established settings where Duf function as a cell-cell adhesion molecule is critical (e.g- myoblast

fusion and/or ommatidia morphogenesis), and if so, provide some degree of tissue-level analysis explaining the basis for the phenotypic abnormalities.
3. A related issue has to do with the embryonic expression patterns of duf and pyd. The authors suggest that the relatively late temporal onset of pyd expression in nephrocytes is significant (perhaps to avoid interference with tissue morphogenesis). Does forced earlier expression of pyd have deleterious effects? Are these specific to the P isoform?

With regards to the initial set of results presented in the manuscript (Figs 1-5 and related text)-my sense is that much of this material fails to shed significant new light on the relevant processes, and can be substantially condensed or left out altogether. Thus, the nephrocyte phenotypes observed in pyd mutants are- as expected- very similar to those already described for duf and sns; the zebrafish phenotypes resulting from ZO-1 knockdown are expected as well, given the established conservation of molecular nephrocyte functions between flies and mammals; and the detailed analysis of Pyd isoforms is not really informative, beyond establishing that a specific, alternative isoform (whose structural characteristics are only superficially explored) is utilized in the context of fly kidney development.

Considering all of the above, I believe that the manuscript will greatly benefit from a major reorganization in presentation of the study. Namely, the authors should place the data supporting their model for "junctional remodeling" and SD formation front and center, adding only minimal additional data necessary for introducing the system. Such an approach is critical, to my mind, in order to turn the paper into a significant cell-biological study of interest to the JCB readership.

Minor figure issues
1. There is some confusion in the presentation/labeling of some of the supplementary figures and their mention in the main text. Thus, there are no panels I-K in figure S1, the dominant negative phenotype pf Pyd-P appears in Fig S3 and not S2, etc.

2. In Fig 1, panel designations G and G' should be switched (G should be the lowest magnification panel).

3. In Fig. 6, colored labels of the antigens visualized (Duf, Pyd, etc) should be added to the panels, as in other figures.

Mark Peifer and Tim Spencer
Editors of JCB

March 21st, 2019

Dear Editors,

We are submitting the revised version of the JCB manuscript 201810171, entitled, "A specific isoform of Pyd/ZO-1 mediates junctional remodelling and formation of slit diaphragms", in which all the suggestions and queries of the reviewers have been addressed. We are grateful for the reviewers' and editors' constructive comments and suggestions that greatly helped us to improve the quality of our manuscript. We hope the present version of the manuscript will be acceptable for publication.

All co-authors have seen and approved the resubmitted version of the manuscript. Please find below our detailed point-by-point response to the reviewers' comments.

Thank you very much for your consideration,

Yours sincerely,

Mar Ruiz-Gómez

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Reviewer 1

Major comments

1: It would be helpful to better define the junctions present between cells in the pyd mutants. Are these junctions possibly some version of SDs between neighboring cells-does Nephrin/Sns also accumulate at these contacts? Or, if they are an adherens-like junction, do classic junctional proteins localize at these structures-perhaps E-Cadherin, which is presumably absent from normal nephrocyte SDs?

Authors' response:

Although not presented in the previous version of the manuscript, we had looked for the presence of several components of adherens and septate junctions at the contacts

between agglutinated *pyd* nephrocytes. We couldn't detect the AJ markers E-Cad or Arm/ß-catenin, neither N-Cad in these ectopic junctions. On the other hand, three (Fas3, Dlg and Lgl) out of the five proteins associated to septate junctions that we checked, did accumulate with Duf at the nephrocyte junctions. The other two were either not expressed (Cora) or did not accumulate at the junctions (Nrg). Regarding additional SD components, we found that Sns did accumulate at the junctions (Fig. 7A). This information is now shown in the revised Figs S2A-C and is mentioned in the results subsection "Mutual requirement of Duf and Pyd for SDs development".

2: Reintroducing Pyd restores SD formation (Fig 6D), but does it lead to loss of the adherens junctions? The confocal images suggests this, but some images indicate Duf remains at cell-cell contacts (Fig6D'), implying there may still be adherens junctions present. Even when Pyd has been reintroduced for sufficient time to allow SD formation (i.e., 24hrs, Fig 6D), significant agglutination remains, suggesting the adherens junctions may still be present. This conversion of junctions is an important aspect of the proposed model. TEM with quantification of adherens-junction number and size at a couple different time points would be more convincing.

Authors' response:

Reintroduction of Pyd eventually leads to an almost complete loss of junctions among nephrocytes. However, after 24h of recovery regions of cell-cell contacts still remain, as was shown in Fig.6 and mentioned in the Results section. We agree with the reviewer that quantitation of junctional complexes at different times will strengthen our interpretation of a process of junctional conversion driven by Pyd. Therefore, we quantified in TEM micrographs the percentage of membrane engaged in septate-like junctions among nephrocytes at 15h and 48h of recovery, and have added this information to Fig. S4H and to the Results subsection "Pyd-P mediates the transition from septate-like junctions to SDs".

3: The authors interpret the presence of subcortical puncta/vesicles containing Duf and Pyd near cell-cell contacts as evidence they are "emanating" from those contacts. However, it also seems plausible that those vesicles could be on their way to the membrane, perhaps from the Golgi or other vesicle sorting bodies. To better define these vesicles, they could co-stain for different markers of the endocytic and exocytic pathways.

Authors' response:

We thank the reviewer for these suggestions and have done these experiments that are technically challenging. In our hands stainings with antibodies against the core Rabs 5, and 11 do not work in heat-fixation conditions, needed for anti-Duf stainings, and worked poorly after formaldehyde-fixation that is suboptimal for detection of the recovery vesicles with anti-Duf (anti-MIP or anti-Pyd stainings work well with FA-fixation, but not all Pyd-positive vesicles are loaded with SD components). Despite the poor quality of these stainings, we haven't found any preferential co-staining of Duf with any of these Rab markers in vesicles. To obtain better resolution, we also performed these co-stainings using endogenously tagged YFP-Myc-Rabs (Dunst et al., 2015). Due to the complicated genetic combinations needed to perform the rescue experiments in these genetic backgrounds, we could only check Rabs located on the

second chromosome such as the core Rabs 5 and 6. In both cases there was no co-expression of these markers and Duf in the vesicles. Regarding the exocyst markers, again we failed to detect co-localisation of Duf and Sec 8 in the vesicles. These data are now included in Fig. S5 D-E and mentioned in the Result subsection "Pre-SD complexes accumulate in non-clathrin-coated vesicles found close to cell junctions and the external nephrocyte membrane".

4: The authors infer a process of junctional transition based on the location and prevalence of Duf+Pyd containing vesicles near sites of cell-cell contact, and later moving to regions where SD form. Because the images indicate the number and location of these puncta can be quite variable, this progression needs to be quantified to be convincing.

Authors' response:

We understand the reviewer's concern and in the revised version of figure 6 we provide this quantification (Fig. 6B).

5: The authors conclude the SD proteins are trafficked in a clathrin-independent manner. However, the evidence for this seems limited. Lack of a clathrin coat may be due to normal shedding of clathrin after internalization. Zip colocalization is presented as a marker of clathrin-independent trafficking, but no reference is provided that actin and myosins are uniquely involved in clathrin-independent endocytosis. Both actin and non-muscle Myosins have been implicated in clathrin mediated endocytosis (CME):
https://www.ncbi.nlm.nih.gov/pubmed/22663081
https://www.ncbi.nlm.nih.gov/pubmed/24443954
The larger size and irregular shape of the vesicles might reflect fusion of smaller vesicles to one another or with larger vesicles present in nephrocytes. More convincing evidence for the claim of clathrin-independence would require knockdown of CME-specific components (e.g., Chc or AP-1), or showing that Clathrin never colocalizes with these puncta (IF of anti-Chc in Drosophila is published).

Authors' response:

We thank the reviewer for this constructive comment. We are aware of the proposed implication of organised actomyosin cytoskeleton in CME and didn't intend to imply an exclusive role in clathrin independent endocytosis (CIE), we just mentioned that CIE requires actomyosin motors. The reason to propose that a CIE mechanism is at work in the Pyd-mediated junctional remodelling process is based on the careful observation of the vesicles loaded with SD components. First, clathrin-coated vesicles have been described as remarkably uniform in size and shape, consisting in spherical vesicles with an average diameter of ~90nm (Miller, 2015 doi:10.1016/j.devcel.2015.03.002). As presented in figures 6, 7 and S5, both in confocal and TEM images, the SD-containing vesicles are larger and vary in size and shape. And second, although one could always argue that these variations in size and shape may be due to fusion of smaller coated vesicles, in our immune-EM experiments we found no coincidence of gold-labelled Duf or Pyd with coated vesicles or pits. Now, following the reviewer suggestion, we are

including a double staining with anti Duf and anti-Chc in revised Fig. 7C to further show that the SD-containing vesicles are devoid of Clathrin, and are mentioning this in the Results subsection "Pre-SD complexes accumulate in non-clathrin-coated vesicles found close to cell junctions and the external nephrocyte membrane".

Minor comments:

1: Does loss of Pyd after initial SD establishment lead to adherens-like junction formation? It is not clear from the confocal images if this is the case (Fig S4 E and F, for example). TEM images would be informative on this point.

Authors' response:

Again, we appreciate this constructive comment. We understand that it is not easy to distinguish between nephrocyte agglutination and apposition by looking at single confocal sections. We had chosen the sections in Figs. S4E-F because they allowed distinguishing agglutination in F (Duf and no Pyd in the ectopic junctions) from apposition in E (Duf and Pyd at the external membrane, and increased fluorescent intensity due to close apposition among two external membranes). Of course, our conclusions are based in the observation of series of consecutive sections for several nephrocyte strings, precluding any kind of confusions. We found that junctional complexes are more abundant after 5 days at 17ºC, and to show that more clearly, following the reviewer's suggestion we have added additional panels to Fig.S4 showing TEM images of these septate-like junctions (Fig. S4, G-G").

2: pyd mutants have increased nephrocyte agglutination, and Duf accumulates at these cell-cell contacts in adherens-like junctions. Wildtype larval nephrocytes can sometimes also make cell-cell contacts (Fig1C shows at least one), presumably this is where FasIII accumulates. Do those wildtype cell-cell contacts also contain similar junctions-what do these contacts look like by TEM?.

Authors' response:

During embryonic stages wild-type nephrocytes make cell contacts and Fas3 accumulate at these junctions. At larval stages we have not observed cell contacts among wild-type nephrocytes, which do not express detectable Fas3. It is true that sometimes closely apposed cells could look like agglutinated, and this is what happens in the string of cells presented in Figure 1C. However, detailed examination of consecutive confocal planes allows distinguishing between agglutination and apposition. We are including a Figure for the reviewers (Fig.R1 https://babia.cbm.uam.es:5001/sharing/7QO8vfp24) showing 32 consecutive images for the string of nephrocytes displayed in figure 1C proving that there is no agglutination or junctions between wild-type nephrocytes.

3: More citations should be added throughout the manuscript, particularly where others have made the same or related observations, for example, Duf localization pattern by IF and EM (Weavers et al., Nature 2009; Zhuang et al., Development 2009), Duf perturbation following Pyd knockdown (Na et al., Cell Reports 2015), BM thickening when nephrocyte SDs are disrupted (Weavers et al., Nature, 2009), etc...

Authors' response:

We apologise for these unintended omissions. We tried to cite all authors that made similar observations and in the revised manuscript we are including the mentioned citations.

4: The study finds that the coiled-coil domain in fly Pyd is important in nephrocyte SD formation. Do any vertebrate ZO-1 isoforms possess a similar coiled-coil domain?

Authors' response:

We checked all predicted human ZO-1 isoforms. Some of them have a stretch 93 amino acids long at the N-terminal region at the same position as the Pyd-P exon 5 domain. However, there is no conservation in primary or secondary structure. Moreover, no predicted coiled-coil motif was present in this region.

5: It would be helpful to include more detailed information on the CRISPR-induced mutations for the new pyd alleles. It is also important to show protein expression data (i.e., Western) to demonstrate the expressed truncated proteins are of the predicted size.

Authors' response:

We agree with the reviewer and the molecular characterisation of the 4 novel *pyd* alleles has been added to the Material and Methods section. Regarding the data about protein expression, we understand that it would be ideal to show the presence of the truncated forms by Western Blots. Unfortunately some technical issues preclude this analysis. First, both pyd^{CC6} and pyd^{S10} produce truncated proteins that lack the epitopes used for the generation of all available anti-Pyd antibodies. Second, we have performed WBs from wild-type samples, and even the monoclonal anti Pyd antibody recognises many bands, making it impossible to distinguish specific isoforms and consequently to identify novel truncated proteins that would be most probably not abundant. We are including a figure to the reviewers (Fig.R2 https://babia.cbm.uam.es:5001/sharing/7QO8vfp24) showing anti Pyd staining in pyd^{CC} late embryos. No staining is present in nephrocytes.

6: Figure 3 shows the presence of a non-SD type junction in the fish zo-1 mutant podocyte, but there is only one image showing a single junction. This should be quantified in control and mutants to determine if this is a common feature of ZO-1 loss.

Authors' response:

Following the reviewer suggestion we have analysed several TEM micrographs taken from ultrathin sections encompassing the whole glomerular area in two morphant and two control fish embryos and registered the number of ectopic junctions present in each sample. We found no ectopic junctions in images from control samples, except for a single instance. In contrast, we observed 21 ectopic junctions in three ultrathin sections from morphants. These numbers are now included in the legend to figure 3.

7: Figure 3 F+G-red boxes don't appear to match the inset images.

Authors' response:

We thank the reviewer for pointing this out. Although the insets were well allocated, in Figure 3F the image was rotated 90º to the left. We have corrected this mistake in the revised figure 3. In addition, we are including a figure for the reviewers (Fig.R3 https://babia.cbm.uam.es:5001/sharing/7QO8vfp24) showing the sequence of TEM images of increasing magnification for all the insets shown in figure 3.

8: Figure 6, some of the ROI boxes in B,C,D don't seem to match to the images below them. Please clarify in the images which box matches which panel below.

Authors' response:

Again we apologise for that. In the previous version of figure 6 we flipped some of the insets to better accommodate them into the final figure frame. In the revised figure we have corrected this, and have labelled the boxes as suggested by the referee to better allocate each inset within their panel.

9: Please show genotypes in Figures 6,7,S4.

Authors' response:

We have included the genotypes in the figures as suggested by the reviewer.

Reviewer 2

Major comments

1: In the first section of the Results, the authors state that it is the delayed expression of Pyd in nephrocytes, that drives relocalisation of Duf from intercellular junctions to SDs. To bolster these conclusions, please provide an image to show that Duf initially localises to intercellular junctions in stage 11/12 nephrocytes before the onset of Pyd expression. Furthermore, if delayed Pyd expression is responsible for late SD formation in wild-type nephrocytes, does ectopic Pyd expression driven earlier in development lead to faster SD formation? A minor point - the authors describe the onset of Pyd expression in nephrocytes as 'considerably later' (at stage 12) than Duf/Sns at stage 11 - please adjust text to be more modest.

Authors' response:

We thank the reviewer for indicating this. We now include at the beginning of the Results section a reference to the Nature paper by Weavers et al, 2009 that shows co-expression of Duf and Sns at regions of contacts in stage 11 wildtype nephrocytes (Fig.S1) (https://media.nature.com/original/nature-assets/nature/journal/v457/n7227/extref/nature07526-s1.pdf) This reference is now included at the beginning of the Results section. Regarding the possibility of supplying

Pyd earlier in order to accelerate SD formation, we thought about it, but unfortunately there is no Gal4 line available to drive expression in garland cells before late stage 12/stage 13, when *pyd* is already expressed in nephrocytes (even using *sns-GCN-Gal4* due to the delay intrinsic to the Gal4 system). As suggested by the reviewer we have toned down the indicated statement and eliminated the adverb "considerably".

2: In the zebrafish experiments, were the control images taken from 'wild-type' fish or a suitable injected control? Ideally, the control images should be taken from fish injected with a control or 'scrambled morpholino', to confirm that the observed defects in the tjp1a morphants are due to knock-down of Tjp1a and not a side-effect of the MO injection. If possible, it should also be demonstrated the MOs successfully reduce Tjp1a expression.

Authors' response:

We thank the reviewer for pointing this out. The image in figure 3A is a wild-type fish, but the quantifications in figure 3C and the confocal and TEM images (figures 3D, D', F and F') correspond to animals injected with a control morpholino. This was specified in the Materials and Methods section but not clearly indicated in the figure legend. To avoid misunderstandings this is corrected in the corresponding panels and in the legend to revised figure 3. Regarding the efficacy of the injected morpholinos to reduce Tjp1a expression, this was verified by RT-PCR from total RNA of injected embryos. We show these data in Fig S1 for morpholino MOex4 (corresponding to the specimens in figure 3) that targets the splice donor site of exon4, and that results in an altered splicing, visible as an increase in the amplicon size (from 683bp to 815bp) leading to a truncation of the protein at amino acid 106 after misincorporation of two amino acids. This is also mentioned in the Results and Materials and Methods sections of the revised manuscript.

3: In the experiments using the TARGET system to assess continued requirement for Pyd, Pyd and Duf were observed in vesicles within the nephrocytes. Please comment on (or ideally provide additional images) whether similar vesicle localisation is observed during normal embryonic development in wild-type nephrocytes.

Authors' response:

We appreciate this valuable comment. We observed vesicles loaded with Pyd and Duf in late embryonic stages, although due to the small size of embryonic nephrocytes it is difficult to obtain high quality images. We have now included a panel in revised Figure S4 (Fig. S4 I) to show some of these vesicles close to the nephrocyte external membrane, where SDs are forming.

4: Although I agree that the observations of vesicles with Duf/Pyd adjacent to different nephrocyte membrane zones are striking, care needs to be taken when making bold statements regarding the directionality with which these vesicles are trafficking. To fully support these claims, the authors would need to provide live-imaging data to show vesicles moving in specific directions - although I realise this

might be technically challenging. Such imaging data would also be informative to strengthen the claims that Duf relocalises from intercellular junctional complexes to SDs. If such experiments are outside the scope of this manuscript, please tone down the language used to be more speculative about the directionality of vesicles observed.

Authors' response:

These experiments would be ideal, but unfortunately they are technically unviable with the tools currently available. However, we have observed a clear tendency to find more Duf-Pyd containing vesicles close to the junctions or to the external membrane at early and later stages of recovery respectively, as this is now quantified in Fig. 6B. Nonetheless, we agree that we have not provided definitive evidence proving the directionality of the vesicles. Therefore, following the reviewer suggestion we have used a more speculative tone when describing the molecular mechanism underlying junctional remodelling both in the results and the discussion sections.

Minor comments:

1: Please include references to previous work in the Introduction to Drosophila nephrocytes (such as Zhuang et al. 2009 and Weavers et al., 2009) earlier in paragraph 2, page 3. Please also include references to support your statement on page 4 that "these discoveries allowed the use of nephrocytes to model kidney diseases" such as Na et al., 2015.

Authors' response:

As we mentioned in our response to reviewer 1, we apologise for these unintended omissions. We tried to cite all authors that made similar observations and in the revised manuscript we are including the mentioned citations.

2: Please comment on how duf null mutant nephrocytes might aggregate in the absence of Duf or Pyd-mediated adhesion, do they require Fas3?

Authors' response:

This is an interesting question. We have evidence that Fas3, Sns Dlg and Lgl accumulate at cell contacts in *duf* deficient nephrocytes. Therefore it is quite possible that the adhesive properties of Fas3 and Sns might mediate nephrocyte agglutination. In addition, the presence of the septate junction proteins Dlg and Lgl might contribute to the organisation of junctional complexes. However, we have not performed analysis in double mutant conditions to investigate their relative contribution.

3: Please provide a higher magnification inset in Figure 3F' to show the intact 'wild-type' SDs in control pronephros samples.

Authors' response:

We have substituted panel 3F' for a higher magnification as requested.

4: Please comment on which domain of Drosophila or vertebrate ZO-1 is known to

mediate the interaction with Duf and whether this domain is present in all isoforms (particularly Pyd-P) of Drosophila Pyd. Please also comment on what is known about ZO-1 isoforms of vertebrates and whether Pyd-P is likely to be conserved.

Authors' response:

In vertebrates the association of ZO-1 with Neph1 is mediated through ZO-1 PDZ1 motif and the PDZ-binding domain of Neph1. The three PDZ domains are conserved in all Pyd isoforms described so far, including Pyd-P, and Duf also contains a PDZ-binding domain. Preliminary results from our laboratory using Pyd-B isoform indicated that its association with Duf is also mediated through the PDZ1 domain, but so far we haven't done these experiments with Pyd-P. Regarding vertebrate ZO-1 isoforms, the only ones investigated in some detail differed in the alternative inclusion of the alpha domain, being the isoform lacking this domain the one enriched in podocytes. This isoform, that shares with Pyd-P the exclusion of the alpha domain, has been described as preferentially expressed in structurally dynamic junctions (Balda & Anderson 1993, doi: 10.1152/ajpcell.1993.264.4.C918). Concerning the relevant coiled-coil domain, as stated in our responses to reviewer 1, we checked all predicted human ZO-1 isoforms. Some of them have a stretch 93 amino acids long at the N-terminal region at the same position as the Pyd-P coiled-coil domain. However, there is no conservation in primary or secondary structure to Pyd-P coiled-coil motif. Taking all these data into account, the conservation of Pyd/ZO-1 function in junctional remodelling could either depend on the N-terminal region present in some ZO-1 isoforms, or more plausibly on the ZO-1-mediated recruitment of another adaptor to the SD complex to compensate for the absence of the CC-domain. Further experiments are needed to answer this important question.

5: Please correct the first sentence of the section "Pre-SD complexed are sorted to non-clathrin....." as I think some words are missing.

Authors' response:

We thank the reviewer for pointing out this error that has been corrected in the revised manuscript.

Reviewer 3

<u>Major comments</u>

1: The model suggests that the nephrocyte "agglutination" phenotype observed in pyd mutants/knockdown results in part from abnormal accumulation of Duf at nephrocyte cell-cell contacts. However, the same phenotype is observed in duf null mutants (i.e., in the absence of Duf- Fig 2C)- how can this be explained?

Authors' response:

This is an interesting question raised also by reviewer 2. We have evidence that Fas3, Sns Dlg and Lgl accumulate at cell contacts in *duf* deficient nephrocytes. Therefore it is quite possible that the adhesive properties of Fas3 and Sns might mediate nephrocyte agglutination. In addition, the presence of the septate junction proteins Dlg and Lgl

might contribute to the organisation of junctional complexes. However, we have not performed analysis in double mutant conditions to investigate their relative contribution.

2: The data suggests that the described mode of action of Pyd, which functions in a variety of tissues, is performed primarily (exclusively?) in nephrocytes, and is based on the use of the "P" isoform. This could imply that the nephrocyte-specific activity would be detrimental in other settings. Indeed, ectopic expression of the P isoform in thoracic sensory organs results in apparently dominant-negative effects (Fig. S3G), but the analysis does not explore the cellular basis for this phenotype. The authors should examine whether expression of Pyd-P is detrimental in established settings where Duf function as a cell-cell adhesion molecule is critical (e.g- myoblast fusion and/or ommatidia morphogenesis), and if so, provide some degree of tissue-level analysis explaining the basis for the phenotypic abnormalities.

Authors' response:

We speculate in the manuscript that of Pyp-P function depends both on the exclusive presence of this isoform in nephrocytes and on the fact that they are the only cell type in which Sns and Duf are co-expressed in *Drosophila*. Indeed, we found that ectopic expression of Pyd-P is detrimental but according to our results only in cells expressing *pyd* endogenously. Thus, although not mentioned in the manuscript we have observed that ectopic overexpression of both Pyd-B and Pyd-P in the mesoderm (where *pyd* is not expressed) has not effect on myoblast fusion, further indicating that Pyd-P is not detrimental for the cell adhesion function of Duf in founder myoblasts. Moreover, following the reviewer suggestion, we now analysed the effect of Pyd-P ectopic expression during ommatidia morphogenesis (*GMR-Gal4* driver). We found a rough eye phenotype associated to disorganisation of ommatidial cells, including an increase in the number of interommatidial and cone cells, very much alike the loss of function phenotypes described for *pyd* in the eye (Seppa, 2008). Thus, Pyd-P ectopic overexpression has a dominant negative effect in the postmitotic cells of the eye discs (some of them expressing *duf*). We also found a dominant negative effect in the wing disc cells (devoid of *duf* and expressing *pyd*). Therefore, we suggest that the dominant negative effect might be unrelated to Duf and rather due to the sequestering of endogenous Pyd-B partners. To clarify this query, we have changed the Results and Discussion sections. In addition, we are including extra panels in Fig.S3 (Fig. S3 M-P) showing the effects of Pyd-P overexpression in the mesoderm and the eye disc.

3: A related issue has to do with the embryonic expression patterns of duf and pyd. The authors suggest that the relatively late temporal onset of pyd expression in nephrocytes is significant (perhaps to avoid interference with tissue morphogenesis). Does forced earlier expression of pyd have deleterious effects? Are these specific to the P isoform?

Authors' response:

This is a very interesting suggestion. We also thought that anticipating *pyd* expression could accelerate SD formation. Unfortunately, this analysis was precluded by the fact that there is no Gal4 line available to drive expression in garland cells before late stage 12/stage 13, when *pyd* is already expressed in nephrocytes (even using *sns-GCN-Gal4* due to the delay intrinsic to the Gal4 system).

Other statements:

With regards to the initial set of results presented in the manuscript (Figs 1-5 and related text)-my sense is that much of this material fails to shed significant new light on the relevant processes, and can be substantially condensed or left out altogether. Thus, the nephrocyte phenotypes observed in pyd mutants are- as expected- very similar to those already described for duf and sns; the zebrafish phenotypes resulting from ZO-1 knockdown are expected as well, given the established conservation of molecular nephrocyte functions between flies and mammals; and the detailed analysis of Pyd isoforms is not really informative, beyond establishing that a specific, alternative isoform (whose structural characteristics are only superficially explored) is utilized in the context of fly kidney development.

Considering all of the above, I believe that the manuscript will greatly benefit from a major reorganization in presentation of the study. Namely, the authors should place the data supporting their model for "junctional remodeling" and SD formation front and center, adding only minimal additional data necessary for introducing the system. Such an approach is critical, to my mind, in order to turn the paper into a significant cell-biological study of interest to the JCB readership.

Authors' response:

This manuscript investigates the role played by Pyd/ZO-1 in SD formation using the fly nephrocyte as a model and thus benefitting of the matchless genetic toolkit available in *Drosophila*. Although we agree that the key finding of this work is the role played by Pyd in promoting junctional remodelling leading to SD formation (a function that is probably conserved in vertebrates), we strongly feel that it is worthwhile leaving the experimental evidence describing *pyd* loss-of-function phenotype in the manuscript, since it was its close examination what allowed us to propose a molecular mechanism to explain *pyd* function in nephrocytes. Moreover, it represents a highly detailed analysis that is expected to be of interest not only to specialists in the cell biology field, but also to a more general audience.

Minor figure issues:

1: There is some confusion in the presentation/labeling of some of the supplementary figures and their mention in the main text. Thus, there are no panels I-K in figure S1, the dominant negative phenotype pf Pyd-P appears in Fig S3 and not S2, etc.

Authors' response:

We apologise for these oversights that have been corrected in this revised version of the manuscript.

2: In Fig 1, panel designations G and G' should be switched (G should be the lowest magnification panel).

Authors' response:

We thank the reviewer for indicating this error that has been corrected in the revised Fig. 1.

3: In Fig. 6, colored labels of the antigens visualized (Duf, Pyd, etc) should be added to the panels, as in other figures.

Authors' response:

The corresponding coloured labels have been added in the revised Fig. 6 as suggested by the reviewer.

April 18, 2019

RE: JCB Manuscript #201810171R

Dr. Mar Ruiz-Gómez
Centro de Biología Molecular Severo Ochoa, CSIC-UAM
Nicolás Cabrera, 1
Madrid 28049
Spain

Dear Dr. Ruiz-Gómez:

Thank you for submitting your revised manuscript entitled "A specific isoform of Pyd/ZO-1 mediates junctional remodelling and formation of slit diaphragms". As you will see, the reviewers are generally satisfied by the changes that you made. Both Reviewer 2 and Reviewer 3 would like you to address in the text the desirability of in the future testing earlier expression of Pyd-P. We'd also like you to state whether you tried ubiquitous early drivers, and if so what result you obtained. You should also modify the text to address the issues Reviewer 3 raises about your view of the role of Duf. Please make these changes and include with your revision a highlighted version of the text that illustrates these. Assuming the changes are made as suggested, this should not require further review.

If these issues are successfully addressed, we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

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Sincerely,

Mark Peifer, PhD
Monitoring Editor
JCB

Tim Spencer, PhD
Deputy Editor
Journal of Cell Biology
ORCiD: 0000-0003-0716-9936

Reviewer #1 (Comments to the Authors (Required)):

Through additional experimentation, quantification, and text revisions, the authors have done an excellent job of addressing my comments and concerns. I find the revised manuscript appropriate for publication in the JCB.

Reviewer #2 (Comments to the Authors (Required)):

In this revised paper by Carrasco-Rando et al., the authors have now addressed all of the points raised in my initial review, either by performing additional quantification, making reference to previous work or (where experiments could not be performed in the available time) toning down some claims in the text.

Whiilst it is a shame that the authors could not force earlier expression of Pyd-P to address mine and Reviewer 3's comments regarding premature SD formation, perhaps this might be technically possible in the future if more Gal4 lines are generated. Although not 'clean', it might have been interesting to try using an early ubiquitous Gal4 driver to see whether this had any effect on the timing of SD formation, however I realise it may be that these embryos are too disrupted to gain useful insight.

Reviewer #3 (Comments to the Authors (Required)):

In my initial review of Carrasco-Rando et al. I recommended a reorganization of the manuscript, so that the focus would be placed on the cellular mechanism by which Pyd-P-mediated trafficking of Duf governed transitions between nephrocyte cell-cell attachments and establishment of slit diaphragms. As the authors have chosen not to follow this suggestion (which I still believe to be worthwhile), and were not asked to do so by the editors, I will limit my comments to the specific data issues I raised (also brought up in some instances by the other reviewers) and the manner by which they were addressed in the revised manuscript.

1. In fact, I would like to focus on a single outstanding issue which emerges, namely, understanding the balance between physiological kidney development and the abnormal (diseased) state observed in pyd mutants, whose current presentation is unclear. To my understanding, the progression of events that is put forward by the authors is that Duf initially participates in establishing connections between nephrocytes, and is later trafficked (via a Pyd-P-mediated process) to sites where it will contribute to SD formation.
• How then do we interpret the nephrocyte "agglutination" phenotype that is observed when this process is impaired (by mutation and, by inference, in relevant kidney disease)? Is it a consequence of a simple failure to dismantle the (previously-established) cell-cell contacts, or is a different/additional mechanism at play?
• And what then is the role of Duf in all of this? Data added to the revised manuscript suggests, according to the authors' response, that adhesion proteins other than Duf (eg, Fas3 and Sns), which accumulate at nephrocyte cell-cell interfaces in duf mutants, may mediate "agglutination". Does this imply that Duf accumulates at cell contacts (from where it is later trafficked) but is not critical for their formation?
While definitive answers to these queries are not currently available, I believe that they should be brought up and discussed directly in the manuscript.

2. I also wish to add that I am perplexed about the authors' response to my suggestion of examining fly embryo kidney development following premature expression of Pyd-P (also suggested by reviewer #2), as a means of testing whether such expression leads to SD formation at an (abnormally) early stage. The authors state that no GAL4 line is available for driving expression of UAS-based constructs in garland cells prior to the stage at which Pyd-P is normally expressed-but

why can't this be attempted using ubiquitous or general ectodermal drivers rather than garland-cell specific drivers?