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Drosophila ATP6AP2/VhaPRR functions both as a novel planar cell polarity core protein and as a regulator of endosomal trafficking

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1st Editorial Decision 28 June 2012

Thank you for your patience while your manuscript has been reviewed and please accept my apologies for the delay in responding. We have just now received the set of reports from the two referees that were asked to evaluate your study, which I copy below. As referee reports are quite explicit and mainly asking for clarifications and control experiments I will not repeat their arguments here, but as you will see below, they all think that your manuscript is highly interesting and their comments are very positive.

Given the referees' recommendations, I would like to invite you to submit a revised version of the manuscript. Please be aware that your revised manuscript must address the referees' concerns and their suggestions should be taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is 'The EMBO Journal' policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process iniciative, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you

foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you again for the opportunity to consider your work for publication. I look forward to your revision.

Please, do no hesitate to contact me in case you have any further question, need further input or any problem arises during the revision process.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

In this paper, the authors report that VhaPRR, which the authors and others have previously linked to the V-ATPase, interacts with the protocadherin Fmi through its extracellular domain and co-localizes with PCP proteins during all pupal wing stages. They also report that this localization depends on intact PCP domains, and vice-versa, suggesting that VhaPRR is itself a PCP core protein. Finally, they report that VhaPRR also plays a role in acidification and endosomal transport, and conclude that VhaPRR is a key factor in epithelial morphogenesis.

I find the paper potentially interesting and I have only a few comments on the possible role of VhaPRR in acidification and traffic - it seems to me that this role remains somewhat obscure. It is remarkable that, except acidic vesicles, all markers related to endo-lysosomes that have been tested are increased in the VhaPRR mutant cells (Lamp1, Armadillo, Notch, endocytosed E-cad), including Rab5, Rab 11 and Rab7. This may support the view that VhaPRR mutant cells exhibit an acidification defect, as proposed by the authors. However, beyond the difference in the distribution of apical vesicles, the lysotracker staining intensity of individual vesicles whether wt/mutant or apical/basal looks very similar (Fig 6). This argues against a defect in the acidification properties, at least at the level of lysotracker detection. Do Rab proteins also accumulate in V-ATPase mutants? Also, is the pH of endo-lysosomes or Lamp1 degradation affected after Fmi or Fz disruption?

Also, in VhaPRR mutant clones, there is little difference in the basal lysotracker staining, but the number of apical vesicles stained with the dye is reduced. Is the cell shape changed in the mutant? This is not easy to see in the x-y projection in Fig 6D, because nuclei are not in the focal plane of mutant cells. Is the number of endosomes and lysosomes reduced in the apical portion of the cell? Conversely, do authors know whether the basal, Lamp1-positive vesicles, which accumulate in the mutant, are acidic?

Referee #2

The Hermle et al manuscript has 2 parts. The first one is an extension of the characterization of the role VhaPRR in the regulation of PCP (Hermel et al., 2010, Buechling et al., 2010 and Cruciat et al., 2010). The novel findings include 1) VhaPRR affects the trafficking of Fmi; 2) VhaPRR is planar polarized; 3) VhaPRR can form a complex with Fmi. In a second part the authors analyse the role of VhaPRR in vesicular trafficking. They show that the loss of VhaPRR function perturbs vesicle acidification as well as Notch and E-Cadherin trafficking. Although the authors' findings suggest a role of VhaPRR in Fmi trafficking, the connection between the function of VhaPRR in vesicular trafficking and its role in PCP is not explored. It also remains unclear why the loss of function of VhaPRR drastically perturbs Stbm and Fz localizations, while it only mildly affects Fmi localization.
Furthermore, the following points should be addressed:
1. The loss of VhaPRR drastically affects Fz and Stbm localization, whereas it has a milder effect on the one of Fmi. Is the trafficking of Fz and/or Stbm also affected by the loss of VhaPRR function?

Do Fmi, Fz or Stbm colocalize in intercellular (intracellular?) structures?
2. Upon Fmi antibody incubation at 4{degree sign}C, the amount of Fmi is lower at the apical AJs. The authors therefore need to quantify the reduction of Fmi membrane localization upon chase at 25{degree sign}C to demonstrate that there is indeed a stronger reduction of Fmi at VhaPRR apical junction. A better characterization of the dynamics of Fmi internalization might be relevant (see Strutt 2011).
3. The authors state that VhaPRR is localized both at the distal and proximal cell junction. However, VhaPRR is absent from many proximal or distal junctions facing the mutant clones in figure 2D. A far better experiment would be to use a GFP::VhaPRR construct to demonstrate that VhaPRR localizes both at the distal and proximal junctions.
4. In figure 7B, it is unclear how the antibody can access the extracellular E-Cad in the absence of detergent since a cuticle is likely present on the apical side of the wing pupal cells at 32APF.
5. It is unclear how the role of VhaPRR in lysosomal degradation might lead to an accumulation of E-Cad at the apical cell junction. Could the authors demonstrate that the increase in E-cadherin is indeed due to more recycling to the apical domain?

1st Revision - authors' response 06 October 2012

Point-by-point response:

Referee #1

We thank the reviewers for their helpful comments and suggestions for the improvement of our manuscript. We respond to the criticisms and suggestions in italicized text below:

In this paper, the authors report that VhaPRR, which the authors and others have previously linked to the V-ATPase, interacts with the protocadherin Fmi through its extracellular domain and co-localizes with PCP proteins during all pupal wing stages. They also report that this localization depends on intact PCP domains, and vice-versa, suggesting that VhaPRR is itself a PCP core protein. Finally, they report that VhaPRR also plays a role in acidification and endosomal transport, and conclude that VhaPRR is a key factor in epithelial morphogenesis.

I find the paper potentially interesting and I have only a few comments on the possible role of VhaPRR in acidification and traffic - it seems to me that this role remains somewhat obscure. It is remarkable that, except acidic vesicles, all markers related to endo-lysosomes that have been tested are increased in the VhaPRR mutant cells (Lamp1, Armadillo, Notch, endocytosed E-cad), including Rab5, Rab 11 and Rab7. This may support the view that VhaPRR mutant cells exhibit an acidification defect, as proposed by the authors. However, beyond the difference in the distribution of apical vesicles, the lysotracker staining intensity of individual vesicles whether wt/mutant or apical/basal looks very similar (Fig 6). This argues against a defect in the acidification properties, at least at the level of lysotracker detection.

We are happy to see that reviewer #1 finds our paper interesting. We have now more precisely addressed the role of VhaPRR in acidification and traffic, including a detailed comparison of VhaPRR *clones with clones mutant for another V-ATPase, Vha68-2, and clones mutant for PCP proteins. These new results can be found in Figure 8 and Supplementary Figure S7. They are also summarized in the schematic model of Figure 9.*
We find that Vha68-2 *clones do not show the same degree of PCP phenotypes as* VhaPRR *clones, but they do share the trafficking defects (see below). There is also a decrease in Lysotracker uptake, but unlike in* VhaPRR *clones Lysotracker is reduced in both apical and basal portions of the cell. Thus, the removal of this subunit has more general effects on V-ATPase activity and organellar acidification. This may also explain why these clones are less viable than VhaPRR clone. It also suggests that VhaPRR regulates a specific V-ATPase subset, e.g. in early endosomes.*
Our experiments were performed by applying the Lysotracker probe to live pupal wings. Lysotracker is an acidotrophic dye that accumulates in compartments with low pH due to its weakly basic nature. This allows an evaluation of the size and number of acidic compartments. However, it is not possible to assess the degree of acidification based on Lysotracker intensity. Lysosensor is better for pH-dependent fluorescence. However, we were not able to obtain satisfying signals with this probe.

Do Rab proteins also accumulate in V-ATPase mutants?

Thank you for suggesting this experiment. We have stained Vha68-2 *clones for Rab5, Rab7 and Rab11 (new Supplementary Figure S7). As for VhaPRR, we see increases in all of these proteins. These results support our view that VhaPRR performs overlapping functions with the V-ATPase in the endolysosomal pathway.*

Also, is the pH of endo-lysosomes or Lamp1 degradation affected after Fmi or Fz disruption?

We did not see any change in the Lysotracker pattern in Fmi *RNAi and* fz^{P21} *clones (Figure 8J and Supplementary Figure S7D). LAMP1 degradation was assessed by expressing Fmi RNAi with dpp-GAL4 in a tub-GFP-LAMP1 background (Supplementary Figure S7G). Also here, no effects on LAMP1 localization pattern was detected. As VhaPRR is strongly reduced in cells with disrupted Fmi or Fz, it can therefore be concluded that the PCP core proteins (particularly Fmi) control the junctional but not the membrane trafficking pool of VhaPRR.*

Also, in VhaPRR mutant clones, there is little difference in the basal lysotracker staining, but the number of apical vesicles stained with the dye is reduced. Is the cell shape changed in the mutant? This is not easy to see in the x-y projection in Fig 6D, because nuclei are not in the focal plane of mutant cells. Is the number of endosomes and lysosomes reduced in the apical portion of the cell? Conversely, do authors know whether the basal, Lamp1-positive vesicles, which accumulate in the mutant, are acidic?

As GFP-LAMP1 accumulates in vesicular compartments in apical and basal parts of the cell and Rab proteins, particularly Rab11, localizes to apical compartments, we do not think that there is a general reduction of apical compartments. In some clones there is a slight misorganization of the epithelium, which may be due to compromised cell viability. We have replaced the images in Figure 6 with a proper x-z projection (as opposed to wing margin cells; inset in Figure 6B') that show normal nuclear organization inside the clones. We also included co-labeling with E-Cadherin to show outlines of cells accumulating LAMP1 (Figure 6E'').
As requested, we also performed the combined Lamp1 visualization and Lysotracker uptake. The results suggest that LAMP1 does accumulate in apical compartments. These compartments are smaller compared to basal LAMP1-positive compartments and are mostly negative for the Lysotracker probe. The overlap of LAMP1 and Lysotracker is stronger in basal compartments, suggesting a specific defect in acidification of apical compartments (now in included in Figure 6 as panel F and G).

Referee #2

The Hermle et al manuscript has 2 parts. The first one is an extension of the characterization of the role VhaPRR in the regulation of PCP (Hermle et al., 2010, Buechling et al., 2010 and Cruciat et al., 2010). The novel findings include 1) VhaPRR affects the trafficking of Fmi; 2) VhaPRR is planar polarized; 3) VhaPRR can form a complex with Fmi. In a second part the authors analyse the role of VhaPRR in vesicular trafficking. They show that the loss of VhaPRR function perturbs vesicle acidification as well as Notch and E-Cadherin trafficking. Although the authors' findings suggest a role of VhaPRR in Fmi trafficking, the connection between the function of VhaPRR in vesicular trafficking and its role in PCP is not explored.

We have now significantly expanded our studies on the connection of the trafficking function of VhaPRR and its role in PCP. An important milestone was the successful generation of somewhat viable V-ATPase loss-of-function clones in the pupal wing using the Vha68-2 *allele and an altered heat-shock protocol (Vaccari et al, 2010). As stated in the first version of the manuscript and in Hermle et al, 2010, this has proved difficult in the past due to cytotoxic effects caused by impaired V-ATPase activity. Mutant clones or RNAi expression domains are mostly viable only for a short time before they are eliminated.*

In Vha68-2 *clones we observed similar trafficking defects as in* VhaPRR *clones (Lysotracker reduction, Rab protein increase, E-Cadherin increase), but PCP protein localization and wing hair orientation is mostly unaffected. To compare the localization of the V-ATPase with VhaPRR, we have also used a Vha55-GFP trap line. Vha55-GFP can be used to assess endogenous Vha55 localization (and function), because we demonstrate that the GFP signal can be eliminated by expression of Vha55 RNAi (Figure 8I). As with other tools we have used (Vha44 antibody (Figure S4B) as well as other GFP trap lines and antibodies (not shown)), we failed to detect a PCP-like localization pattern. Moreover, as opposed to VhaPRR, Vha55-GFP is not stabilized by Fmi overexpression (Supplementary Figure 4E).*
We have also used several genetic approaches (fz^{P21} *and* fmi^{E59} *(not shown) as well as* Fmi *RNAi clones) to study the role of the PCP pathway in membrane trafficking. Our results suggest that our trafficking readouts (Lysotracker, Notch, E-Cadherin and LAMP1) are largely unaffected in clones with disrupted PCP signaling.*
Taken together, we now have more evidence to suggest that VhaPRR performs two functions, one in PCP (Fmi-independent, not V-ATPase dependent) and one in trafficking (not Fmi-dependent, V-ATPase dependent). Most of the new data is presented in Figure 8 and Supplementary Figure 7. They are also summarized in the schematic model of Figure 9.

It also remains unclear why the loss of function of VhaPRR drastically perturbs Stbm and Fz localizations, while it only mildly affects Fmi localization.

See Point 1.

Furthermore, the following points should be addressed:

1. The loss of VhaPRR drastically affects Fz and Stbm localization, whereas it has a milder effect on the one of Fmi. Is the trafficking of Fz and/or Stbm also affected by the loss of VhaPRR function? Do Fmi, Fz or Stbm colocalize in intercellular (intracellular?) structures?

Thanks for pointing this out. We are happy to clarify. A major feature of PCP proteins is that they show a strong and rather complicated relation to each other. Generally, the contribution of each individual PCP factor for formation of and signaling from the PCP complex is very poorly understood. While Fz is a signaling receptor that can recruit cytosolic components such as Dsh, Fmi is a protocadherin with homophilic binding activity. Fmi is reduced at apical junctions in fz *clones and vice versa. However, Fmi is reduced by Fz overexpression, while Fz is stabilized by Fmi overexpression (our data and Strutt et al, Curr Biol 2008).*
We see a stronger reduction in Fz than in Fmi stability within PCP domains upon VhaPRR removal. However, at this point we cannot say whether this effect is direct or indirect (e.g. due to the slight Fmi instability). Fmi might also be more stable due to its homophilic trans-binding activity. Future work is needed to understand the order of events during formation and disassembly of PCP domains and also to elucidate the precise contribution of VhaPRR to these processes. Unfortunately, this is where the pupal wing faces technical limitations. It would require multi-colour imaging of live pupal wing tissue with high temporal and spatial resolution, preferrably including signaling or FRET sensors for protein-protein interaction, but this has not been performed yet and is also beyond the scope of this paper.
Nonetheless, we have used a Fmi antibody uptake assay in prepupal wings to demonstrate reduced Fmi stability at the cell surface (Figure 1). This technique has helped us to uncover Fmi effects that were not readily visible in fixed tissue. Unfortunately, we are not able to perform a Fz or Stbm uptake experiment, because there are no suitable antibodies against their extracellular domains required for this kind of studies. Instead, we tested a number of organellar markers, but they failed to show any significant overlaps with Fz, Stbm or Fmi. The fate of these proteins in VhaPRR *clones is therefore currently unclear. We have rephrased the discussion on p.15 to clarify this issue.*
As requested, we have now also performed co-immunostainings for Fz and Stbm in VhaPRR mutant clones. Both proteins co-localize in the residual PCP domains but also partially in intracellular structures. The images for this result are now included in Figure 1. We also found a partial co-localization of Fz-GFP and Fmi in residual PCP domains and intracellular structures (of note, it was not possible to use the Fz antibody together with the Fmi antibody because of overlapping antibody species for the clonal marker b-gal and Fz). We decided not to include this experiment in the paper due to space limitations. But we are happy to provide the data if needed.

2. Upon Fmi antibody incubation at 4°C, the amount of Fmi is lower at the apical AJs. The authors therefore need to quantify the reduction of Fmi membrane localization upon chase at 25°C to demonstrate that there is indeed a stronger reduction of Fmi at VhaPRR apical junction. A better characterization of the dynamics of Fmi internalization might be relevant (see Strutt 2011).

Thanks for this suggestion. We have now performed a quantification of the Fmi uptake experiment, which shows that there is a small, but significant increase of Fmi uptake inside the mutant clone compared to the neighboring wild-type tissue (see Figure 1I).

3. The authors state that VhaPRR is localized both at the distal and proximal cell junction. However, VhaPRR is absent from many proximal or distal junctions facing the mutant clones in figure 2D. A far better experiment would be to use a GFP::VhaPRR construct to demonstrate that VhaPRR localizes both at the distal and proximal junctions.

As we have shown in Supplementary Figure S3C, the overexpression of VhaPRR fails to localize to PCP domains. We have tried many different constructs (untagged and tagged) as well as many different drivers (including flp-out clones) but without success. As pointed out in the discussion, endogenous PCP domains may have saturating binding sites for VhaPRR blocking access of exogenous protein. Alternatively, there may be missing post-translational modifications in the exogenous protein required for recruitment to PCP domains.
The absence and presence of VhaPRR from both proximal and distal junctions may be explained by the uneven recruitment of sPRR from the neighbouring tissue. Even at the stage shown here (28hrs APF), the reappearance of VhaPRR (described in Figure 4) is possible. Our experience with this experiment is that not all residual PCP domains have the capacity to bind sPRR, particularly at earlier stages. It is also possible that sPRR can help to stabilize or re-form PCP domains in a non-autonomous manner, making the proximal/distal localization issue even harder to explore. This is supported by our observation that PCP phenotypes decline with later stages (Figure 1B and Supplementary Figure S1C).

In figure 7B, it is unclear how the antibody can access the extracellular E-Cad in the absence of detergent since a cuticle is likely present on the apical side of the wing pupal cells at 32APF.

A cuticle is indeed present at this stage of pupal wing development. However, during dissection we physically remove it, so it does not prevent antibody binding to E-Cadherin on the cell surface. This can only be done with fixed samples. Therefore, the antibody uptake assay is not possible at this stage.

4. It is unclear how the role of VhaPRR in lysosomal degradation might lead to an accumulation of E-Cad at the apical cell junction. Could the authors demonstrate that the increase in E-cadherin is indeed due to more recycling to the apical domain?

In our antibody uptake assay, we see an increase in intracellular E-Cadherin upon chasing with the antibody, so endocytic uptake does not seem to be affected. However, at the same time there is an enhanced signal at the apical junctions. Therefore, enhanced recycling is to us the most likely explanation. E-Cadherin has been shown to recycle via the Rab11-dependent pathway in Drosophila *epithelial cells (Langevin et al, 2005). Thus, we co-expressed a dominant-negative form of Rab11, Rab11SN, with* VhaPRR *RNAi. The results, indeed, show a suppression of the E-Cadherin increase at apical junctions (new Supplementary Figure S5). We also detected a strong increase of Rab11 staining in apical compartment upon VhaPRR elimination, which may reflect an activated recycling pathway. A positive correlation between junctional E-Cadherin and apical Rab11 is supported by our Vha68-2 experiments (Figure 8R and Supplementary Figure S7A). Nevertheless, this remains indirect evidence for an inhibitory role of VhaPRR in recycling. Also here, there are technical limitations when using pupal wing because the recycled pool cannot readily be distinguished from the non-internalized pool. To yield more insight, one would have to resort to an experimental system that allows specific recycling and transcytosis assays etc (such as filter-grown MDCK cells).*

