PxdA interacts with the DipA phosphatase to regulate endosomal hitchhiking of peroxisomes

John Salogiannis, Jenna Christensen, Lívia Songster, Adriana Aguilar-Maldonado, Nandini Shukla, and Samara Reck-Peterson

Corresponding author(s): Samara Reck-Peterson, University of California San Diego

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|------------------|------------|
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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 Dr. Reck-Petersen,

Your manuscript has been evaluated by two experts in the field. They find your data interesting, and in general the experiments well executed. However, they also raised some points, which in my view are easy to deal with. One major point deals with the localisation of DipA in the PxdAR2044P mutant and the identity of the structures on which DipA accumulated in pxd mutants.

If you think you can address the reviewers’ points, I am happy to handle a revised version of your manuscript.

Sincerely yours,

Anne Spang
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Reck-Peterson,

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.

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).

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To submit the rebuttal letter, revised manuscript, and figures, use this link: Link Not Available

Please contact us with any questions at mboc@ascb.org.

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
mbc@ascb.org

Reviewer #1 (Remarks to the Author):
Summary: Salogiannis et al examine the roles of PxdA and the DipA phosphatase in the regulation of peroxisome hitchhiking on endosomes. Overall, the paper is tidy, well written and makes significant advances, particularly in identifying DipA as a PxDA interactant. The authors also examine other classes of organelles and find that in Aspergillus, lipid droplets, mitochondria and autophagosomes do not hitchhike on endosomes. Thus, Aspergillus and Ustillago appear to differ significantly in their use of endosomal hitchhiking. Please find a few suggestions for improvement below.

Major points:

1. Figure 3C. The PxdAR2044P-mKate appears to be accumulating at the hyphal tip. Is it accumulating on peroxisomes or attaining this localization through some other means? Since this mutation is in a region known to be associated with endosome association, is the protein accumulating on peroxisomes through another domain? Showing the split channels and commenting on the localization would be helpful.

2. Figure 5D-F. DipA is dependent on PxdA for endosome localization. The diffused foci that are being followed in the pxdA mutant are unlikely to be endosomes. What do the authors believe these correspond to?

3. Figure 5D-F. DipA clearly depends on PxdA for endosome association. What happens to DipA localization in the PxdAR2044P variant? This point relates to point #1.

4. How do DipA and PxdA loss-of-function phenotypes compare? DipA loss-of-function results in a variety of defects related to growth and morphogenesis. If PxdA phenocopies DipA then its tethering role would appear to be essential to DipA function. By contrast, if PxdA displays weaker or a subset of PxdA phenotypes, then this interaction would account for a subset of DipA’s regulatory roles. Commenting on this would help place the findings in a broader biological context.

5. Based on this work it appears that Ustillago and Aspergillus employ endosomal hitchhiking for fundamentally different reasons. In Ustillago many species of cargo hitchhike with a general function that appears akin to mixing by cytoplasmic streaming. By contrast, in Aspergillus the phenomenon seems restricted to Peroxisomes. Woronin body biogenesis is a major peroxisomal activity in the apical hyphal compartment, and this may comprise a case where regulated cytoskeletal transport and Woronin body maturation are intimately linked. A brief mention of the known physiological differences between Ustillago and Aspergillus would help to round-out the discussion concerning the general biology of hitchhiking.

Minor points:

1. Figure 2A. It was hard to find what the full-length names for the domains. UR1 and UR2 are not defined in the paper. “Unstructured Region”? Insertion of a legend into the figure would be helpful.

2. Figure 2C-E. The symbols for the statistics results don’t seem to be on the bar graphs (*p <0.05, ****p< 0.0001 (Krustal-Wallis test with Dunn’s multiple comparisons test compared to WT strain)).

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4. Page 7. “and/ or or“ Delete one of the ors.

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Reviewer #2 (Remarks to the Author):

The MS by Slogiannis et al is a succinct, well-executed paper that identifies one more component of the adaptor linking peroxisomes to early endosomes in the filamentous fungal model Aspergillus nidulans. DipA is a protein phosphatase that might have a catalytic role or behave as a moonlighting protein that in its ‘second life’ serves as a scaffold of a protein complex that contains PdxA and links peroxisomes to EEs. The authors further demonstrate that lipid droplets move independently of EEs and describe a novel pdxA missense allele that uncouples peroxisomes from EEs and that will be very useful for future investigations. I would be happy to recommend acceptance once the authors have dealt with a few minor points listed below.

Comments

The loss of image quality during pdf conversion makes difficult to judge the quality of some of the display items, in particular the color combinations in Figure 3C, the kymos in Fig4E and the negative control in Figure 5E. Please pay special attention to these items.
Please add page numbering for resubmission, as their absence makes rather cumbersome to refer to specific paragraphs in the text.

Introduction

plus ends oriented outward: this term is misleading, as it would include apical or basipetal domains. I suggest the term 'tip-wards' used by Ron Morris a few decades ago.

uncA and uncB: the right reference is not Peñalva, 2012 please cite Zekert and Fischer, 2009, MBoC 20: 673

Last paragraph intro: 'Surprisingly', why was it surprising?

Results:

First paragraph of results: important: GFP-Atg8 does not label autophagosomes in the standard nutrient-replenished medium used here; it labels pre-autophagosomes (PASs), which appear as cytosolic puncta. Please clarify. The authors might consider citing Autophagy 2013 9(7): 1024-43 in this particular regard.

Late endosomes hitchhiking on late endosomes in Ustilago, please clarify this sentence

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Discussion

Autophagosome distribution ... not affected. Please consider my previous comment, PAS distribution?

M&Ms

title genome FGSC_A4 please give a web site reference

Due to the weak ...colocalized with DipA’ This whole sentence might be better placed in the corresponding results subheading

A. nidulans Uniprot database please give reference
We thank the reviewers for their time spent reviewing our manuscript. As described in detail below, we have addressed every reviewer comment, and the reviews and corresponding edits have improved the manuscript. Of note, a graduate student in the Reck-Peterson lab, Livia Songster, performed many of the revisions and has been added as second author on the revised manuscript.

Reviewer #1 (Remarks to the Author):

**Summary:** Salogiannis et al examine the roles of PxdA and the DipA phosphatase in the regulation of peroxisome hitchhiking on endosomes. Overall, the paper is tidy, well written and makes significant advances, particularly in identifying DipA as a PxDA interactor. The authors also examine other classes of organelles and find that in Aspergillus, lipid droplets, mitochondria and autophagosomes do not hitchhike on endosomes. Thus, Aspergillus and Ustilago appear to differ significantly in their use of endosomal hitchhiking. Please find a few suggestions for improvement below.

**Major points:**

1. **Figure 3C.** The PxdAR2044P-mKate appears to be accumulating at the hyphal tip. Is it accumulating on peroxisomes or attaining this localization through some other means? Since this mutation is in a region known to be associated with endosome association, is the protein accumulating on peroxisomes through another domain? Showing the split channels and commenting on the localization would be helpful.

   We now provide the separate channel images of PxdA(WT)/PxdA(R2044P)-mKate and GFP-RabA/5a-labeled early endosomes in Figure 3C and D. The PxdA(R2044P) signal is predominantly diffuse, with a few motile puncta still associating with early endosomes (Figure 3B,D). As the signal is not distinct, it is difficult to interpret the exact nature of the diffuse population. However, we do not believe that PxdA is directly associating with peroxisomes nor that a peroxisome binding site is “uncovered” after removing PxdA’s endosomal association, since our endosomal binding mutants failed to colocalize with peroxisomes in our previous study (Salogiannis et al., 2016 JCB).

2. **Figure 5D-F.** DipA is dependent on PxdA for endosome localization. The diffused foci that are being followed in the pxdA mutant are unlikely to be endosomes. What do the authors believe these correspond to?

   We see that the vast majority of DipA no longer forms foci that associate with moving endosomes when PxdA is depleted. As for the weak signal that persists, it may correspond to the DenA-associated puncta previously characterized (Schenke et al., 2016). One other possibility is that a very small fraction of DipA-GFP bypasses PxdA and can associate with EEs in a PxdA-independent manner. This latter point is supported by data demonstrating that a small fraction of DipA puncta accumulating at the hyphal tip in a HookA deletion do not colocalize with PxdA (revised Supplementary Figure S4). Future work on DipA may elucidate the nature of these diffuse foci.

3. **Figure 5D-F.** DipA clearly depends on PxdA for endosome association. What happens to DipA localization in the PxdAR2044P variant? This point relates to point #1.

   We performed the suggested experiment and now show that there are fewer moving DipA foci (~50% reduction) in a strain in which the PxdA(R2044P) mutant has replaced the endogenous PxdA (Supplementary Figure S4E and S4F). This data further supports our finding that DipA depends on PxdA for association with endosomes.
4. How do DipA and PxdA loss-of-function phenotypes compare? DipA loss-of-function results in a variety of defects related to growth and morphogenesis. If PxdA phenocopies DipA then its tethering role would appear to be essential to DipA function. By contrast, if PxdA displays weaker or a subset of PxdA phenotypes, then this interaction would account for a subset of DipA's regulatory roles. Commenting on this would help place the findings in a broader biological context.

As demonstrated by dipAΔ colony morphology (Supplementary Figure S4A), DipA loss-of-function results in a growth defect. On the other hand, PxdA has only a very subtle growth defect (Salogiannis et al. 2016). Therefore, it is likely that PxdA-dependent localization represents only a subset of DipA’s functionality. In congruence with this, DipA is a phosphatase and likely has many downstream targets (Schinke et al. 2016) and functions that are independent of its interaction with PxdA. For example, DipA loss-of-function also affects septal compartmentalization (Schinke et al. 2016).

5. Based on this work it appears that Ustilago and Aspergillus employ endosomal hitchhiking for fundamentally different reasons. In Ustilago many species of cargo hitchhike with a general function that appears akin to mixing by cytoplasmic streaming. By contrast, in Aspergillus the phenomenon seems restricted to Peroxisomes. Woronin body biogenesis is a major peroxisomal activity in the apical hyphal compartment, and this may comprise a case where regulated cytoskeletal transport and Woronin body maturation are intimately linked. A brief mention of the known physiological differences between Ustilago and Aspergillus would help to round-out the discussion concerning the general biology of hitchhiking.

We thank the reviewer for this insightful comment. Indeed, we have also noted these differences and are currently exploring whether Woronin body function/maturation is linked to peroxisome hitchhiking, and/or whether Woronin bodies themselves also hitchhike. We have included a brief mention of the differences in cargo transport in Aspergillus and Ustilago in the discussion as suggested. On lines 291-298: “Furthermore, while multiple cargos have been demonstrated to hitchhike in U. maydis (Guimaraes et al. 2015, Baumann et al., 2012, 2014; Higuchi et al., 2014), thus far, we have identified peroxisomes as the only hitchhiking cargo in A. nidulans. Peroxisomes in A. nidulans can mature into Woronin bodies, organelles crucial for septal pore blocking in A. nidulans and other filamentous Ascomycota species (Markham and Collinge, 1987; Jedd and Chua, 2000; Steinberg et al. 2017). One possibility is that peroxisome-specific hitchhiking promotes the even distribution of peroxisomes along the hyphae, allowing proper septal pore blocking.”

Minor points:

1. Figure 2A. It was hard to find what the full-length names for the domains. UR1 and UR2 are not defined in the paper. "Unstructured Region”? Insertion of a legend into the figure would be helpful.

   Thank you for catching this. We updated the Figure 2 legend to include the definition of UR as “uncharacterized region”.

2. Figure 2C-E. The symbols for the statistics results don't seem to be on the bar graphs (*p <0.05, ****p< 0.0001 (Krustal-Wallis test with Dunn’s multiple comparisons test compared to WT strain).

   We updated Figure 2 to reflect this.
3. Figure 5E. For those readers unaccustomed to looking at Kymographs, it would be good to consistently label the time and distance axes.

   We added arrows indicating time and distance in both Figure 5B and 5E.

4. Page 7. "and/ or or" Delete one of the ors.

   Thank you for catching this.

5. The Discussion would be easier to follow if relevant figures were called out when the results were mentioned, especially in the paragraph on "Lipid droplets do not hitchhike on early endosomes in Aspergillus" where LD velocities are compared.

   Thank you for the suggestion. We updated the manuscript to include references to relevant figures in the Discussion section.

Reviewer #2 (Remarks to the Author):

The MS by Slogiannis et al is a succinct, well-executed paper that identifies one more component of the adaptor linking peroxisomes to early endosomes in the filamentous fungal model Aspergillus nidulans. DipA is a protein phosphatase that might have a catalytic role or behave as a moonlighting protein that in its 'second life' serves as a scaffold of a protein complex that contains PdxA and links peroxisomes to EEs. The authors further demonstrate that lipid droplets move independently of EEs and describe a novel pdxA missense allele that uncouples peroxisomes from EEs and that will be very useful for future investigations. I would be happy to recommend acceptance once the authors have dealt with a few minor points listed below.

Comments
The loss of image quality during pdf conversion makes difficult to judge the quality of some of the display items, in particular the color combinations in Figure 3C, the kymos in Fig4E and the negative control in Figure 5E. Please pay special attention to these items.

   Thank you for the comment. We have added separate single-channel image panels for the two-channel experiments in Figure 3C and D and have made sure that the pdfs are of high quality.

Please add page numbering for resubmission, as their absence makes rather cumbersome to refer to specific paragraphs in the text

   We apologize for this oversight. We have included line numbers in the resubmission and reference those line numbers in this response.

Introduction
plus ends oriented outward: this term is misleading, as it would include apical or basipetal domains. I suggest the term 'tip-wards' used by Ron Morris a few decades ago.

   We updated this term on line 69.

uncA and uncB: the right reference is not Peñalva, 2012 please cite Zekert and Fischer, 2009, MBoC 20: 673
Thank you for the suggestion. We added Zekert and Fischer 2009.

Last paragraph intro: ‘Surprisingly’, why was it surprising?

We found this surprising because HookA is required for lipid droplet movement in *Ustilago maydis*. For clarity, we changed the sentence on lines 119-120 to read: “In contrast to *U. maydis*, we found that the movement and distribution of lipid droplets was also independent of HookA.”

Results:
First paragraph of results: important: GFP-Atg8 does not label autophagosomes in the standard nutrient-replenished medium used here; it labels pre-autophagosomes (PASs), which appear as cytosolic puncta. Please clarify. The authors might consider citing Autophagy 2013 9(7): 1024-43 in this particular regard.

We apologize for this oversight and changed the nomenclature to reflect the true labeled GFP-Atg8 vesicle population as “pre-autophagosomes” and cited Pinar et al., 2013.

Late endosomes hitchhiking on late endosomes in Ustilago, please clarify this sentence

We are not quite sure what the reviewer means by this comment, but we removed the comparison between pre-autophagosomes and late endosomes to avoid confusion. The sentence on line 141-142 now reads: “Our findings that proper distribution of mitochondria does not require PxdA is consistent with the lack of EE-mediated hitchhiking of mitochondria observed in *U. maydis*.”

Heading 'Identification' ... 'to further understand' please correct this split infinitive

We changed this “to understand further” on line 158.

- Heading 'PdxA interacts...' dipAΔ exhibits perturbed colony growth >>> dipAΔ impairs colony growth (or just growth?)

We made this change on line 213.

highly motile puncta: please spell out, 2.5 um/sec according to (Fig 4B)

We made this textual change on line 216-217.

EEs accumulate near the hyphal tip 'displays a similar accumulation' (Fig 4). Perhaps accumulation is not the best word, aggregation or coalescence might do a better job.

We believe accumulation is the correct terminology here, particularly since this terminology is now standard in the field and has been previously used by our lab (Tan et al., 2014; Salogiannis et al., 2016), the Steinberg lab (Bielska et al., 2014; Guimereas et al., 2015) and the Xiang lab (Zhang et al., 2014) for the same phenomenon.

Perhaps the authors might wish to include a double label of EEs and DipA in hookAΔ, if available?

We agree that this is an important experiment, and we now include a micrograph demonstrating that the majority of PxdA-marked EEs colocalize with DipA in a hookAΔ background (Supplementary Figure S4).
- imaged the dynamics >> studied the dynamics
  Thank you. We made this change on line 231.

we find that ... >> we found that (twice in the same paragraph)
  We updated the manuscript to reflect this change.

Fig 4A from this and genetic data it seems pretty clear that DipA interacts with PdxA, but I wonder if the authors might include a 'canonical IP' experiment in which epitope tagged DipA is detected by western blotting of the PdxA IPs.

  We agree that this is a great way to validate our "hit" from the mass spec experiments. We now include a co-immunoprecipitation experiment followed by Western Blotting demonstrating that DipA-GFP is enriched in a PdxA-HA pull-down compared to a non-HA tagged strain (Supplementary Figure S4C).

Discussion
Autophagosome distribution ... not affected. Please consider my previous comment, PAS distribution?

We changed the language when discussing GTP-Atg8 puncta to “pre-autophagosomes” as stated above.

M&Ms
reference genome FGSC_A4 please give a web site reference
  We updated this on lines 452 and reference AspGD.

Due to the weak ...colocalized with DipA' This whole sentence might be better placed in the corresponding results subheading
  Thank you for that suggestion. We migrated this sentence into the Results section on lines 220-221 when discussing the DipA/PxdA colocalization.

A. nidulans Uniprot database please give reference
  We updated this on lines 602-603.
Dear Dr. Reck-Peterson, dear Sam,

I read your revised manuscript and am happy to report that I think that you addressed all the reviewers comments and concerns satisfactorily. Therefore, I accept your manuscript for publication in Molecular Biology of the Cell.

Thank you for the submission of this interesting piece of work to MBoC.

Best wishes,
Anne Spang

Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Reck-Peterson:

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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