Journal of Cell Science

Vacuolar H^+-ATPase dysfunction rescues intralumenal vesicle cargo sorting in yeast lacking PI(3,5)P2 or Doa4

Zachary N Wilson, Dalton Buysse, Matthew West, Daniel Ahrens and Greg Odorizzi
DOI: 10.1242/jcs.258459

Editor: Mahak Sharma

Review timeline
Original submission: 25 January 2021
Editorial decision: 23 February 2021
First revision received: 14 June 2021
Accepted: 25 June 2021

Original submission

First decision letter

MS ID#: JOCES/2021/258459

MS TITLE: V-ATPase dysfunction rescues ILV cargo sorting in yeast lacking PI(3,5)P2 or Doa4

AUTHORS: Zachary N Wilson, Dalton Buysse, Matthew West, and Greg Odorizzi
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

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

As you will see, the reviewers found the study interesting and well organized and they also appreciated that experiments were well controlled and carefully quantified. From the reviewers' comments, you will see that there are few important points that will require additional experiments and changes to the manuscript text. In particular, both reviewers 1 and 2 find that more experiments are required to explain, "how loss of acidification could compensate for Doa4 loss"¤. Further reviewer 1 has raised concern about "how slowing endosome-vacuole fusion compensates for such a specific targeting defect"¤ of Cps1 cargo. Additional experiments and/or textual changes to address this concern are recommended. Reviewers 2 and 3 also suggest to test whether deletion of Ypt7 and or Vps41 rescues the Fab1 (or Doa4) defects. Reviewer 2 has also questioned about the morphological differences in vacuole of different mutants (pt #5) as well as about the use of LUCID approach in Vph1 deletion mutant (pt #6).

If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this manuscript, the authors describe a set of unexpected genetic interactions between mutations in two different processes (PI(3,5)P2 formation and the Doa4-mediated deubiquitination) implicated in MVB targeting and mutations that disrupt vacuolar (and presumably endosomal) acidification. It was shown previously that biosynthetic sorting of some cargoes such as carboxypeptidase S (CPS) to the vacuolar lumen requires sorting into ILVs, and when this sorting is disrupted by mutations such as fab1Δ, doa4Δ, or multiple ESCRT protein mutations, CPS ends up on the vacuolar membrane (Odorizzi et al, 1998). However, in this work, the authors find that combining fab1Δ or doa4Δ mutations with a mutation that abolishes vacuolar acidification restores CPS sorting to the vacuolar lumen. The authors suggest that ILV formation and fusion with the vacuole may be coordinated.
Thus, in the absence of PI(3,5)P2 or failed ubiquitin removal from CPS destined for the ILV, fusion with the vacuole occurs without ILV insertion resulting in CPS mistargeting. However, if endosome-vacuole fusion is slowed, CPS targeting is restored. The results are potentially interesting and the experiments overall well-done, but the underlying mechanism remains unclear. The paper would be strengthened and would be of more general interest if some of the gaps in the model could be filled.

Comments for the author

The authors show that the vph1Δ mutant has more and larger MVBs than wild-type cells, and from this, along with previous evidence of defective homotypic vacuole fusion in the mutant, conclude that endosome-vacuole fusion is slowed. Further supporting this conclusion, recruitment of Ypt7 and HOPS subunit Vps41 to the vacuolar membrane are reduced, though not eliminated, in the vph1Δ mutant.

However, other cargoes targeted to the MVB are unaffected in a fab1Δ mutant and ILV number is also normal, so it appears there is a rather specific defect in CPS targeting defect in this mutant. It is not clear how slowing endosome-vacuole fusion would compensate for such a specific targeting defect, and this needs to be discussed. Similarly, it is not clear how loss of acidification could compensate for Doa4 loss, and the authors find no evidence of a direct connection between Fab1 kinase and Doa4 activity other than the suppression of both mutations by vph1Δ. While it may be difficult to fully establish these connections in CPS sorting, more effort should be made to try to explain them experimentally, or at least in a well-justified final model.

Minor points to be addressed:
1. More detail about how the percentage of missorted GFP-Cps1 is calculated for Figure 1B, 2C, and 5B should be included. Some indication of the percentage of cells showing GFP-Cps1 at the vacuolar membrane in Figures 1 and 2.

2. In proximal tubule, recruitment of small GTPase Arf6 to the endosomal membrane responds to luminal acidification (Marshansky et al. (2006) Nature Cell Biology 8:124). Could something like this account for the failure of Ypt7 recruitment in the vph1Δ mutant?

Reviewer 2

Advance summary and potential significance to field

This paper by Wilson et al. investigates the molecular mechanisms by which deletion of subunits of the late endosomal/vacuolar V-ATPase is able to rescue the temperature-sensitive growth defects of yeast cells with defective Fab1 kinase function, an earlier finding of the same authors. In this study, the authors find that the vacuolar degradation of carboxypeptidase 1 (Cps1), a ubiquitin-dependent biosynthetic cargo, which is grossly impaired in cells deleted in fab1 (fab1delta), is restored when the Vph1 subunit of the V-ATPase is simultaneously deleted. The authors also show that the sorting defect of Cps1 in cells defective in the ubiquitin hydrolase, Doa4, is also alleviated by simultaneous deletion of Vph1, although in the latter case the growth defect is not corrected. Using TEM analysis, the authors find no major changes in the number of MVBs or in the number of their intralumenal vesicles (ILVs) in Fab1delta cells and only a slight increase in these numbers in the double fab1/vph1 deleted cells. Lastly, the authors find that Ypt7 and one of its effectors, Vps41, decrease their vacuolar localization in vph1-deleted cells. Several other control experiments have been done to corroborate these findings. The authors conclude that Vph1 deletion impedes MVB-Vacuole fusion and most likely through this mechanism it rescues the Cps1 sorting defect caused by Fab1 deletion. They suggest that their findings indicate a co-ordination between MVB-vacuole fusion with the generation and maturation of ubiquitinated biosynthetic cargoes.

These are potentially interesting studies that deal with the important topic of PI(3,5)P2 regulated step(s) in the vacuolar degradative pathway and its connection to ILV formation and MVB fusion with the vacuoles. The study is well done, with clear results and presentation yielding important new insights into the process.

Comments for the author

My main criticism is the preliminary feel for the Doa4 aspect of the study and some inconsistencies between the data and their interpretation. Most of my comments can be addressed by textual changes, although some extra experiments sure would significantly strengthen the study.

1. The connection between the Doa4 deletion and Vph1 is much less characterized compared to that of Fab1 and Vph1. It would be important to know what the TEM findings and statistics are with Doa4 deletion. There appear to be major differences between the fab1 and Doa4 defects, most strikingly the lack of enlargements of the vacuoles in the Doa4 deleted cells. One would assume that the biggest effect of Doa4 deletion would be on the sorting of Cps1 into ILVs at the level of the MVB. This is also suggested by the largest increase in the LUCID assay (Fig. 4D). How would that be corrected by the impaired MVB-Vacuole fusion caused by Vph1 deletion?

2. The simplest explanation for the rescuing effect of Vph1 deletion in the Fab1 deletion case is that by limiting the fusion between the MVBs and the vacuole, there is more time for the sorting of the ubiquitinated Cps1 cargo into the ILVs that appears to be defective but may be not incomplete in the Fab1 deleted cells. If this is the case, it may not indicate an active level of integration between these steps in the normal cell that would require some sort of signaling coordination between these steps.

3. I think it would be important to discuss in more detail the fact that ILVs are still generated in the MVBs in Fab1 deleted cells and only the sorting of the ubiquitinated biosynthetic cargoes into those ILVs is affected by the lack of PI(3,5P2. I am not certain that this nuance has received enough attention in the literature, and it is important to understand for the conclusion of this study. If there is a molecular explanation for this distinction, that would be important to cite.

4. If loss of Ypt7 or its HOPS effector, Vps41from the vacuole is responsible for the fusion defect and this is a major reason for the rescue, would Ypt1 or Vps41 deletion also rescue the Fab1 (or Doa4) defect?

5. There are striking differences between the wild-type and mutants in the electron densities of their vacuoles. Wild-type is dark, all mutants are light. Is this a known phenomenon in the yeast literature? Even if so, this should be commented on when describing the EM results.

6. If I correctly interpret, the LUCID assay monitors the loss of FLuc luminescence once the protein is inside the MVB but the exact reason why this happens is not entirely clear. If this loss is at least

partially due the acidic pH how would the deletion of Vps1 and less acidification affect these measurements?

Minor points:
1. Spell out ILV in the title.

2. Vac14 deletion does not yield the same extent of defect as Fab1 deletion. This would need some explanation in the text.

Reviewer 3

Advance summary and potential significance to field

I have reviewed the manuscript entitled "V-ATPase dysfunction rescues ILV cargo sorting in yeast lacking PI(3,5)P2 or Doa4" by Wilson and colleagues. This work nicely shows that defects in biosynthetic cargo sorting into endosome intralumenal vesicles caused by inactivation of PI(3,5)P2 generation (FAB1, VAC14 and VAC7 deletion) is suppressed by V-ATPase inactivation. Moreover, rescue is specific to inactivation of V-ATPase at late endosomes/vacuoles (VPH1 deletion) but not at the Golgi/early endosomes (STV1 deletion). Thin section transmission electron microscopy demonstrated intralumenal vesicles are still formed in MVBs of FAB1 and VPH1 deletion mutants although vacuole size/numbers are altered.

Additionally, MVBs and vacuoles are more abundant in VPH1 deletion mutant, which is explained by the deficient recruitment of GFP-Ypt7 and its effector the HOPS tethering/SNARE regulator complex (visualized via imaging of its subunit GFP-Vps41) that mediates MVB-vacuole fusion. Finally, missorting of biosynthetic cargo in cells carrying a deletion of the DOA4 deubiquitinase associated with the ESCRT III machinery is also rescued by VPH1 deletion.

Overall this work advances our understanding of endosome maturation and late endosome-lysosome transition especially with regards to biosynthetic ubiquitinated membrane protein cargo. Mechanistic understanding of this transition is still incomplete but results from this this paper suggest a model that will be of interest to the field and can be further explored. Thus, this research will have implications for a broad spectrum of cell biologist in the protein trafficking and organelle biology field. The work is also technically sound. Fluorescence microscopy of GFP-Cps1 cargo and electron microscopy observation of MVBs/vacuoles are a well-established methods to asses this pathway. The data is nicely complemented by use of the newer LUCID approach.

Comments for the author

Major points
1) Given the proposed model in which rescue by VPH1 deletion works through impeding Ypt7/HOPS recruitment/function, I would suggest testing if mutation of YPT7 or a HOPS subunit also rescues the cargo missorting phenotype of FAB1 and DOA4 deletion.

Minor points
1) The phenotype in figure 1 is clear. However, the quantification in figure 1 B shows no error bars although it indicates n>100. Some indication of reproducibility should be provided. The same applies to Figure 2C and 5B. Along the same lines, M&M should better clarify how was missorting of GFP-Gps1 determined for each cell, was it simple visual inspection or was a specific measurement obtained?

2) Supplemental Figure 2, legend to panels B and C are swapped.

First revision

Author response to reviewers' comments

Reviewer 1 Comments:

1. It is not clear how slowing endosome-vacuole fusion would compensate for such a specific targeting defect [specific to Cps1], and this needs to be discussed.
> We now discuss at the end of the first paragraph of the Discussion section (p. 11) how Cps1, unlike many other ILV cargoes, is not subject to iterative sorting and, thus, useful for revealing minor defects in ILV cargo sorting efficiency.

2. Similarly, it is not clear how loss of acidification could compensate for Doa4 loss, and the authors find no evidence of a direct connection between Fab1 kinase and Doa4 activity other than the suppression of both mutations by vph1Δ. While it may be difficult to fully establish these connections in CPS sorting, more effort should be made to try to explain them experimentally, or at least in a well-justified final model.

> We now provide additional justification that V-ATPase disruption restores ILV cargo sorting in doa4Δ cells by delaying endosome-vacuole fusion. This idea is supported by an increase in vacuole number observed in doa4Δvph1Δ cells (Figure 5A and C) and that removal of Ypt7 in doa4Δ cells, which causes a severe inhibition of vacuole fusion, partially restores Cps1 sorting measured through the LUCID assay (Figure 5E). We only provide evidence in this manuscript that vph1Δ and ypt7Δ are epistatic to doa4Δ in relation to ILV cargo sorting. We do not know the exact nature of the V-ATPase activity that functions downstream of Doa4, and we think it is likely more complex than just a role in endosome or vacuole acidification.

Minor points:

1. More detail about how the percentage of missorted GFP-Cps1 is calculated for Figure 1B, 2C, and 5B should be included. Some indication of the percentage of cells showing GFP-Cps1 at the vacuolar membrane in Figures 1 and 2.
> This has been added both in the figure legends and mentioned within Materials and Methods (p.14).

2. In proximal tubule, recruitment of small GTPase Arf6 to the endosomal membrane responds to luminal acidification (Marshansky et al. (2006) Nature Cell Biology 8:124). Could something like this account for the failure of Ypt7 recruitment in the vph1Δ mutant?
> We have added to the Discussion section (p. 12) this commentary on a potential role for Vph1 in directly recruiting Ypt7 to the yeast endosome or vacuole membrane. As support for this possible mechanism, we highlighted the publication mentioned above and recent work in osteoclasts demonstrating a role for the V-ATPase in recruiting Rab7 to secretory lysosomes.

Reviewer 2 Comments:

My main criticism is the preliminary feel for the Doa4 aspect of the study and some inconsistencies between the data and their interpretation. Most of my comments can be addressed by textual changes, although some extra experiments sure would significantly strengthen the study.

1. The connection between the Doa4 deletion and Vph1 is much less characterized compared to that of Fab1 and Vph1. It would be important to know what the TEM findings and statistics are with Doa4 deletion.
There appear to be major differences between the fab1 and Doa4 defects, most strikingly the lack of enlargements of the vacuoles in the Doa4 deleted cells. One would assume that the biggest effect of Doa4 deletion would be on the sorting of Cps1 into ILVs at the level of the MVB. This is also suggested by the largest increase in the LUCID assay (Fig. 4D). How would that be corrected by the impaired MVB- Vacuole fusion caused by Vph1 deletion?

> We have included TEM results for both doa4Δ and doa4Δvph1Δ in Figure 3. While not to the same extent as fab1Δ cells, doa4Δ cells often contain enlarged vacuoles. Removing VPH1 in doa4Δ cells increased vacuole number and increased the mean ILVs observed per MVB, indicative of a similar block in vacuole fusion. Our previous work on Doa4 has demonstrated that Doa4 both directly

stabilizes Snf7 polymerization at the endosome surface (Johnson et al., 2017) and delays ILV membrane scission by inhibiting interactions between the Snf7 polymer and the Vps2/Vps24 sub-complex (Buysse et al., 2020). Furthermore, doa4Δ cells contain reduced levels of monomeric ubiquitin, which can be restored by some ESCRT-III mutations (Amerik et al., 1999) and have defects in sorting biosynthetic ILV cargo but not in forming ILVs (this manuscript and Richter et al., 2007). A presumption from these observations is that Doa4 delays ILV scission to allow for efficient ubiquitin recycling prior to ILV scission. Our new observations also suggest that Doa4 may delay ILV scission to allow for efficient cargo ensnarement. Thus, we propose that a delay (vph1Δ) or severe inhibition (ypt7Δ) in endosome – vacuole fusion allows for the inefficiency in ILV cargo sorting in doa4Δ cells to be rectified (p. 13).

2. The simplest explanation for the rescuing effect of Vph1 deletion in the Fab1 deletion case is that by limiting the fusion between the MVBs and the vacuole, there is more time for the sorting of the ubiquitinated Cps1 cargo into the ILVs that appears to be defective but may be not incomplete in the Fab1 deleted cells. If this is the case, it may not indicate an active level of integration between these steps in the normal cell that would require some sort of signaling coordination between these steps.
> We have introduced this idea in the Discussion section (pp. 11-12).

3. I think it would be important to discuss in more detail the fact that ILVs are still generated in the MVBs in Fab1 deleted cells and only the sorting of the ubiquitinated biosynthetic cargoes into those ILVs is affected by the lack of PI(3,5P2. I am not certain that this nuance has received enough attention in the literature, and it is important to understand for the conclusion of this study. If there is a molecular explanation for this distinction, that would be important to cite.
> We have more thoroughly highlighted this distinction in the text and provide additional citations where this disconnect between ILV cargo-sorting and formation have been observed. Because PI(3,5)P2 has been demonstrated to regulate ion export we have also suggested that our observations argue against Fab1 or PI(3,5)P2 as only regulating lumenal content removal to support ILV formation (pp. 11-12). This is an important observation as it does suggest that PI(3,5)P2 performs a role in the ILV cargo-sorting step; however, our study does not provide evidence on whether that is a direct or indirect role.

4. If loss of Ypt7 or its HOPS effector, Vps41 from the vacuole is responsible for the fusion defect and this is a major reason for the rescue, would Ypt1 [the reviewer means Ypt7] or Vps41 deletion also rescue the Fab1 (or Doa4) defect?
> Because vacuoles become extremely fragmented, we could not assess GFP-Cps1 sorting in fab1Δypt7Δ or doa4Δypt7Δ cells. Instead, we used the LUCID assay to assess FLuc-Cps1 sorting and show that the FLuc-Cps1/RLuc ratio is significantly reduced in doa4Δypt7Δ cells compared to doa4Δ yeast (Figure 5E), implying that a YPT7 deletion can also rescue the ILV cargo-sorting defect of doa4Δ cells. This was not the case for fab1Δypt7Δ cells, although we observed a similar starting FLuc-Cps1/RLuc ratio in both the fab1Δ and ypt7Δ single mutants.

5. There are striking differences between the wild-type and mutants in the electron densities of their vacuoles. Wild-type is dark, all mutants are light. Is this a known phenomenon in the yeast literature? Even if so, this should be commented on when describing the EM results.
> The difference in vacuole electron densities is attributed to a reduction in stored polyphosphate which complexes with electron-dense cations or can capture electron-dense cations used as contrast stains in TEM analyses. Proton pumping by the V-ATPase is required for polyphosphate storage in the vacuole (Gerasimaitė, et al., 2014) and V-ATPase mutants contain electron translucent vacuoles (Keuenhof et al., 2021); we have mentioned this in the text (p. 7). We found that fab1Δ cells also contain lower total phosphorous levels (Wilson et al., 2018), and the extreme vacuole enlargement may be contributing to the electron translucence of vacuoles in fab1Δ cells. Along with V-ATPase mutants, both fab1Δ and ypt7Δ yeast were identified as mutant strains that contained lower total polyphosphate in a screen using the yeast knockout collection (Freimoser et al., 2006).

6. If I correctly interpret, the LUCID assay monitors the loss of FLuc luminescence once the protein is inside the MVB but the exact reason why this happens is not entirely clear. If this loss is

at least partially due the acidic pH how would the deletion of Vps1 [Vph1] and less acidification affect these measurements?
> If an acidic pH is partially responsible for the loss of FLuc luminescence, one would expect an increase in FLuc luminescence in vph1Δ cells, increasing the FLuc/RLuc ratio in vph1Δ cells. Such a result would make it appear that vph1Δ cells have a major FLuc-Cps1 sorting defect when compared to wild-type cells. However, we do not observe this result, as highlighted on p. 9. Our interpretation of the LUCID assay is that FLuc sequestration into the MVB physically impairs FLuc from accessing the added luciferin substrate, which is added after incubating cells in a passive lysis buffer to allow substrate to enter the cytosol (Nickerson and Merz, 2015). Because the FLuc-Cps1/RLuc ratio was similar between wild-type cells and the vph1Δ single mutant, which matched our observations of GFP-Cps1 sorting by fluorescence microscopy in vph1Δ cells, we had confidence in using the LUCID assay in vph1Δ mutants.

Minor points:

1. Spell out ILV in the title.
> Corrected.

2. Vac14 deletion does not yield the same extent of defect as Fab1 deletion. This would need some explanation in the text.
> We have explained this in the text (p. 6), highlighting that a vac14Δ deletion still contains a small amount of PI(3,5)P2 synthesis.

Reviewer 3 Comments for the author

Major points

1. Given the proposed model in which rescue by VPH1 deletion works through impeding Ypt7/HOPS recruitment/function, I would suggest testing if mutation of YPT7 or a HOPS subunit also rescues the cargo missorting phenotype of FAB1 and DOA4 deletion.
> Please see (Figure 5E) and our response above to Reviewer 2 comment #4.

Minor points

1. The phenotype in figure 1 is clear. However, the quantification in figure 1 B shows no error bars although it indicates n>100. Some indication of reproducibility should be provided. The same applies to Figure 2C and 5B. Along the same lines, M&M should better clarify how was missorting of GFP-Gps1 [the reviewer means GFP-Cps1] determined for each cell, was it simple visual inspection or was a specific measurement obtained?
> This oversight was addressed to demonstrate the level of reproducibility with the number of experiments and number of cells quantified mentioned in the respective figure legends. See Materials and Methods section under Fluorescence Microscopy where we describe how GFP-Cps1 missorting was determined.

2. Supplemental Figure 2, legend to panels B and C are swapped.
> Corrected.

Second decision letter

MS ID#: JOCES/2021/258459

MS TITLE: Vacuolar H^+-ATPase dysfunction rescues intralumenal vesicle cargo sorting in yeast lacking PI(3,5)P2 or Doa4

AUTHORS: Zachary N Wilson, Dalton Buysse, Matthew West, Daniel Ahrens, and Greg Odorizzi

ARTICLE TYPE: Research Article

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

Reviewer 1

Advance summary and potential significance to field

This manuscript addresses the coordination of recruitment of cargo (newly synthesize CPS) into intralumenal vesicles (ILVs) and fusion of the late endosome/MVB with the vacuole. In this revised version, the authors provide evidence that both the endolysosomal lipid PI(3,5)P2 and the deubiquitinase Doa4 play a role in cargo selection for ILVs, and if their roles are not completed ILV cargoes end up at the vacuolar membrane. However, a vph1 deletion or non-functional vph1 mutant can suppress these defects, potentially by slowing endosome-vacuole fusion and allowing additional time for an inefficient process.
This work will be of interest to researchers in the trafficking field because it starts to address how dynamics of different steps affect fidelity. However, the manuscript is still somewhat narrow in scope.

Comments for the author

The authors have addressed my previous comments. The revised manuscript is more cohesive than the earlier version.

Reviewer 2

Advance summary and potential significance to field

This is a revised version of a previously reviewed manuscript. Advances made in this paper have been outlined in the original review.

Comments for the author

The authors have addressed all of my comments satisfactorily. They have included new experimental data and made several changes to the text. These changes have significantly improved the manuscript making it suitable for publication in JCS.

Reviewer 3

Advance summary and potential significance to field

Overall this work advances our understanding of endosome maturation and late endosome-lysosome transition especially with regards to biosynthetic ubiquitinated membrane protein cargo. Mechanistic understanding of this transition is still incomplete but results from this this paper suggest a model that will be of interest to the field and can be further explored. Thus, this research will have implications for a broad spectrum of cell biologist in the protein trafficking and organelle biology field. The work is also technically sound. Fluorescence microscopy of GFP-Cps1 cargo and electron microscopy observation of MVBs/vacuoles are a well-established methods to asses this pathway. The data is nicely complemented by use of the newer LUCID approach

Comments for the author

The authors have satisfactorily responded to the comments. The manuscript is now ready for publication.