A lysosomal biogenesis map reveals the cargo spectrum of yeast vacuolar protein targeting pathways

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Re: JCB manuscript #202107148

Dr. Florian Fröhlich
Osnabrück University
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Germany

Dear Dr. Fröhlich,

Thank you for submitting your manuscript entitled "A yeast lysosomal biogenesis map uncovers the cargo spectrum of lysosomal protein targeting pathways”. The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers’ key concerns, as outlined here.

You must address the comments of Rev 1 regarding the over-interpretation of some of the data and you should provide a table that more clearly summarizes the sorting defects observed in the various mutants tested in this study. You also should address the comments of Rev 2 regarding the need for additional technical details and other assays to enhance the quality of the findings. You also should do a better job clarifying the data presented in Figure 2 as suggested by Rev 2. In addition, we hope that you will be able to address all of the remaining reviewer comments in your revised manuscript.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Tools 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: Tools may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://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. Tools 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.

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers’ comments point by point. Please also highlight all changes in the text of the manuscript.

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 in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

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

Eising et al provide a nice dataset showing changes in the levels of vacuolar proteins between different mutants that have blocks within pathways that lead to the vacuole. This study builds on previous work from the Frohlich group to use proteomics changes to map sorting pathways and clients. Overall, this is nice work and the choices for what mutants to examine are interesting and helpful to the field. The follow-up experiments to show utility of this 'Tools' paper are good, though some are mostly superficial. Still, this meets the criteria set forth by the JCB as the standard for a methods/utility study.

My main reservation about the manuscript as written is that it comes to very broad conclusions that in my mind, overreach the data. It's one thing to conclude that a given pathway is required to have normal levels of a given protein in the vacuole. But it is another to say that any deficit here necessarily means that such a protein is a direct client of the sorting pathway. As the authors state themselves, there is "flexibility regarding the different transport routes." Things like Pho8, a classic AP3 client, reach the vacuole in the absence of AP3. Many of the conclusions about what sorts what in this paper are built on changes in the levels of potential cargos in the vacuole. This may indicate a protein takes a pathway, or it may indicate that a pathway is required indirectly. Here are some instances where this comes into play for this manuscript:

The authors state that their "data reveal that luminal vacuolar proteins are exclusively transported by the Vps10 sorting receptor". Yet, there are instances in their Perseus output where this does not appear to be the case. For instance Prb1 does not change in vps10Δ mutants. What would be helpful here is to have a Table showing all the predicted luminal proteins in vacuoles and their relative levels across the different mutants to allow the readers to make up their own minds easily about the strength of this conclusion.

The authors also conclude that most proteins travel through the AP3 pathway. This is a bold conclusion, especially given the idea that the Vps10 pathway represents a 'default' pathway out of the TGN. The idea is based on changes in vacuolar content in vps45Δ/ggaΔ cells vs ap3Δ cells. Atleast one protein highlighted, Fet5, is depleted in both ap3 and vps45Δ mutants, but not ggaΔ mutants. Same for Vam3, a well known AP3 client. Given such data, is it really clear that one can assign a protein a given route ased on these data alone? The microscopy screen is a potentially nice addition here. Its based on the idea that AP3 cargo go to the vacuole in a vps45Δ mutant (- as an aside, "class D compartment" needs a definition). A more robust assessment, however, is whether cargo can accumulate in enlarged endosomes of escrtΔ mutants, which accumulate the V-ATPse but not proteins such as Pho8 and Vam3. This type of secondary analysis would be needed to make this bold conclusion about AP3 clients.

A similar issue with interpretation comes from examining ggaΔ mutants and vps10Δ mutants that have changes in plasma membrane permeases with the conclusion that these mutants have "a general deficiency in sorting of ubiquitinated cargo to the vacuole". This is confusing because these vacuoles are PEP+ and should not have appreciable clients of the multivesicular body pathway within them. Second, the changes in things like Mup1, Dip5 and Tna1 are different between ggaΔ and vps10Δ such that there is a major increase in the latter. This is likely explained by the depletion of vacuolar proteases that allow these permeases to resist degradation in the vacuole lumen rather than mirror a defect in sorting them to the vacuole as concluded.

A second area that needs addressing is clearly showing how levels of proteins in the vacuole relate to their total amounts in the cell. These data were collected, but I do not see them in the supplemental tables and are shown as a ratio in supplemental figure that only annotates a few. It is important to know whether more of less of a protein in the vacuole is due to that protein sorting somewhere else, or whether its overall levels are different. This is key for membrane proteins, since things like vacuolar hydrolases would be expected go be depleted from both the vacuole and the whole cell due to secretion in a vps10Δ mutant for instance. Including these data and furnishing a figure showing that these vacuolar changes are or are not related to the whole cell changes is important for the reader to make conclusions.

Minor points
The citation of previous work on Npc2 (Berger 2007)is inaccurate. That study showed that loss of GGAs or AP3 perturbed localization of Npc2, consistent with data here showing its delivery thought a Vps45, Gga, Vps10-dependent route.
Secretion data that is represented in Figure 3D does not appear included in the excel sheets.

The authors conclude Vps10 is the only client of Ggas for sorting out of the Golgi. What about ubiquitinated membrane proteins? What about Ycf1 and Bpt1, which the authors discuss as cargo that follows the endosomal route to vacuoles and that are de-enriched in ggaΔ vacuoles in the proteomics data tables?

It is probably something to do with Perseus, but Vps10 shows up as measurable in a vps10Δ cell. This will need explanation in the text and methods.

In Figure S4, what's going on with the images for Vtc3, Scw4, Vhc1, etc in apl6Δ mutants?

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

In their manuscript entitled "A yeast lysosomal biogenesis map uncovers he cargo spectrum of lysosomal protein targeting pathways", Eising et al. use quantitative proteomics to characterize the proteome of yeast vacuoles, and to measure how the vacuole proteome is altered in several yeast mutants defective for specific vacuolar trafficking pathways. Extensive work by many groups over many years has led to the characterization of three main trafficking pathways capable of sorting cargo from the Golgi to the vacuole: the GGA-VPS pathway, the AP-3 pathway, and the CVT pathway. However, these pathways have been dissected using only a handful of model cargo, and for many cargoes sorted to the vacuole the pathways utilized have not been described. The authors view this as an opportunity to apply their quantitative proteomic approach, which they describe in the following narrative:

1. The authors begin by introducing their quantitative proteomics approach (which they term "QPrevail") and they introduce the mutations they will use to disrupt specific vacuole trafficking pathways. This culminates in the presentation of a "vacuole biogenesis map", which illustrates how the proteome of the yeast vacuole is altered when specific vacuole trafficking pathways are abrogated. The remainder of the paper leverages this map to describe the cargo profile of different vacuole trafficking pathways.
2. The authors describe Vps10-dependent cargo, which are validated by examining aberrant secretion in Vps10 mutants. They also analyze the vacuole proteome in yeast harboring deletions in the domain 1 and domain 2 sortillin-like domains of Vps10. This analysis reveals some (but not all) cargo exhibit dependence on either domain 1 or domain 2 of Vps10.
3. The same data are used to identify Gga-dependent cargo. This analysis reveals that Vps10 (and thus all of its cargo) are depleted from vacuoles in the absence of GGAs, along with several PM transporters that traffic to the vacuole via the ESCRT pathway. In contrast, certain t-SNAREs and a PM flippase (Dnf1) accumulate on the vacuolar membrane - indicating aberrant membrane trafficking throughout gga mutant cells.
4. Vacuoles from cells lacking Vps45 are depleted for Vps10 and its cargo, CVT cargo, and the v-ATPase complex. Vacuoles from cells lacking Atg19 are depleted for known cargoes of the CVT pathway. Vacuoles from cells Api5 (the AP-3 pathway) are depleted for many vacuolar transmembrane proteins.

The manuscript is presented as a Tools/Resource paper, and the results provide a potentially valuable resource that could be mined to inspire future studies. The manuscript is well-written, and most of the claims in the paper are justified by the data. However, some claims are not well-substantiated by the data. Here, I offer some suggestions for how the authors might strengthen the paper.

Major Points

1. Although vacuolar purification methods have been used and described previously, the method used by the authors is critical to the robustness and reproducibility of the assay - particularly in a "Tools" paper. Therefore, the authors should provide more details about their vacuole purification protocol. Additionally, since this is a Tools manuscript, the authors should be more rigorous in the quality control assessment of their purified vacuoles. Since the vacuoles were purified by flotation on a density gradient, it is possible that mutations that severely disrupt vacuolar morphology or content could also interfere with purification of vacuoles - either by changing vacuole density or by resulting in vacuoles that are more fragile and prone to fragmentation. (This seems like a particular concern for vacuoles isolated from vps45 mutants.) Adding quality control assays (e.g., EM or fluorescence microscopy analysis) for the purified vacuoles would go a long way to alleviating these concerns. Such QC analysis would improve confidence that the measurements presented in the paper are legitimate "apples-to-apples" comparisons.

2. Figure 2 is the pith of this manuscript, and there are some aspects of this figure that I think could be improved and clarified. First, the authors don't define the gray boxes, which presumably indicate that a protein could not be reliably quantified (i.e., less than 3 peptides were measured in the experiment). The gray boxes dominate a few of the biological replicate experiments - meaning these replicates were not particularly sensitive. The authors should clarify and explain. My concern here is that some of the proteins in this cluster (e.g., Pib1, Gtr2, Avt4) cluster with a group of AP-3 cargo but the data does not provide evidence that they are diminished in vacuoles isolated from ap3 mutants. Second, I am curious about the inclusion of Atg8 as an AP-3-
dependent cargo. This seems like a finding of potential importance, but it is not addressed by the authors. The authors should validate this finding by determining if Atg8 localization is AP-3-dependent. Third, the panel corresponding to HOPS/TORC1 shows a cluster without a defined profile (i.e., they are all black boxes, and thus all classified as "unaffected" in the panel of mutants) which the authors claim predicts protein-protein interactions (lines 198-201). However, these proteins only cluster because they are unaffected in the backgrounds tested, and any other unaffected proteins should cluster similarly. So I think the claim of the authors is not valid for this particular cluster.

3. The authors claim that most Vps10 cargo are depleted from vacuoles of yeast expressing the domain 2 mutant (lines 234-237). However, the data in Figure 3G indicates that domain 2-dependent sorting for Npc2, Yhr202W and Pep4 but not Ape3 and CPY. (CPY is an inconsistent case, because it is depleted in exp 1 but not in exp2.) The authors should adjust their claim to match the data, and discuss accordingly.

4. In the text (lines 282-284) the authors mention that Vps10 is depleted in vps45 mutant vacuoles. In Figure 5B, however, Vps10 does not appear to be significantly depleted (it is classified as "unaffected"). The authors should change the text to reflect this, and adjust their discussion accordingly.

Minor Points

1. In Figure 5G - the authors make the conclusion that Ncr1 and Atg15 are sorted to the vacuole by a mechanism that does not involve cytosolic recognition. For Ncr1, there are a multitude of other cytosolic loops which could harbor sorting signals. For Atg15, this interpretation is complicated by the fact that most GFP-tagged Atg15 appears to be retained in the ER, with only a small portion trafficking to vacuolar membranes. It is unclear if this localization pattern is an artifact of tagging. In fact, there may even be more Atg15 trafficking to the vacuole membrane when the N-terminal segment is deleted - suggesting the N-terminus may be involved in ER retention - but it is difficult to make any conclusion without a quantitative analysis.

2. The authors make the claim that "QPrevail can be used to study post-translational modification dependent protein targeting" (lines 141-143, lines 322-324). However, as far as I can tell the authors do not identify or quantify PTM-modified peptides. So I don't believe this claim is substantiated by the data presented in this paper.

3. In Figure S5: I would predict that cargoes with both sorting signals would be unaffected in single mutants, but cargoes with one or the other would be sensitive to the single mutants. The authors should consider color-coding the plots in panels B and C to reflect the sorting signals indicated in panel A.
Dear Dr. Emr

Thank you very much for serving as editors on our manuscript “A yeast lysosomal biogenesis map uncovers the cargo spectrum of lysosomal protein targeting pathways”. I would like to thank you and the reviewers for their time and effort, as well as the constructive comments. Below is a point-by-point response to the questions raised during the review. This shows which data we have added during the revisions and where we have edited the manuscript. As suggested we have toned down our conclusions and have added quality controls for the vacuolar purifications. We have also improved the description of the clustering. I hope that these changes are sufficient to accept our manuscript for publication in the Journal of Cell Biology. I am looking forward to your response.

Sincerely

Florian Fröhlich

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

Eising et al provide a nice dataset showing changes in the levels of vacuolar proteins between different mutants that have blocks within pathways that lead to the vacuole. This study builds on previous work from the Frohlich group to use proteomics changes to map sorting pathways and clients. Overall, this is nice work and the choices for what mutants to examine are interesting and helpful to the field. The follow-up experiments to show utility of this 'Tools' paper are good, though some are mostly superficial. Still, this meets the criteria set forth by the JCB as the standard for a methods/utility study.

We would like to thank the referee for her/his overall positive feedback.

My main reservation about the manuscript as written is that if comes to very broad conclusions that in my mind, overreach the data. It's one thing to conclude that a given pathway is required to have normal levels of a given protein in the vacuole. But it is another to say that any deficit here necessarily means that such a protein is a direct client of the sorting pathway. As the authors state themselves, there is "flexibility regarding the different transport routes." Things like Pho8, a classic AP3 client, reach the vacuole in the absence of AP3. Many of the conclusions about what sorts what in this paper are built on changes in the levels of potential cargos in the vacuole. This may indicate a protein takes a pathway, or it may indicate that a pathway is required indirectly. Here are some instances where this comes into play for this manuscript:

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proteins in vacuoles and their relative levels across the different mutants to allow the readers to make up their own minds easily about the strength of this conclusion.

We would like to thank the referee for raising this critical point. We have modified our conclusions accordingly and toned it down. We have also generated the requested table and generated plots showing the abundance of the luminal vacuolar proteins in the different mutants. These data can be found in supplementary figures 3a and b and in supplementary table 3.

The authors also conclude that most proteins travel through the AP3 pathway. This is a bold conclusion, especially given the idea that the Vps10 pathway represents a ‘default’ pathway out of the TGN. The idea is based on changes in vacuolar content in vps45Δ/ggaΔ cells vs ap3Δ cells. At least one protein highlighted, Fet5, is depleted in both ap3 and vps45Δ mutants, but not ggaΔ mutants. Same for Vam3, a well known AP3 client. Given such data, is it really clear that one can assign a protein a given route used on these data alone? The microscopy screen is a potentially nice addition here. Its based on the idea that AP3 cargo go to the vacuole in a vps45Δ mutant (-as an aside, “class D compartment” needs a definition). A more robust assessment, however, is whether cargo can accumulate in enlarged endosomes of escrtΔ mutants, which accumulate the V-ATPase but not proteins such as Pho8 and Vam3. This type of secondary analysis would be needed to make this bold conclusion about AP3 clients.

It is well known that some AP-3 cargoes can reach the vacuole via the plasma membrane in AP-3 deficient cells. This has also been used as a readout, e.g. taking a protein chimera consisting of GFP-tagged Nyv1 carrying a C-terminal transmembrane domain of the plasma membrane SNARE Sncl, called GNS (Reggiori et al, 2000). As we show in supplementary figure 4 and 5, Fet5 appears to be a protein of the ER when tagged with a N-terminal-GFP (possibility artificially retained in the ER). We assume that it is the nature of screens that not all observations can be explained. However, we agree that additional experiments would strengthen our results. We therefore used high throughput genetics to cross all our potential AP-3 cargoes, N-terminally GFP tagged, against a strain harboring Vph1-mKate and a deletion of VPS27. We now tested, as suggested by the referee, which proteins colocalize with the class E compartment. This analysis confirmed that the majority of proteins identified as potential AP-3 cargoes are indeed transported to the vacuole without passing through an endosomal compartment. We have now made a list of proteins that are most likely cargoes of the AP-3 pathway. The data can be found in the new supplementary figure 5 and in supplementary table 14.

A similar issue with interpretation comes from examining ggaΔ mutants and vps10 mutants that have changes in plasma membrane permeases with the conclusion that these mutants have "a general deficiency in sorting of ubiquitinated cargo to the vacuole". This is confusing because these vacuoles are PEP+ and should not have appreciable clients of the multivesicular body pathway within them. Second, the changes in things like Mup1, Dip5 and Tna1 are different between ggaΔ and vps10Δ such that there is a major increase in the latter. This is likely explained by the depletion of vacuolar proteases that allow these permeases to resist degradation in the vacuole lumen rather than mirror a defect in sorting them to the vacuole as concluded.

We thank the referee for raising this point. We believe that the vacuoles of vps10Δ cells and gga1 gga2ΔΔ cells should both show a phenotype that is PEP4-. In contrast the vacuoles of WT cells should have a PEP+ phenotype. However, the isolation of vacuoles does not result in pure fractions, as we have shown in our previous manuscript (Eising et al. eLife 2019). This means we most likely also have tethered endosomes in our isolations, thus explaining the ability to detect also plasma membrane proteins on their route to vacuoles.

However, we agree that we were not precise enough in explaining the effects of GGA mutants on protein sorting. As the Mup1 pulse-chase assays show, Mup1 indeed does not reach the vacuole in GGA mutants suggesting there is either an endocytic defect or a sorting defect. This supports the data from our proteomics experiments.

In contrast, in vps10 mutants, Mup1 e.g, appears to be more abundant in vacuoles. This is most likely a phenotype caused by the absence of the vacuolar proteases in VPS10 mutants. However, the GGA mutants also show lower levels of vacuolar proteases. Therefore the absence of Mup1 must appear earlier.

We have now clarified this point in the manuscript to avoid confusion.

A second area that needs addressing is clearly showing how levels of proteins in the vacuole relate to their total amounts in the cell. These data were collected, but I do not see them in the supplemental tables and are shown as a ratio in supplemental figure that only annotates a few. It is important to know whether more of less of a protein in the vacuole is due to that protein sorting somewhere else, or whether its overall levels are different. This is key for membrane proteins, since things like vacuolar hydrolases would be expected go be depleted from both the vacuole and the whole cell due to secretion in a vps10Δ mutant for instance. Including these data and furnishing a figure showing that these vacuolar changes are or are not related to the whole cell changes is important for the reader to make conclusions.

The data can be found in supplementary table 1. This excel sheet has two tabs, on for the total proteome levels and one for the vacuolar proteome levels. The data is presented in supplementary figure 2. There, all vacuolar proteins are represented by the yellow dots in comparison to all identified proteins. As can be seen there, there are no systematic changes in vacuolar protein abundance in the analyzed mutants. As shown in these figures, CPY is enriched in mutants VPS10, GGA and VPS45. While the referee has an important point that due to their secretion they could be depleted this does not appear in the data. One explanation for this could be that the secreted proteins in e.g. Vps10 deletion cells often are “sticking” to the cell wall (personal communication with Elizabeth Conibear liquid invertase assay; Bean et al. 2017 Traffic) and are therefore processed in the sample preparation for the entire cell extracts. We have also included a figure showing the abundance of all soluble vacuolar proteins in supplementary Figure 3b in whole cell extracts.
Minor points

The citation of previous work on Npc2 (Berger 2007) is inaccurate. That study showed that loss of GGAs or AP3 perturbed localization of Npc2, consistent with data here showing its delivery thought a Vps45, Gga, Vps10-dependent route.

We thank the referee for pointing this out. We have adjusted the text accordingly.

Secretion data that is represented in Figure 3D does not appear included in the excel sheets.

We thank the referee for pointing this out. We have now included all proteome data in supplementary tables.

The authors conclude Vps10 is the only client of Ggas for sorting out of the Golgi. What about ubiquitinated membrane proteins? What about Ycf1 and Bpt1, which the authors discuss as cargo that follows the endosomal route to vacuoles and that are de-enriched in ggaΔ vacuoles in the proteomics data tables?

We thank the referee for raising this point. Ycf1 and Bpt1 are only depleted in vps45Δ cells and not in gga12Δ cells. However, we also identify the palmitoyltransferase Akr1 as a depleted cargo as well as two palmitoylated proteins Lsb6 and Lcb4. However, we are not aware of any ubiquitinated membrane proteins in gga12Δ cells. We have added a description of the Akr1 phenotype in the manuscript.

It is probably something to do with Perseus, but Vps10 shows up as measurable in a vps10Δ cell. This will need explanation in the text and methods.

The referee has a valid point. We enabled the re-quant option in MaxQuant that allows the quantification of proteins that yield only one SILAC pair. It quantifies the background to be able to assign a ratio to proteins with only one SILAC pair detectable. Therefore, we can show a ratio for Vps10 even in a strain that is vps10Δ. We have clarified this point in the materials and methods section and in the text.

In Figure S4, what going on with the images for Vtc3, Scw4, Vhc1, etc in apl6Δ mutants?

During the automated crossing of strains some do not yield viable progenitors. Since the entire screening process, including the microscopy is automated the microscope takes pictures of empty wells. Therefore, the images only show background and are unfortunately not useful. Similarly some of the crossing did not make it through for the new vps27Δ screen. These images are not shown in supplementary figure 5.

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

In their manuscript entitled "A yeast lysosomal biogenesis map uncovers he cargo spectrum of lysosomal protein targeting pathways", Eising et al. use quantitative proteomics to characterize the proteome of yeast vacuoles, and to measure how the vacuole proteome is altered in several yeast mutants defective for specific vacuolar trafficking pathways. Extensive work by many groups over many years has led to the characterization of three main trafficking pathways capable of sorting cargo from the Golgi to the vacuole: the GGA-VPS pathway, the AP-3 pathway, and the CVT pathway. However, these pathways have been dissected using only a handful of model cargo, and for many cargoes sorted to the vacuole the pathways utilized have not been described. The authors view this as an opportunity to apply their quantitative proteomic approach, which they describe in the following narrative:

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The manuscript is presented as a Tools/Resource paper, and the results provide a potentially valuable resource that could be mined to inspire future studies. The manuscript is well-written, and most of the claims in the paper are justified.
by the data. However, some claims are not well-substantiated by the data. Here, I offer some suggestions for how the authors might strengthen the paper.

We would like to thank the referee for this positive feedback on our manuscript.

Major Points

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We would like to thank the reviewer for raising this very important point. We have now included a quality controls in figure 1 showing the quantification of vacuole number and size in WT versus mutant cells. We therefore mixed Vph1-mCherry expressing WT cells with Vph1-mNeon expressing mutant cells prior to lysis. After density gradient centrifugation we imaged the isolated vacuoles by confocal microscopy. For apl2Δ cells, vps10Δ cells, gga1∆ggα2Δ cells and atg19Δ cells we did not observe any difference in vacuole size or number. For vps45Δ cells we used the known AP-3 cargo Yck3 as a marker. For vps45Δ cells we see differences in the number of isolated vacuoles.

However, we do not consider this a problem for our proteomics analysis. We are using normalized ratios from the experiments to do the calculations. This means that the distribution of ratios is adjusted by the MaxQuant software to show an average of 1. Therefore it is still possible to determine which proteins are specifically less abundant in WT cells compared to vps45Δ cells. This can be seen by comparing the ratios in supplementary table 1 with the columns for the normalized ratios. We have included a figure for the referee showing the distribution of proteins in one of the vps45Δ vs WT replicates. The normalization does not alter the overall distribution. We have also highlighted all the proteins that we consider, based on our results, highly likely candidates of the AP-3 pathway. As can be seen in the figure, these proteins are only mildly altered in the VPS45 mutants.

We have clarified this point in the results section and in the materials and methods section.

2. Figure 2 is the pith of this manuscript, and there are some aspects of this figure that I think could be improved and clarified. First, the authors don’t define the gray boxes, which presumably indicate that a protein could not be reliably quantified (i.e., less than 3 peptides were measured in the experiment). The gray boxes dominate a few of the biological replicate experiments - meaning these replicates were not particularly sensitive. The authors should clarify and explain. My concern here is that some of the proteins in this cluster (e.g., Pib1, Gtr2, Avt4) cluster with a group of AP-3 cargo but the data does not provide evidence that they are diminished in vacuoles isolated from ap3 mutants.

We thank the referee for raising this important point. The grey boxes are indeed replicates where the protein was not sufficiently quantified. We have now included this point in the manuscript. As the referee describes, some of the replicates where not particularly sensitive. The problem is that many vacuolar proteins are relatively low abundant, making there quantification very difficult. However, we consider it important to show that also these replicates behave very similar amongst the different utilized mutants and should be shown. As figure 2 summarize our results we believe that all the experiments in the following figures are important to highlight which proteins are linked to the specific pathways.

Second, I am curious about the inclusion of Atg8 as an AP-3-dependent cargo. This seems like a finding of potential importance, but it is not addressed by the authors. The authors should validate this finding by determining if Atg8 localization is AP-3-dependent.

The referee has a valid point here. Based on the observed ratios, Atg8 could be classified as a potential AP-3 cargo. However, Atg8 is modified with phosphatidyethanolamine and targeted to membranes from the cytoplasm. This is also clear in our additional microscopy screen. There, we classify Atg8 as a protein that is not localized at the vacuole. We have now clarified this point in the manuscript and generated a list on potential AP-3 cargoes. However, a potential explanation why Atg8 is less abundant in/at vacuoles of AP-3 deficient cells is the depletion of the known AP-3 cargo protein Atg27. Isolation of vacuoles from atg27Δ cells clearly show depletion of known CVT cargo proteins, highlighting again that the CVT pathway operates under these conditions. Indeed, one of the statistically significantly depleted proteins is Atg8 potentially explaining the phenotype we observe in AP-3 deficient cells. We have included this data in supplementary figure 3 and modified the manuscript accordingly.

Third, the panel corresponding to HOPS/TORC1 shows a cluster without a defined profile (i.e., they are all black boxes, and thus all classified as "unaffected" in the panel of mutants) which the authors claim predicts protein-protein interactions (lines 198-201). However, these proteins only cluster because they are unaffected in the backgrounds tested, and any other unaffected proteins should cluster similarly. So I think the claim of the authors is not valid for this particular cluster.
We thank the reviewer for picking this up although we respectfully disagree with the assessment. As shown in figure 2, The HOPS complex (Vps10, Pep3, Pep5, Vps33, Vps41 and Vam6) form a distinct cluster from the TORC1 complex (Tco89, Tor1, Kog1). Even though all proteins show very small changes in their ratios, there still are detectable changes. At this point we cannot make any conclusion if this has any biological relevance. However, the six subunits of the HOPS complex have very similar profiles. Similarly, the three detected subunits of TORC1 have very similar profiles, slightly different from that of HOPS subunits. Since we know for these proteins to physically interact with each other it makes perfect sense that their profiles are very similar. In general it is possible in proteomics experiments to predict protein-protein interactions based on similar behavior of proteins in complex samples. However, since this is not the major point of our manuscript we have adjusted the statement in the text to make this point clearer.

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3. The authors claim that most Vps10 cargo are depleted from vacuoles of yeast expressing the domain 2 mutant (lines 234-237). However, the data in Figure 3G indicates that domain 2-dependent sorting for Npc2, Yhr202W and Pep4 but not Ape3 and CPY. (CPY is an inconsistent case, because it is depleted in exp 1 but not in exp2.) The authors should adjust their claim to match the data, and discuss accordingly.

We agree with the referee that this data does not look convincing. However, since we are showing ratios here on a logarithmic scale, even a small change is still significant. As mentioned before, some replicates have lower peptide identification rates. Lower identification rates can cause larger errors in the quantification. We have now repeated the experiment and show more convincing data for the Vps10 domain mutants supporting our claims. We have replaced the experiments in the supplementary tables and in Figure 3.

4. In the text (lines 282-284) the authors mention that Vps10 is depleted in vps45 mutant vacuoles. In Figure 5B, however, Vps10 does not appear to be significantly depleted (it is classified as "unaffected"). The authors should change the text to reflect this, and adjust their discussion accordingly.

We thank the referee for highlighting this important point. Vps10 is indeed statistically not an outlier in the Vps45 results. As shown it is right at the border of significance and most likely a problem with one of the replicates in Vps45. However, since all the Vps10 cargo proteins appear significantly depleted we think that this is still a biologically relevant phenotype. We have adjusted the manuscript accordingly.

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Minor Points

1. In Figure 5G - the authors make the conclusion that Ncr1 and Atg15 are sorted to the vacuole by a mechanism that does not involve cytosolic recognition. For Ncr1, there are a multitude of other cytosolic loops which could harbor sorting signals. For Atg15, this interpretation is complicated by the fact that most GFP-tagged Atg15 appears to be retained in the ER, with only a small portion trafficking to vacuolar membranes. It is unclear if this localization pattern is an artifact of tagging. In fact, there may even be more Atg15 trafficking to the vacuole membrane when the N-terminal segment is deleted - suggesting the N-terminus may be involved in ER retention - but it is difficult to make any conclusion without a quantitative analysis.

We agree with the referee on this point. Because of the low abundance of the protein we were unable to reliably quantify the abundance of the protein in total cell extracts. This did not work by tagging and western blot as well as by mass spectrometry. We therefore switched to an indirect readout. Deletion of Atg15 resulted in an increase of plasma membrane proteins in isolated vacuoles. This suggests that Atg15 is required for the lysis of intraluminal vesicles from the endocytic pathway. We did not observe such an enrichment in cells where we deleted the N-terminus of Atg15. Together this suggests that Atg15 is still reaching the endo-lysosomal pathway, even if no cytosolic parts are present, thus supporting our hypothesis that it does not contain a cytosolic sorting motif. We have added these analyses in figure 5. We have also modified the manuscript to better explain the observed phenotypes for Atg15 and Ncr1.

2. The authors make the claim that "QPrevail can be used to study post-translational modification dependent protein targeting" (lines 141-143, lines 322-324). However, as far as I can tell the authors do not identify or quantify PTM-modified peptides. So I don't believe this claim is substantiated by the data presented in this paper.

We thank the referee for raising this point. This assumption is based on the detection of less Atg8 in atg4Δ vacuoles. We have now re-phrased this sentence in the introduction.

3. In Figure S5: I would predict that cargoes with both sorting signals would be unaffected in single mutants, but cargoes with one or the other would be sensitive to the single mutants. The authors should consider color-coding the plots in panels B and C to reflect the sorting signals indicated in panel A.

We thank the referee for raising this important point. We have no changed the color coding according to the referees suggestion. While we see a very convincing effect for Atg27 with its two YXXL motifs the data appear to be more convoluted for the dileucine motifs. However, the mutations in the APS3 are predictions based on the available crystal structure of the AP-2 complex. In summary our results suggest that the binding of cargoes to the AP-3 complex is rather complicated and can probably only solved by structural analysis of AP-3 in complex with different cargo molecules.
January 5, 2022

RE: JCB Manuscript #202107148R

Dr. Florian Fröhlich
Osnabrück University
Biology/Chemistry
Barabarastrasse 13
Osnabrück 49076
Germany

Dear Dr. Fröhlich:

Thank you for submitting your revised manuscript entitled "A lysosomal biogenesis map uncovers the cargo spectrum of lysosomal protein targeting pathways in yeast". 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,

Scott Emr
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Senior Scientific Editor

Journal of Cell Biology

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

The authors have included an analysis with vps27Δ mutants that helps buttress the analysis of cargo trafficking along an AP3 dependent pathway. These supplementary data along with the more reserved conclusions in the text offer room to readers to reinterpret the data as tools for future studies. This work will be of interest to JCB readers and is satisfactory, in my view, in its current state.

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

In their revised manuscript, Eising et al. have significantly improved upon their original submission. The authors were receptive to reviewer feedback and incorporated new data and changes to the text that have strengthened the overall manuscript. Notably, several aspects of the paper were made more rigorous and the authors have toned down their interpretations and addressed limitations, resulting in a manuscript that makes it easier for readers to interpret the data and draw conclusions.

Significant improvements include:
• The authors have improved quality assessment of their vacuole purification method by adding quantitative analysis comparing WT and mutant vacuole abundance and diameter (Figure 1C). This provides evidence that there is not significant changes to vacuole density or fragmentation associated with mutant strains. (Except in the case of the vps45 mutant, which is appropriately discussed in the manuscript.)
• The authors have clarified important points regarding Figure 2, such as the meaning of gray boxes. In particular the text added in lines 209-213 is very helpful. I also felt the revisions to the discussion of inset 6 (lines 201-208) were appropriate and clarified the interpretation of this particular cluster of complexes.
• The follow-up analysis characterizing the N-terminal deletion of Atg15 relative to atg15 deletion strains (Figure 5H) is a nice addition to the manuscript, and it supports the authors’ interpretation that N-terminal deletion of Atg15 does not impede its function.

As explained in their point-by-point response the authors have also toned down some interpretations of the data where appropriate. In general, the revised manuscript is very much improved and will be of interest to the membrane trafficking community.