



ESCRT, not intraluminal fragments, sorts ubiquitinated vacuole membrane proteins for degradation

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February 2, 2021

Re: JCB manuscript #202012104

Dr. Ming Li
University of Michigan-Ann Arbor
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1105 N. University Ave.
Ann Arbor, MI 48109

Dear Dr. Li,

Thank you for submitting your manuscript entitled "ESCRT, not intraluminal fragments, internalizes vacuole membrane proteins for degradation". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. Thank you for your patience with the peer-review process. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that the reviewers all feel that the paper provides important new data to address the controversy around the mechanism of vacuole membrane protein recycling for degradation. They do have some constructive comments that we feel will strengthen the work.

Rev#1 takes issue with how ILF/vacuole fusion events are defined (#1), and Rev#2 recommends including more info about the stat analyses (Rev#3 agrees), showing all the data, and ideally testing what you think may be the technical factors that led to the ILF model by McNally and Brett (e.g., could you test whether indeed it was the use of purified vacuole systems lacking the ubiquitin ligase machinery and free ubiquitin). Rev#1 is concerned that the rapamycin-inducible degradation system may drive ESCRT over-accumulation at the vacuole surface (#2).

We have discussed their points, which are valid. The issues raised by Reviewer #1 in our view need to be addressed for publication. Please also tackle the reviewers' comments regarding the reporting and use of statistical analyses and please also show as much of the data as possible. Concerning the scope issue raised by Rev #2, these experiments could make yours a definitive study, and a follow-up on your study that contains just this information will probably not be deemed as important. We look forward to your response on this point. While we encourage you to address this point, we will not require it for publication in JCB of this Report. Please let us know if you have any questions or anticipate any issues addressing the reviews. We would be happy to discuss as needed.

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.

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Text limits: Character count for a Report is < 20,000, 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: Reports may have up to 5 main text figures. To avoid delays in production, figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, <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. Reports may have up to 3 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 the Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Ira Mellman, Ph.D.
Editor, Journal of Cell Biology

Melina Casadio, Ph.D.
Senior Scientific Editor, Journal of Cell Biology

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

This study examines how yeast vacuole and plasma membrane transmembrane proteins are inducibly degraded. Two contrasting models of this degradation system have recently emerged: an ESCRT-dependent turnover model at the vacuole surface, and the intraluminal fragment (ILF)

pathway that is proposed to mediate ESCRT independent transmembrane cargo turnover (Karim 2018; McNally 2017). Here, the authors test whether cargoes previously reported to rely on the ILF pathway can be detected as ILF substates. Through Western blotting and time-lapse imaging, they conclude that proteins previously reported as taking the ILF pathway are in fact degraded via ESCRT-dependent turnover. They also show data indicating ESCRTs can act directly on Fth at the vacuole surface for turnover.

The study is essentially a deep investigation of the cues and mechanisms underlying protein turnover and the proposed ILF pathway from previous work. This present work here fails to reproduce several key observations from the ILF work, and instead finds examples where these cargoes undergo ESCRT dependent degradation at the vacuole surface in vivo. A key observation is that Zrc co-localizes with Fth at ILFs, indicating that there is no selective cargo sorting at ILFs.

Strengths of this study include careful mechanistic analysis, and use of immunoblotting and time-lapse imaging to interrogate protein turnover in real time. The experiments are well controlled and conclusive. This is also an important study because it addresses an emerging controversy in the field as to how these two opposing pathways could operate in yeast.

The only major concerns relate to how an ILF is operationally defined in this study compared to the original Karim/McNally works. Those studies utilized in vitro reconstitution and some in vivo imaging approaches, but this study identified ILF folds in vivo at the vacuole primarily by single-plane microscopy. There are parts (see below) where this may not be enough to conclude that these structures are bone fide ILFs. Secondly, the later part of the manuscript which uses a rapamycin-inducible degradation system may also artificially drive ESCRT accumulation at the vacuole surface, and should be dissected more closely.

Major concerns:

- 1) A concern is how to operationally define vacuole fusion events. In Figure 2I you show homotypic fusion events that either become ILFs or remain attached. From the data presented, it seems hard to make this distinction without at least some indication of the 3D shape of the vacuole. Using phrases like "detached" ILFs seem over-conclusive based on only this imaging.
- 2) Figure 5: The Rapa inducible degradation system is used here to demonstrate that ESCRTs can act on the vacuole surface to degrade Fth1-GFP. This is an elegant experiment, but this forced ESCRT recruitment is somewhat artificial. Can a more brief recruitment, then Rapa washout, be attempted so not so much ESCRT machinery is forced onto the vacuole surface? Alternatively, can a system be used with only mono-ubiquitin to reduce the affinity of ESCRT recruitment? These may help the experiment be a bit more physiological.

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

In the manuscript entitled "ESCRT, not intraluminal fragments, internalizes ubiquitinated vacuole membrane proteins for degradation," Yang et al show that sorting in the yeast vacuole is largely dependent on ESCRT machinery. The authors use multiple yeast strains to show that proteins previously shown to be sorted via intraluminal fragments, including Fth1 and Cot1, are actually sorted independent of these fragments but instead degraded via ESCRT-mediated sorting. This work provides detailed experimentation to prove their conclusions and is an important finding for

the community. However, there are some critical improvements that I think need to be included to increase the value of these findings: namely the authors should add in all the data they discuss but do not show and they should include more statistical analyses. Please see below for more detailed comments regarding concerns/suggestions that I think the authors should consider in order to improve the quality and value of this manuscript:

1. There are multiple times throughout the manuscript where the authors describe experimental results but do not show this data in the figures. I find this unacceptable and particularly in the case when the authors are attempting to prove previously published data to be flawed, I think it is important to show all the supporting data.

2. While the authors provide some hypothesis at the end of the manuscript to explain the discrepancy between their work and the previously published data supporting the ILF model, I think it would add to the quality of the work here if they could test some of these hypotheses. For example, the authors suggest that the previous papers used artificial systems that did not have important factors like Rsp5, Pib1, E1 or E2 enzymes. Could the authors use yeast strains lacking these proteins to see if that disrupts degradation of the membrane proteins via ESCRT mediated sorting and causes ILF-dependent sorting. This would prove that this is in fact why their model is superior to the previously published data.

3. The entire manuscript is lacking statistical analysis. Also, instead of showing bar graphs it would be nice to see the spread of the data by showing individual data points for each cell when graphing puncta/cell.

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

This study addresses an ongoing question in the field of regulated vacuolar membrane protein turnover and provides more compelling evidence in favour of an ESCRT mediated internalization of ubiquitinated receptors. In developing a new microfluidic chamber to monitor the turnover of vacuolar proteins over several hours in different conditions (genetics/drugs) they were able to distinguish between the two pathways *in vivo*. In following some of the core ILF clients Fth1, Fet5, Vph1, and the PM transporter Hxt3 they demonstrate the dependence on the ESCRT machinery and did not observe selective sorting into the ILF during vacuole fusion events. Much of the evidence for the ILF fragment is based on reconstitution experiments where the ubiquitination pathways are not active. The authors conclude that, while these fragments occur during fusion (~20% of events), they appear to refuse with the vacuolar membrane, and are not a functional pathway of degradation. The ILF pathway was previously shown to be activated during heat stress and cycloheximide treatment, but these authors could not validate in systems that included internal controls. An extensive analysis of ESCRT-independent intraluminal fragments also carried the negative control Zrc1, indicating that this would not be a selective mechanism for degradation. The data strengthens previous work delineating this pathway, adding new insights as well. The combination of the RapiDeg system along with a series of genetic mutations and treatments (in 2 different strains), all taking advantage of their new long term imaging assays and high resolution imaging is very strong.

I have no major concerns with the data as presented. Just 2 minor comments

- In 4C and S1 the wt and vps4D strains are separated by a dotted line in the image. Does this

mean they cut images from the 2 wells together? It wasn't clear in the legend how this was done.

- Statistical analysis wasn't described.

Reviewer #1:

Major concerns:

1) A concern is how to operationally define vacuole fusion events. In Figure 2I you show homotypic fusion events that either become ILFs or remain attached. From the data presented, it seems hard to make this distinction without at least some indication of the 3D shape of the vacuole. Using phrases like "detached" ILFs seem over-conclusive based on only this imaging.

We completely agree with this reviewer. Without a Z stack to cover the entire vacuole, we cannot say with confidence that some ILFs are "detached." In the original capturing for Fig. 2H-J, we took a stack of 2x0.4 μm at each time point, which was not enough to cover the entire vacuole. The normal size of a vacuole is 2-4 μm in diameter. The reasons for taking only two steps were two-fold: 1) To minimize photodamage. At every 2 minutes interval, taking a complete Z stack across the vacuole resulted in severe photobleaching, and cell growth stopped. 2) In the original ILF studies, MacNally et al. also only showed a 1 μm cross-section of vacuoles, which was not enough to cover the entire vacuole either (McNally et al., 2017).

Because we are not confident about the ratio of detached fragments in figure 2I and taking z-stacks caused severe photodamage in the time-lapse movie, we removed the quantification in the original figure 2J. Instead, we took z-stacks for the Fth1-Zrc1 colocalization experiment and quantified the ratio of detached intraluminal fragments. Our data indicated that 22% of intraluminal fragments are detached from the vacuole membrane (**Figure 2L**, n=205 ILFs from four biological repeats), which confirms that the formation of detached intraluminal fragments is rare.

Lastly, we wish to emphasize that the most critical points for figures 2H-L are: even though vacuoles undergo normal fusion and fission, and ILFs do form during this process, there is no selective sorting of Fth1 into ILFs, and Fth1 is not constitutively degraded.

2) Figure 5: The Rapamycin inducible degradation system is used here to demonstrate that ESCRTs can act on the vacuole surface to degrade Fth1-GFP. This is an elegant experiment, but this forced ESCRT recruitment is somewhat artificial. Can a more brief recruitment, then Rapamycin washout, be attempted so not so much ESCRT machinery is forced onto the vacuole surface? Alternatively, can a system be used with only mono-ubiquitin to reduce the affinity of ESCRT recruitment? These may help the experiment be a bit more physiological.

Thank you for your kind words on the Rapideg system. Again, we completely agree that Rapideg-induced ESCRT recruitment is somewhat artificial. It was designed based on our knowledge that vacuole membrane protein degradation is initiated by polyubiquitination. In our previous study, we have used ubiquitin blots to demonstrate that VM proteins are polyubiquitinated, not mono-ubiquitinated, before their degradation (Li et al., 2015a; Li et al., 2015b; Yang et al., 2020). This was the reason to use 3xUb, instead of 1xUb.

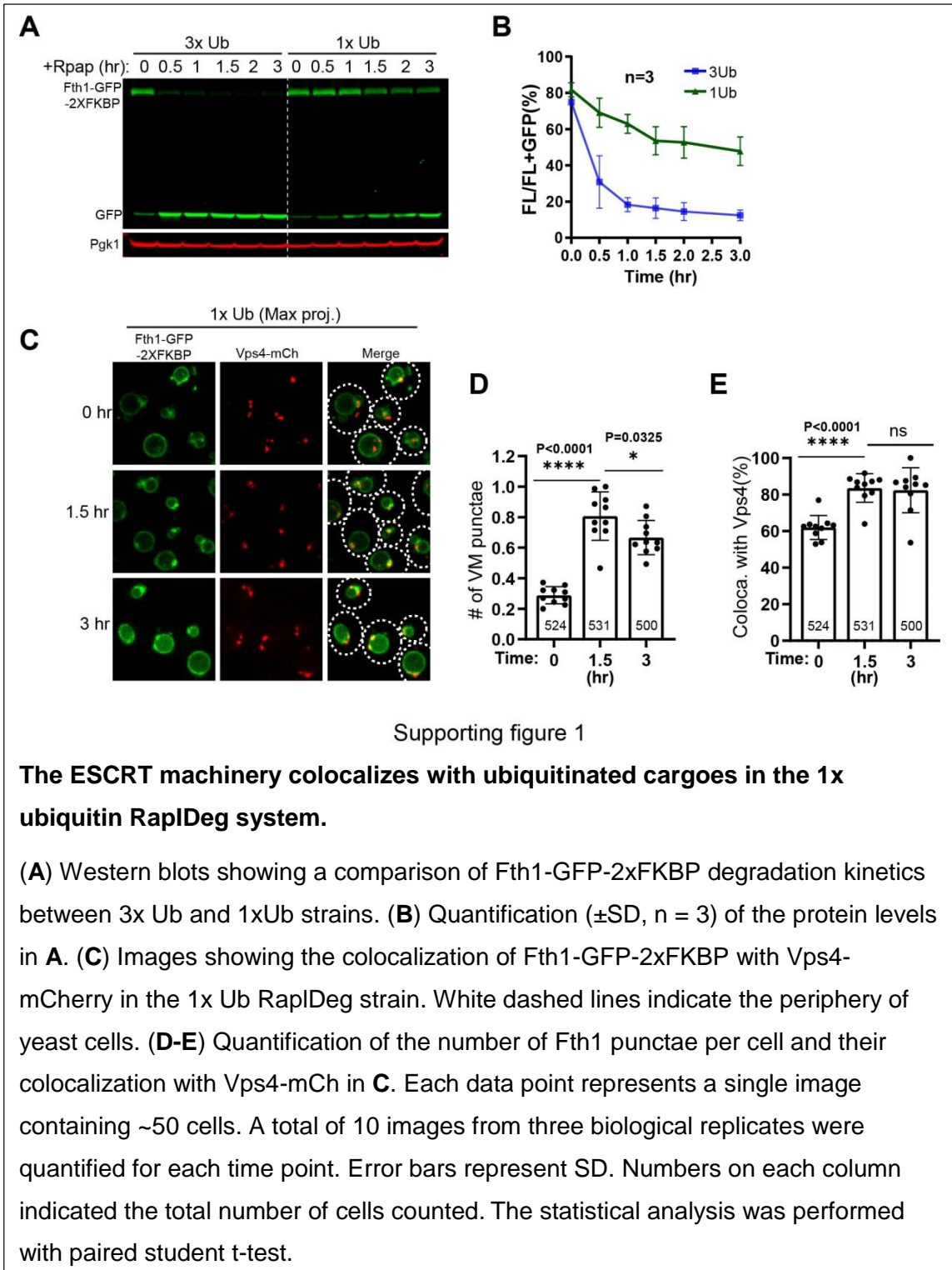
In our opinion, the 3xUb system demonstrated that the rate-limiting step in VM protein degradation is protein ubiquitination. Once proteins are ubiquitinated, ESCRTs can be quickly recruited onto the vacuole membrane to sort cargoes into the lumen for degradation. Under physiological conditions, the ESCRT machinery **can** act very efficiently. For example, the plasma membrane methionine transporter Mup1 is a very abundant protein (22,000 copies per cell)(Ho et al., 2018). It will be quickly degraded through endocytosis if yeast cells are exposed to a high concentration of methionine (Henne et al., 2012; Lin et al., 2008; MacDonald et al., 2015). The degradation can be completed within 45-60 minutes. During this process, ESCRTs can efficiently internalize all ubiquitinated Mup1 from the endosome surface. In comparison, Fth1 has ~5,000 copies per cell (Ho et al., 2018). Once Fth1 is "ubiquitinated" by the Rapideg system, it is not entirely surprising to see ESCRTs being quickly assembled on the vacuole membrane to sort Fth1 into the lumen for degradation.

We also tested the rapamycin washout experiment and could not reduce the protein degradation rate this way (data not shown). In hindsight, this result made sense because the FKBP-rapamycin-FRB ternary complex's affinity is very high (kd~10 nm)

(Banaszynski et al., 2005). Once the complex is formed, it will hardly dissociate. It can even withstand the force generated by the ESCRT machinery during cargo sorting and membrane deformation. In essence, attaching ubiquitin to cargo proteins through the Rapideg system can mimic the covalent bond of protein ubiquitination.

We then created a yeast strain that overexpresses FRB-1xUb. The 1xUb system degrades much slower than the 3xUb system, with ~ 50% of the cargo protein (Fth1) degraded after 3 hours of the rapamycin treatment (**supporting figure 1**). After 1.5 hours of rapamycin treatment, Fth1-GFP was sorted into punctate structures in most cells. Over 80% of these sorting structures colocalized the Vps4. Compared to the 3xUb system in **Fig. 5D-5F**, we observed fewer sorting structures (0.8 punctae/cell vs. 2.4 punctae/cell), and their sizes were much smaller. All these data were consistent with the idea that the affinity between 1xUb and ESCRT is much weaker. However, they still support the hypothesis that the ESCRT machinery is recruited to the vacuole membrane to sort ubiquitinated cargo proteins.

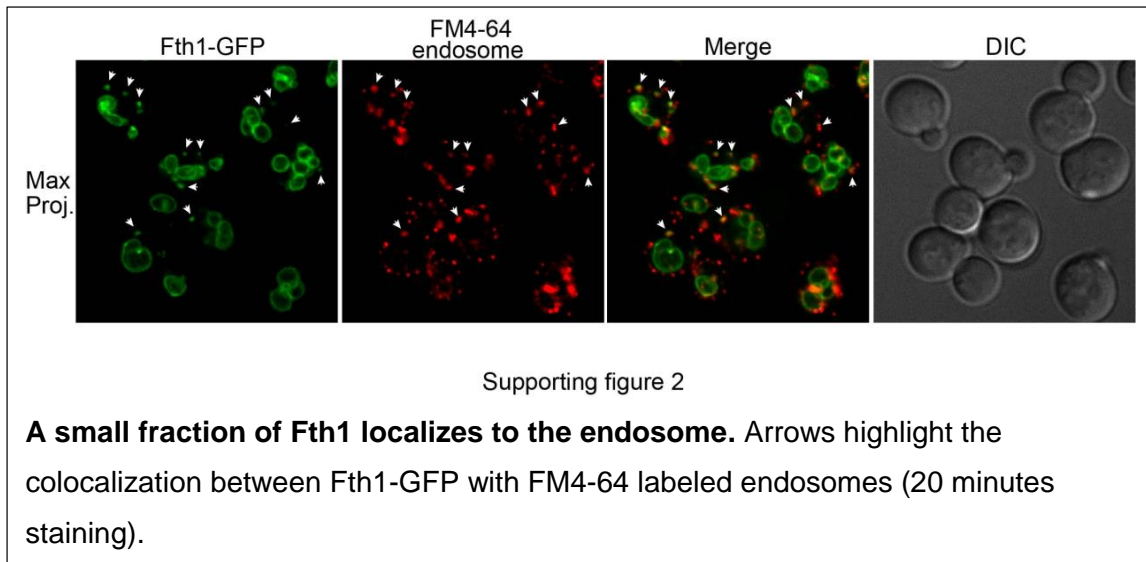
Because we reached the same conclusion with both 1xUb and 3xUb systems and cargo membrane proteins are poly-ubiquitinated before their degradation, we think it is reasonable to keep the data collected with the 3x Ub system. Although we will not include the 1xUb system in our manuscript, we will publish this rebuttal letter to show this valuable discussion. Thank you!

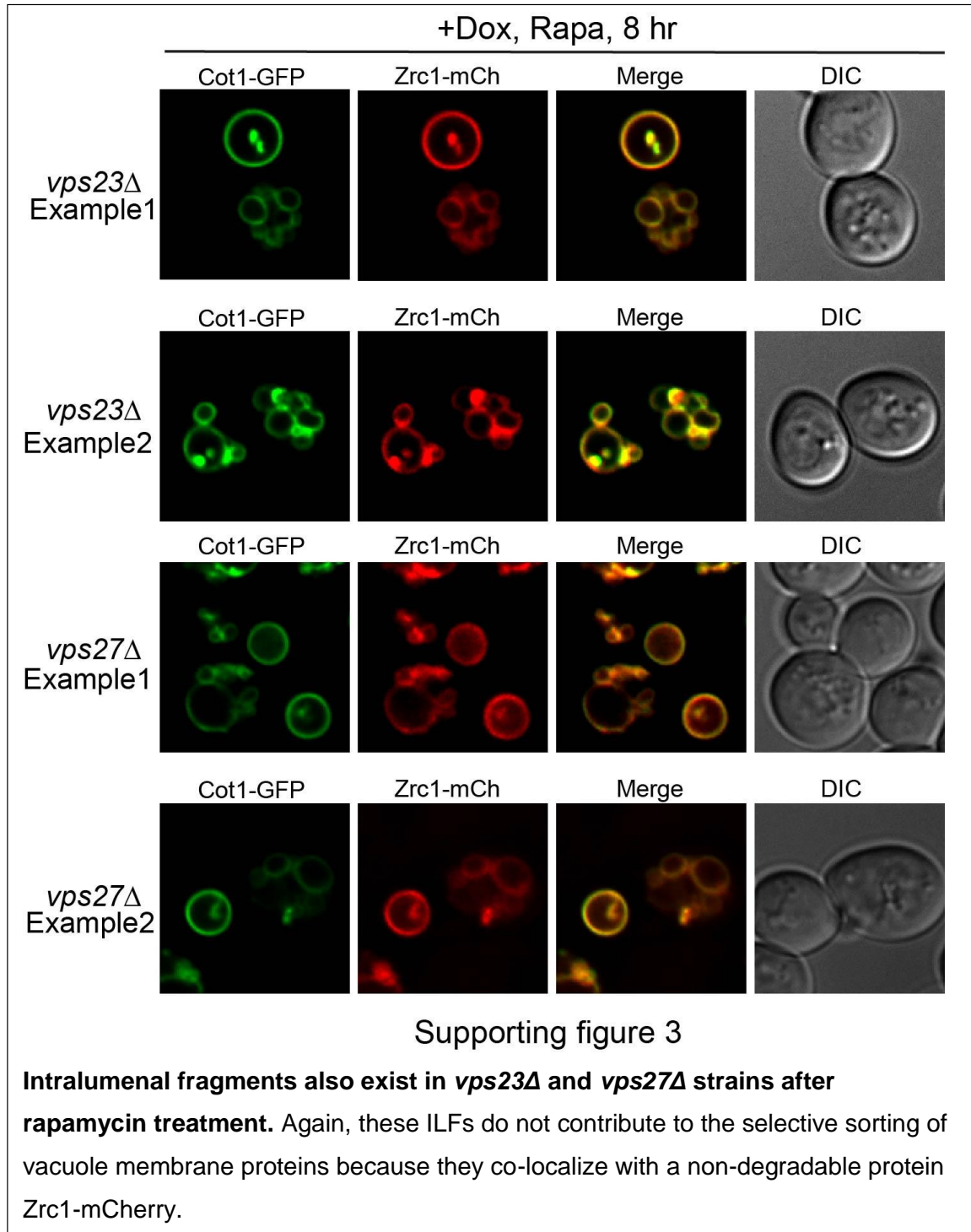


Reviewer #2:

1. There are multiple times throughout the manuscript where the authors describe experimental results but do not show this data in the figures. I find this unacceptable and particularly in the case when the authors are attempting to prove previously published data to be flawed, I think it is important to show all the supporting data.

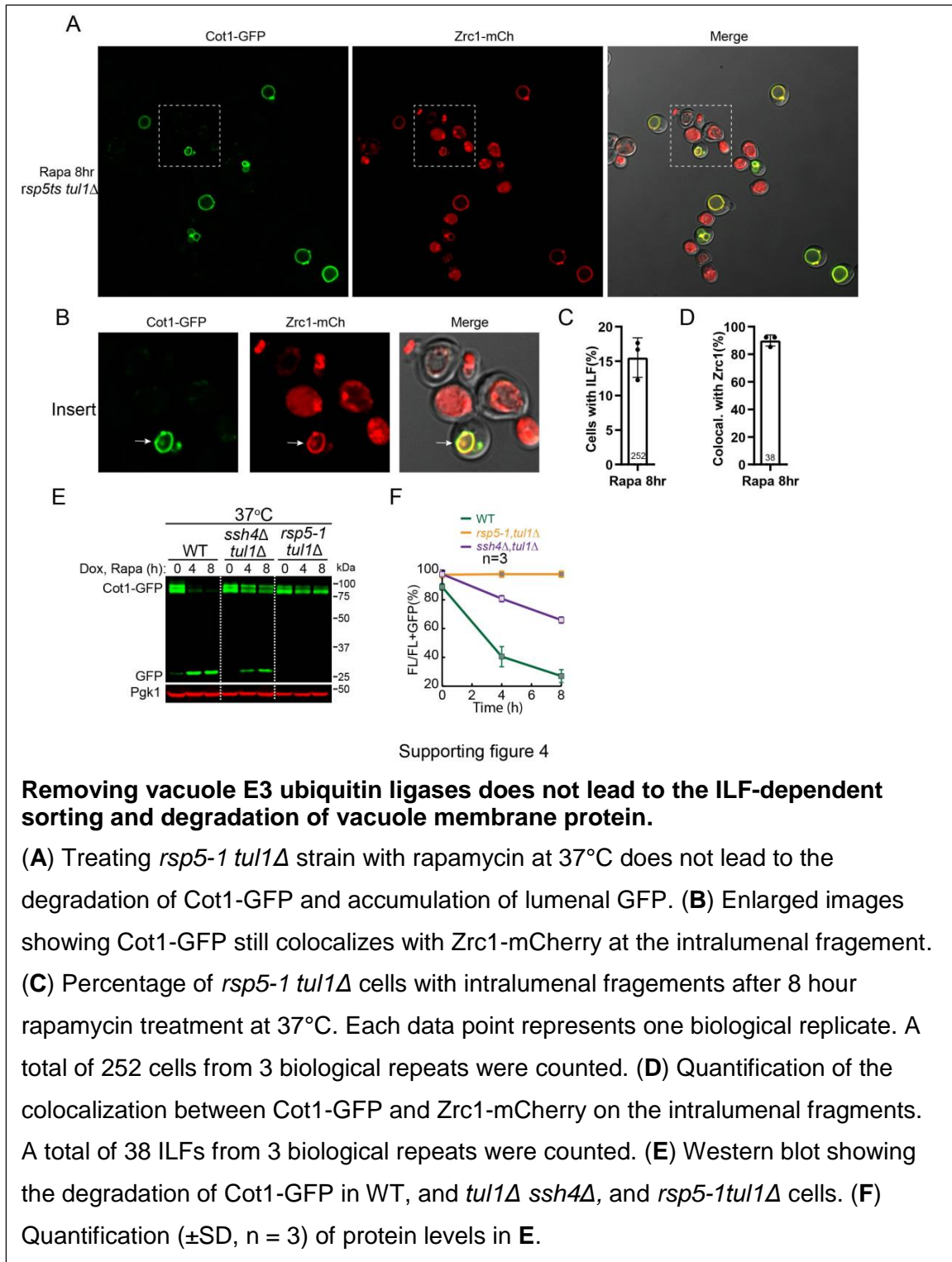
Thank you for pointing out this issue. We could not fit all the data in a JCB Report. In the previous submission, we used "data not shown" four times. We are now showing all of them as either supporting figures or supplemental figures. They include: 1) a small fraction of Fth1 localizes to the FM4-64 stained endosomes (**Supporting figure 2**); 2) neither heat nor CHX can trigger the degradation of vacuole membrane proteins in BY4741 (**figure S1**); 3) Intraluminal fragments also exist in *VPS23* and *VPS27* deletion strains after rapamycin treatment (**Supporting figure 3**); and 4) ESCRT deletion (*vps27 Δ* and *vps36 Δ*) blocked the degradation of Hxt3 in BY4741 (**figure S6**). We will publish this rebuttal letter to show all the data.





2. While the authors provide some hypothesis at the end of the manuscript to explain the discrepancy between their work and the previously published data supporting the ILF model, I think it would add to the quality of the work here if they could test some of these hypotheses. For example, the authors suggest that the previous papers used artificial systems that did not have important factors like Rsp5, Pib1, E1 or E2 enzymes. Could the authors use yeast strains lacking these proteins to see if that disrupts degradation of the membrane proteins via ESCRT mediated sorting and causes ILF-dependent sorting. This would prove that this is in fact why their model is superior to the previously published data.

Thank you for this interesting suggestion. We have performed two independent experiments to test the involvement of protein ubiquitination in the ILF pathway: **1)** we purified vacuoles and probed for ubiquitin, E1, E2, and E3 ligases. As shown in **figure S7** of the updated manuscript, only the transmembrane E3 ligase (represented by Ubx3, a Dsc complex component) was co-purified with the vacuole. We could not detect other necessary ubiquitination machinery, including free ubiquitin, E1, E2, Pib1, and Rsp5. This data confirmed that purified vacuoles could not carry out *in vitro* ubiquitination due to the lack of necessary components; **2)** we used a *tul1Δrsp5-1* strain to test if there is indeed a ubiquitin-independent ILF sorting of Cot1-GFP. This yeast strain is very sick. Over 60% of cells died and lost the GFP fluorescence after rapamycin treatment at 37°C (**supporting figure 4**). For cells that did survive the treatment, we can observe intraluminal fragments in 38 cells out of 252 cells from three biological repeats (15% cells). This is consistent with the idea that ILF formation is ubiquitin-independent. However, these ILFs still colocalized with Zrc1-mCherry (92% positive, 35 out of 38 counted ILFs). Most importantly, there is no accumulation of free GFP in the vacuole lumen even though we observed ILFs by imaging. Western blot also showed that Cot1-GFP degradation was blocked in the *tul1Δrsp5-1* strain (**supporting figure 4**) (Yang et al., 2020). All these data further confirmed that ILF is not involved in the selective sorting and degradation of vacuole membrane proteins.



3. The entire manuscript is lacking statistical analysis. Also, instead of showing bar graphs it would be nice to see the spread of the data by showing individual data points for each cell when graphing puncta/cell.

Thank you for pointing out this issue. All western blots in this manuscript were carried out three times. We quantified them, and the error bars in the graphs represented standard deviations. Some of the error bars were too small to be displayed. Furthermore, we included individual data points and performed the statistical analysis for most of the bar graphs in this resubmission.

Reviewer #3:

I have no major concerns with the data as presented. Just 2 minor comments

Thank you for the positive feedback!

- In 4C and S1 the wt and vps4D strains are separated by a dotted line in the image. Does this mean they cut images from the 2 wells together? It wasn't clear in the legend how this was done.

They were taken in the same well. The design was to show that, under precisely the same conditions, WT and *vps4* Δ respond differently to the rapamycin treatment. We added the dotted lines to separate the WT group from the *vps4* Δ group. In this revision, we added the following sentence in the figure legend: "WT and *vps4* Δ cells were imaged in the same chamber" Thank you!

- Statistical analysis wasn't described.

We added the statistical analysis. Please see the response to Reviewer 2, point 3, for details.

References:

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- Yang, X., Zhang, W., Wen, X., Bulinski, P.J., Chomchai, D.A., Arines, F.M., Liu, Y.Y., Sprenger, S., Teis, D., Klionsky, D.J., *et al.* (2020). TORC1 regulates vacuole membrane composition through ubiquitin- and ESCRT-dependent microautophagy. *J Cell Biol* *219*.

May 6, 2021

RE: JCB Manuscript #202012104R

Dr. Ming Li
University of Michigan-Ann Arbor
Molecular, Cellular, and Developmental Biology
Rm 3214, Biological Sciences Building
1105 N. University Ave.
Ann Arbor, MI 48109

Dear Dr. Li,

Thank you for submitting your revised manuscript entitled "ESCRT, not intraluminal fragments, internalizes ubiquitinated vacuole membrane proteins for degradation". We and the reviewers commend you for a thorough revision effort that clearly strengthened the study. We also agree with Rev#1 that adding the 1xUb system validation data to the paper would be valuable for readers (this would allow the data to be more easily accessed and more discoverable to all readers of the papers, as not all would read the peer review correspondence). We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

1) eTOC summary: A 40-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

- Please include a summary statement on the title page of the resubmission. It should start with "First author name(s) et al..." to match our preferred style.

2) JCB Reports are typically limited to 5 main and 3 supplementary figures. We can allow additional supp figures if they are needed. Still, we would greatly appreciate your efforts to try to combine some of the supp data to limit the number of figures please (perhaps down to 4 or even 5). Each figure can span up to 1 entire page as long as all panels fit on the page.

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends.

4) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.

- For all cell lines, vectors, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic

features *even if described in other published work or gifted to you by other investigators*

- Please include species and source for all antibodies, including secondary, as well as catalog numbers/vendor identifiers if available.
- Sequences should be provided for all oligos: primers, si/shRNA, gRNAs, etc.
- Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
 - a. Make and model of microscope
 - b. Type, magnification, and numerical aperture of the objective lenses
 - c. Temperature
 - d. imaging medium
 - e. Fluorochromes
 - f. Camera make and model
 - g. Acquisition software
 - h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

5) A summary paragraph of all supplemental material should appear at the end of the Materials and methods section.

- Please include one brief descriptive sentence per item.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

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-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, <https://jcb.rupress.org/fig-vid-guidelines>.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in the Journal of Cell Biology.

Sincerely,

Ira Mellman, Ph.D.
Editor, Journal of Cell Biology

Melina Casadio, Ph.D.
Senior Scientific Editor, Journal of Cell Biology

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

The revised manuscript addresses the majority of issues, and adds new experimental and supportive data that further examine the proposed model for vacuole protein turnover, while further comparing it to ILF-dependent sorting.

In particular, new experimental data presented in the Response to Reviewers letter (Supp Figure 1) uses a 1x Ubiquitin tagging system to dissect the influence of ESCRT-mediated turnover of Fth1-GFP (this 1xUb is compared to the 3x Ub tag in the original manuscript). This experiment nicely shows a slower turnover of Fth1-GFP that likely correlates with reduced ESCRT recruitment to the vacuole from the 1xUb tag. The new experiment also addresses the concern that the 3xUb recruitment may have been too artificial, and nicely supports the overall finding that ESCRTs can act on the vacuole surface. Other new additions include more supportive data comparing the previous ILF studies with this present study.

The only remaining suggestion is that the dataset in Supporting figure 1 should be included in the final manuscript version. This data helps validate the Fth1 FKBP-FRB system, and shows that a lower affinity, more physiologically-relevant recruitment of ESCRTs to the vacuole surface is still functional for Fth1 turnover. Therefore, it strengthens the manuscript, and should be included in the revision.

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

In the manuscript entitled "ESCRT, not intraluminal fragments, internalizes ubiquitinated vacuole membrane proteins for degradation," Yang et al show that sorting in the yeast vacuole is largely dependent on ESCRT machinery. The authors use multiple yeast strains to show that proteins previously shown to be sorted via intraluminal fragments, including Fth1 and Cot1, are actually

sorted independent of these fragments but instead degraded via ESCRT-mediated sorting. This work provides detailed experimentation to prove their conclusions and is an important finding for the community. In this revised version, the authors have included additional statistical analysis and added additional supplementary data to the manuscript to support many of their conclusions. The data presented in the current version provide convincing evidence to support the authors' claims and will be an important addition to the field.