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VPS37A directs ESCRT recruitment for phagophore closure

Yoshinori Takahashi, Xinwen Liang, Tatsuya Hattori, Zhenyuan Tang, Haiyan He, Han Chen, Xiaoming Liu, Thomas Abraham, Yuka Imamura-Kawasawa, Nicholas Buchkovich, Megan Young, and Hong-Gang Wang

Corresponding Author(s): Hong-Gang Wang, Penn State College of Medicine and Yoshinori Takahashi, Penn State College of Medicine

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March 25, 2019

Re: JCB manuscript #201902170

Prof. Hong-Gang Wang
Penn State College of Medicine
500 University Dr.
Hershey, PA 17033

Dear Prof. Wang,

Thank you for submitting your manuscript entitled "VPS37A recruits CHMP2A and VPS4 to orchestrate phagophore closure". 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 will see that the reviewers - and we agree - found the results interesting. They provided suggestions that we find constructive and important to ensure that the conclusions are robustly supported by the data.

In particular, Rev#2 requests more evidence that VPS37A recruits CHMP2A and is found inside the phagophore after closure, independently of the Halo-tag approach (point #2), that VPS37A recruits CHMP2A and VPS4 (#3) and that VPS37A's role Is independent of EGFR degradation but solely relies on the autophagy role (#1). We editorially find these points essential to address rigorously in a revision to directly support the conclusions. We feel that addressing these points will strengthen the study significantly.

Rev#1 provided comments that seem addressable via text edits and clarifications in the manuscript text, and these clarifications and additions would in our view help make the manuscript a compelling contribution to the field. Please consider and address all of this referee's comments as technically possible.

Rev#3 is concerned that the work fails to pinpoint how VPS37A recruits CHMP2A to phagophores, whether CHMP2A acts in isolation or needs the rest of ESCRT-III. Like Rev#1, the ref wants more info about the screen and hit prioritization (#1), they also request clarifications about the distribution of VPS37A and CHMP2A (#2), additional quantifications (#3). They suggest exploring further how CHMP2A is recruited by VPS37A (#4-5). Rev#3 additionally asks for evidence that ESCRT recruitment to autophagosomes is different from ESCRT recruitment to damaged membranes (#6) and stresses that the data must be robust and reproducible (#7). We feel that Rev#3's points are important and would encourage you to tackle all these points in revision experimentally -- with the exception of part of point #4 and point #5. Addressing point #4 subpoint a) "is the UBAP also dispensable for autophagosome closure?" and subpoint b) "Does CHMP2A get recruited directly by VPS37A or are intervening components of ESCRT-I, -II, and -III needed?" would help deepen the understanding of your proposed mechanism and we feel that these questions should be a priority in the revision process. On the other hand, subpoint c) "the authors should show the mechanistic link between VPS37's UEV and CHMP2A" should be addressed to your best ability and also by addressing the prior subpoints a) and b), but, beyond that, further mechanistic

understanding is not absolutely necessary. In our view, what to do exactly to assess how VPS37A controls CHMP2A is not clear, and thus, although advised, it is not absolutely necessary beyond addressing Rev#3 comment #4, subpoints a) and b). Regarding Rev#3 point #5, you could simply tone down the claim of ubiquitin as also pointed out by Rev#1, since, from the reviewers' comments, it is likely the UEV domain does not bind ubiquitin. Lastly, to address point #7, please ensure that you have used an appropriate sample size for the statistical analyses performed.

Please let us know if you would anticipate any issue addressing these points; we would be happy to discuss the revisions further 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.

GENERAL GUIDELINES:

Text limits: Character count for an Article 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: Articles 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, http://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. Articles 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.

The typical timeframe for revisions is three months; if submitted within this timeframe, novelty will not be reassessed at the final decision. 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,

Richard Youle, PhD
Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

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

Here, Takahashi et al. follow up on their recent paper Nature Comms. paper, which had previously carried out an imaging based screen of a small set of candidate genes using membrane permeable and impermeable Halo ligand to find that the ESCRT-III subunit CHMP2A and the ESCRT-III-binding ATPase VPS4 carry out autophagosome closure. ESCRT-III and VPS4 are part of a "generic" membrane scission machinery that functions in many areas of biology, and the N. Comms. paper left unanswered the question as to what specifically targets ESCRTs to unclosed phagophores. In the current paper, the screen was modified such that it can be carried out by FACS with genome-wide coverage. Among the strongest hits, ESCRT-I subunit VPS37A was identified, which fits with the upstream role of ESCRT-I in the pathway. There are four human VPS37 genes, and the authors show that VPS37A is dispensable for canonical sorting of ubiquitinated cargo, whilst required for phagophore closure. Moreover, the specialized function of VPS37A in phagophore closure was mapped to its unique N-terminal domain, which is probably a UEV domain. While the ligand for the VPS37A UEV domain is still unidentified, overall the advance is a substantial addition to our understanding of phagophore closure, the data (in particular the exceptionally robust VPS37A phenotypes in Figs. 2-6) are convincing, and the manuscript is recommended for publication following fairly minor improvements.

1. UEV nomenclature and discussion, para. 2. The evidence that the VPS37A domain is a UEV is a bit spare. Stuchell et al. 2004 noticed that this region was annotated as a "ubiquitin E2 conjugating enzyme" in SwissProt, and Stuchell et al. changed the nomenclature to the non-catalytic "UEV" on the grounds that there is no Cys in this region, which is required for E2 activity. While Stuchell et al. are correct that this region cannot be an active E2 enzyme, the sequence homology to the E2 is very low, and to the TSG101 UEV, nonexistent. The bottom line is the literature and evidence as to the identity of this domain are weak. The authors avoid speculating that this region of VPS37A binds to ubiquitin, but readers are likely to make this inference. In the absence of a better idea, I suggest calling it a "putative UEV domain" the first time it is referred to, and adding a brief mention in the discussion (para. 2) that the structure, function, and binding partners of this domain remain to be elucidated, and to emphasize there is no evidence available as to whether this domain of VPS37A binds to ubiquitin or PTAP motifs, or not. The reference to the binding properties of the TSG101 UEV domain, which has no sequence homology to VPS37A, is misleading in this part of the discussion.

2. Hit prioritization. More explanation was needed as to how hits were selected for secondary screening. What was the rationale for secondary screening of myocillin and SCAP, which are not known to be involved in membrane scission or autophagosome biogenesis? Do the negative results for MYOC and SCAP in Fig. 1D suggest that about half of the p2 hits in Fig. 1C are noise? Table S1 hits include all of the other ESCRT-I subunits TSG101, VPS28, VPS37B-D, MVB12A/B and UBAP1/2(L). What does their position lower down on the list say about their role in phagophore closure? It is hard to imagine that VPS37A is working without forming a complex with TSG101 and VPS28, plus some subset of the MVB12/UBAP subunits. Some secondary follow-up on at least one

or two of these would have been interesting.

3. Pg. 8, call-out to Fig. 3E, F for expression of VPS37A splice variants- Fig. 3E, F do not show these data, nor do they seem to be shown elsewhere.

4. Fig. 4B uses Baf clamping to measure flux, but it is becoming more standard to use the tandem LC3 red/green reporter for this. It would strengthen the conclusions to use the red/green reporter assay, although I don't consider it strictly essential.

5. The data in Fig. 7 are not as compelling as the rest of the manuscript. This is a hard experiment, because VPS4-DN is so potent at trapping assemblies of ESCRT-III such as CHMP2A. The effect of VPS37A KO is statistically significant but not as impressive as the data in earlier figures. The authors may want to give some thought to either omitting these data or adding some discussion about why the effects are not so dramatic.

6. The possibility that EPG5 is the ligand for the VPS37A UEV domain is worth one sentence in the discussion.

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

This manuscript provides a follow up discovery to the authors recent discovery that CMHP4 and Vps4 act during phagophore closure. Crucial to the previous discovery and this subsequent follow up was the development of a clever assay using membrane permeable and membrane impermeable ligands which bind halo-tag LC3. Using this assay the authors perform a genome wide screen to identify new genes involved in phagophore closure. The previous assay was modified to allow a high-throughput analysis. A few top hits from the screen were selected and further validated. Amongst these were Epg5, CHMP2A and Vps37A. The authors focused on Vps37A and demonstrated it was required for closure, and this was attributed to the UEV domain. Vps37A appears to recruit CHMP2A and may remain inside the phagophore after closure.
Overall the manuscript is well written, and the data clearly presented. The authors use the Halo-tag system extensively, and have good biochemical and morphological data to support their conclusions. However, there are points which are not sufficiently addressed by their investigation.
Major points
1. Using EGFR degradation the authors conclude that Vps37A is not affecting endocytic function and therefore the inhibition is due to a specific effect on phagophore closure. In Figure 4 the authors should present the data as a time course as it is not clear the differences they report are significant, or how they have calculated the half-life. In addition, the decreased degradation in the KO should result in increased recycling of the receptor. Furthermore, they should demonstrate that the rescue is inhibited by expression of DN-Vps4.
2. The conclusion that Vps37A recruits CHMP2A and is found inside the phagophore after closure requires further validation independently from the Halo-tag. The authors should demonstrate directly that Vps37A is found inside closed autophagosomes using for example a tandem-fluorescence tag on Vps37A. Further biochemical analysis is needed to support the degradation of Vps37A sequestered by the closed autophagosome.
3. The authors should provide more evidence to strengthen the conclusion stated in the title that Vps37A recruits CHMP2A and Vps4. Mutation or deletion of the binding domain between Vps37A and CHMP2A should be tested biochemically and morphologically.

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

Takahashi et al., present a follow up manuscript to their recent Nat Comms publication describing a new assay for autophagosome closure that, using siRNA, revealed a role for CHMP2A in closing autophagosomes. In this assay, halo-tagged LC3 is selectively illuminated with saturating doses of a membrane impermeant probe (MIL) to label internal and external faces of open autophagosomes. Subsequent labelling with a membrane permeant probe (MPL) will allow the illumination of LC3 on the internal face of closed autophagosomes, but will not bind to LC3 on open autophagosomes as binding sites would have been pre-saturated by the MIL. MPL would also illuminate LC3 present on autolysosomes, but here, LC3 would be inaccessible to MIL. As such, but discriminating between the relative MIL and MPL signals, the authors can interpret the extent of autophagosome closure.

Their previous publication identified only CHMP2A as a requirement for AP closure, which was a somewhat surprising result. Here, the authors employ genome wide CRISPR screening to identify further candidates including the ESCRT-I component VPS37A (and EPG5 (a protein previously implicated in autophagy), MYOC and SCAP). The authors focus on VPS37A and identify a role for the putative VPS37A UEV domain (absent from other VPS37 proteins) in localisation to the phagophore, recruitment of CHMP2A and autophagosome closure.

The data are generally convincing and I find this an interesting manuscript. The findings, however, are little incremental as the role of the ESCRT machinery was previously identified using this assay. This study adds just one extra protein to the list, but barring some requirement for the UEV domain, doesn't demonstrate how VPS37A recruits CHMP2A to this structure, nor whether CHMP2A acts in isolation or needs the rest of ESCRT-III.

Specific points related to the manuscript's findings follow below:

1. The gates used for the FACS based assay are rather large, the MIL+MPL- (reflecting presumably unclosed autophagosomes) is 10% of the population. It wasn't clear how many of these hits were involved in AP closure, or how the authors narrowed their screen to 5 genes of interest. The provided table S1 lists all the target genes in the library and no legend is provided or discussed in the text as to how these hits were settled on. After selecting these genes, the secondary screen was performed in the absence of BafA1 - I wasn't sure why this was as BafA1 was present for the primary screen.
2. Quantification (and indication of N numbers) of the EM in Figure 2 is needed. The APs have large voids, which is surprising. Is this a fixation artefact? The autophagosomes themselves have very large gaps suggesting a defect in formation, rather than the final closure. Where does VPS37A localise during this process? Figure 6 just shows localisation to the whole round autophagosome. Is this consistent with CHMP2A?
3. The enhancement of LC3-II in crVPS37A cells upon starvation was not greatly obvious (Figure 3E) and needs quantification.
4. It is not clear what the UEV domain is doing to allow autophagosome closure to occur. That it is dispensable for EGFR degradation is interesting, but doesn't explain mechanistically what this domain is doing. In light of the dispensability for MVB sorting, is UBAP (which forms a MVB-specific ESCRT-I complex) also dispensable for autophagosome closure? Does CHMP2A get recruited directly by VPS37A, or are intervening components of ESCRT-I, -II and -III needed? As now a genome wide CRIPSR screen and a genome wide siRNA screen have failed to identify these intermediates, I think the authors should show the mechanistic link between VPS37's UEV and

CHMP2A.
5. Regarding point 4, the authors speculate ubiquitin may be recruiting VPS37A via its UEV domain. However, residues required for ubiquitin binding are thought to be absent in VPS37A's UEV domain (Stuchell, JBC, 2004). The authors should show whether this domain binds ubiquitin if they want to propose something in contrast to the literature.
6. Recently, ESCRT-III has been proposed to localise to damaged endomembranes. As the HT-LC3 assay employs a membrane permeabilization and cytoplasmic extraction to allow illumination of membrane-bound LC3 with MIL, it would be good to demonstrate ESCRT recruitment to APs was distinct from ESCRT recruitment to damaged membranes.
7. In general, many of the data were n = 2 or not stated. I think the authors should ensure n = 3 for as many studies as possible.

1st Revision - Authors' Response to Reviewers: June 25, 2019

Point-By-Point Discussion (Manuscript #201902170)

We thank the referees for their positive assessment of our study and constructive criticisms to further improve our manuscript. We have endeavored to attend to each of the issues raised through additional experimentation and revisions to the text and supplemental information. A point-by-point description follows:

Reviewer #1

1-1) *UEV nomenclature and discussion, para. 2. The evidence that the VPS37A domain is a UEV is a bit spare. Stuchell et al. 2004 noticed that this region was annotated as a "ubiquitin E2 conjugating enzyme" in SwissProt, and Stuchell et al. changed the nomenclature to the non-catalytic "UEV" on the grounds that there is no Cys in this region, which is required for E2 activity. While Stuchell et al. are correct that this region cannot be an active E2 enzyme, the sequence homology to the E2 is very low, and to the TSG101 UEV, nonexistent. The bottom line is the literature and evidence as to the identity of this domain are weak. The authors avoid speculating that this region of VPS37A binds to ubiquitin, but readers are likely to make this inference. In the absence of a better idea, I suggest calling it a "putative UEV domain" the first time it is referred to, and adding a brief mention in the discussion (para. 2) that the structure, function, and binding partners of this domain remain to be elucidated, and to emphasize there is no evidence available as to whether this domain of VPS37A binds to ubiquitin or PTAP motifs, or not. The reference to the binding properties of the TSG101 UEV domain, which has no sequence homology to VPS37A, is misleading in this part of the discussion.*

Response: We thank the reviewer for the thoughtful suggestions. As he/she mentioned, there is less homology between the TSG101 UEV and the VPS37A UEV-like domain and the residues responsible for the interaction with ubiquitin and the PT/SAP motif in the TSG101 UEV domain are not conserved in the VPS37A PUEV domain (PMID: 15218037; 15240819). In the revised manuscript, we have clearly stated these facts, renamed the domain as 'putative UEV domain (PUEV)' and modified the figures accordingly.

1-2) *Hit prioritization. More explanation was needed as to how hits were selected for secondary screening. What was the rationale for secondary screening of myocillin and SCAP, which are not known to be involved in membrane scission or autophagosome biogenesis? Do the negative results for MYOC and SCAP in Fig. 1D suggest that about half of the p2 hits in Fig. 1C are noise? Table S1 hits include all of the other ESCRT-I subunits TSG101, VPS28, VPS37B-D, MVB12A/B and UBAP1/2(L). What does their position lower down on the list say about their role in phagophore closure? It is hard to imagine that VPS37A is working without forming a complex with TSG101 and VPS28, plus some subset of the MVB12/UBAP subunits. Some secondary follow-up on at least one or two of these would have been interesting.*

Response: We apologize for not clearly describing the hit prioritization. As stated in the revised manuscript, we have chosen the 5 genes for secondary screening based on their highest probability scores and functional implications in autophagy (PMID: 30093494 (VPS37A); 20550938 (EPG5); 24732711 (MYOC); 30462530 (SCAP); 30030437 (CHMP2A)) and found that 2 genes (VPS37A and EPG5; crCHMP2A cells failed to grow and thus were excluded from the analysis) are potentially involved in phagophore closure. Other hits (MYOC and SCAP) could be simply 'noise' as the reviewer mentioned or autophagy suppressors since the secondary screening was performed in the presence of BafA1 to exclude genes whose loss enhances autophagy induction without blocking the flux. Regarding the involvement of other ESCRT components in phagophore closure, while our screening only identified CHMP2A and VPS37A as the potential ESCRT genes required for phagophore closure, we fully agree with the idea that VPS37A functions together with the other ESCRT-I components. We think that the failure to detect other ESCRT components in our screen are attributed to their other functions required for cell growth/survival. Therefore, in the revised manuscript, we performed the HT-LC3 assay after a short-term depletion of ESCRT-I components (TSG101, VPS28, UBAP1) and ESCRT-II VPS25 to examine the effects on phagophore closure. Our data show that depletion of the core ESCRT-I components TSG101 and VPS28, but not the endosome-specific ESCRT-I component UBAP-1 or ESCRT-II VPS25, accumulates MIL^+ immature autophagosomal membranes (**Figure 8A, B**). Moreover, while the loss of TSG101 or VPS28, but not VPS37A and UBAP1, affects the stability of other ESCRT-I components (**Figure 8C**), we find that VPS37A loss impairs GFP-VPS28 localization to the phagophore (**Figure 8D, E**). These results support the idea described above and further strengthen our conclusion.

1-3) *Pg. 8, call-out to Fig. 3E, F for expression of VPS37A splice variants- Fig. 3E, F do not show these data, nor do they seem to be shown elsewhere.*

Response: We apologize for the confusion. While the anti-VPS37A antibody used in this study detects VPS37A regardless of the PUEV domain (**Figure 5B**), we do not observe any VPS37A-specific bands below 37 kDa in our blots (**Figure 4C, F**). As the predicted molecular sizes of the PUEV domain-lacking variants are less than 37 kDa (**Figure 5A**), we conclude that the PUEV-containing variants are the major forms of VPS37A expressed in our system. We have modified the text accordingly to avoid reader's confusion.

1-4) *Fig. 4B uses Baf clamping to measure flux, but it is becoming more standard to use the tandem LC3 red/green reporter for this. It would strengthen the conclusions to use the red/green reporter assay, although I don't consider it strictly essential.*
Response: We have performed the mRFP-GFP-LC3 assay to further demonstrate the importance of VPS37A in autophagic flux (**Figure 4A, B**).

1-5) *The data in Fig. 7 are not as compelling as the rest of the manuscript. This is a hard experiment, because VPS4-DN is so potent at trapping assemblies of ESCRT-III such as CHMP2A. The effect of VPS37A KO is statistically significant but not as impressive as the data in earlier figures. The authors may want to give some thought to either omitting these data or adding some discussion about why the effects are not so dramatic.*
Response: We fully agree with the comment that the inhibitory effect of VPS37A depletion on the phagophore localization of CHMP2A is not as strong as the data in other figures. As the reviewer mentioned, this may be attributed to the overexpression of DN-VPS4 that potently traps ESCRT-III assemblies such as CHMP2A. We propose that the trapping of CHMP2A on perturbed endosomes in DN-VPS4-expressing cells may allow for the delivery of CHMP2A to LC3-postive membranes upon abnormal endosome/lysosome-phagophore fusion (PMID: 30030437). Alternatively, this phenotype may occur by a mechanism that partially bypasses the requirement of ESCRT-I for CHMP2A recruitment. To support the later possibility, during the revision of this manuscript, it has been reported that Rab5-dependent interaction between Snf7 and Atg17 leads to the recruitment of ESCRT-III for autophagosome closure in yeast (PMID: 31010855). These possibilities are now discussed in the revised manuscript.

1-6) *The possibility that EPG5 is the ligand for the VPS37A UEV domain is worth one sentence in the discussion.*
Response: Thank you for providing us an opportunity to explore the link between EPG5 and VPS37A. Unfortunately, our co-immunoprecipitation/GFP-Trap analysis fails to detect the interaction between VPS37A and EPG5 (data not shown). However, this does not exclude the potential involvement of EPG5 in the recruitment of other ESCRT components. Therefore, in the revised manuscript, we simply mention "ESCRT recruitment" as a potential function of EPG5 in autophagosome completion.

Reviewer #2

2-1) *Using EGFR degradation the authors conclude that Vps37A is not affecting endocytic function and therefore the inhibition is due to a specific effect on phagophore closure. In Figure 4 the authors should present the data as a time course as it is not clear the differences they report are significant, or how they have calculated the half-life. In addition, the decreased degradation in the KO should result in increased recycling of the receptor. Furthermore, they should demonstrate that the rescue is inhibited by expression of DN-Vps4.*
Response: We appreciate the reviewer for this comment to improve our manuscript. In the revised manuscript, we have added the time-course data of EGFR degradation shown as nonlinear regression curve, which was used to calculate the half-life of EGFR in the original figure. As shown in **Figure 5E**, depletion of VPS37A significantly delayed lysosomal degradation of EGFR upon EGF stimulation. However, we would like to stress the fact that the effect of VPS37A depletion on EGF-stimulated EGFR degradation is very mild compared to that observed for phagophore closure, likely due to the expression of other VPS37A homologues including VPS37B-D (note that these homologues do not contain the PUEV domain). In agreement with this theory and as described in response *1-2*, unlike TSG101 or VPS28 depletion, VPS37A loss is not sufficient to destabilize other ESCRT-I components. Regarding the suggestion to use DN-VPS4 for the rescue experiment, unfortunately, we were unable to perform the experiment due to the toxic effect of DN-VPS4 expression that is exaggerated during overnight serum starvation required to promote the plasma membrane localization of EGFR. As an alternative approach, we used BafA1 to verify that the restoration of delayed EGFR degradation by both the VPS37A variants requires the intact endolysosomal pathway (**Figure 5F**).

2-2) *The conclusion that Vps37A recruits CHMP2A and is found inside the phagophore after closure requires further validation independently from the Halo-tag. The authors should demonstrate directly that Vps37A is found inside closed autophagosomes using for example a tandem-fluorescence tag on Vps37A. Further biochemical analysis is needed to support the degradation of Vps37A sequestered by the closed autophagosome.*
Response: We have repeated the autophagic flux assay to verify the lysosomal turnover of VPS37A that is enhanced by starvation (**Figure 4E**). In addition, as the reviewer suggested, we have generated VPS37A KO cells that are stably expressing mRFP-GFP-VPS37A FL or ΔPUEV. We find that, in FL-expressing cells, starvation induces further accumulation of $mRFP^{+}GFP^{-}$ foci, in agreement with the observation that VPS37A FL can be sequestered within autophagosomes and degraded by lysosomes (**Figure 6Avii-ix, C**). In contrast, while starvation also induces ΔPUEV foci, these structures are found to be non-degradative ($mRFP^{+}GFP^{+}$) (**Figure S2**) and negative for LC3 (**Figure 7A**). However, unlike mRFP-GFP-LC3 (**Figure 4A**), we also observe many $mRFP^{+}GFP^{-}$ foci in both FL- and ΔPUEV-expressing cells even under untreated conditions (**Figure S2**). Consistently, our HT-LC3 assay result shows that BafA1 can increase the levels of GFP-VPS37A even in the absence of PUEV although the level of ΔPUEV accumulation is much lower than that of FL (**Figure 6C**). Since the delay in EGFR degradation by VPS37A loss can be rescued by the expression of both FL and ΔPUEV (**Figure 5E**), these observations suggest that, in addition to autophagic sequestration which is induced upon starvation, endolysosomal sequestration of VPS37A may also occur via the MVB pathway although we think that this is beyond the focus of this manuscript and requires further experimental demonstration.

2-3) *The authors should provide more evidence to strengthen the conclusion stated in the title that Vps37A recruits CHMP2A and Vps4. Mutation or deletion of the binding domain between Vps37A and CHMP2A should be tested biochemically and morphologically.*
Response: We thank the reviewer for this comment to strengthen the conclusion and apologize for any confusion due to the statement in the original title. We would like to stress the fact that we do not have any evidence that VPS37A can directly bind to CHMP2A nor do we think that the direct association is the mechanism for the recruitment of CHMP2A. As described in response *1-2*, our new data show that other ESCRT-I components including TSG101 and VPS28 are also required for autophagosome completion and that VPS37A is required for the phagophore accumulation of GFP-VPS28 upon CHMP2A depletion. These new results combined with our original data demonstrate that VPS37A functions together with TSG101 and VPS28 to direct ESCRT recruitment for phagophore closure. We thus have edited the title to 'VPS37A directs ESCRT recruitment for phagophore closure'.

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

3-1) *The gates used for the FACS based assay are rather large, the MIL+MPL- (reflecting presumably unclosed autophagosomes) is 10% of the population. It wasn't clear how many of these hits were involved in AP closure, or how the authors narrowed their screen to 5 genes of interest. The provided table S1 lists all the target genes in the library and no legend is provided or discussed in the text as to how these hits were settled on. After selecting these genes, the secondary screen was performed in the absence of BafA1 - I wasn't sure why this was as BafA1 was present for the primary screen.*
Response: We apologize for not clearly describing the hit prioritization. As described in the response *1-2*, we chose the 5 genes based on their scores and functional implications in autophagy. Regarding to the screening conditions, while the primary screen was conducted in the absence of BafA1, the secondary screen was performed in the presence of BafA1 to exclude genes whose loss simply induces autophagy.

3-2) *Quantification (and indication of N numbers) of the EM in Figure 2 is needed. The APs have large voids, which is surprising. Is this a fixation artefact? The autophagosomes themselves have very large gaps suggesting a defect in formation, rather than the final closure. Where does VPS37A localise during this process? Figure 6 just shows localisation to the whole round autophagosome. Is this consistent with CHMP2A?*
Response: Regarding the 'large voids' in AP-like structures in our original electron micrographs (**Figure 2E**), we artificially enlarged the intermembrane spaces of phagophores and autophagosomes by preparing samples in the absence of potassium ferrocyanide to allow us to easily detect immature autophagic structures (PMID: 18425441; 30030437). Using this method, we have quantified the numbers of total autophagic structures and unsealed

autophagosomal membranes in 2D electron micrographs and included the data in the revised manuscript (**Figure 2F**). In addition, by preparing the samples in the presence of potassium ferrocyanide, we have verified the accumulation of oval-shaped phagophore-like immature autophagosomal membranes in starved crVPS37A cells by electron microscopy (**Figure 2D**). These results are consistent with the HT-LC3 assay results and the immunofluorescence microscopy data that show the accumulation of MIL^+ unclosed LC3-positive membranes (**Figure 2A**) and LC3 foci positive for early autophagic markers (GFP-ULK1 and GFP-ATG5) (**Figure 3A, C**), respectively.

Regarding the localization of VPS37A during autophagy, our additional data (magnified images from the original figure) show that, similar to CHMP2A (PMID: 30030437), GFP-VPS37A FL localizes on MIL^+MPL^- phagophores during starvation (**Figure 6Aii-iii**). However, unlike GFP-CHMP2A, which is rarely detected in the luminal side of MPL^+ closed autophagosomes, we observed GFP-VPS37A FL signals on the luminal side of MIL^+MPL^+ and MIL^-MPL^+ closed autophagic structures (**Figure 6Aiv-ix**). Moreover, as described in response *2-2*, VPS37A FL appears to undergo lysosomal degradation (**Figure 3E and S2**). These observations indicate that at least a portion of VPS37A FL is sequestered within autophagosomes upon the membrane closure and delivered to lysosomes. Regarding the GFP-FL signals detected on the whole round LC3-positive autophagosomal membrane in **Figure 7C** (original Figure 6C), we believe that this is simply caused by the overexpression of DN-VPS4, which can exaggerate the accumulation of ESCRT components (discussed in response *1-5*).

3-3) *The enhancement of LC3-II in crVPS37A cells upon starvation was not greatly obvious (Figure 3E) and needs quantification.*

Response: We have repeated the experiment in the original Figure 3E, quantified the levels of LC3-II (and control β-actin), and calculated autophagic flux and autophagy induction as described by Tooze *et al* (PMID: 25702116). In **Figure 4D**, we show that VPS37A depletion severely impaired autophagic flux without affecting autophagy induction. This result is consistent with the HT-LC3 assay, immunofluorescence microscopy, and the mRFP-GFP-LC3 assay results, showing the accumulation of MIL^+, GFP-ULK1/GFP-Atg5-positive, and GFP^+mRFP^+ immature LC3-positive autophagosomal membranes, respectively, in response to starvation (**Figure 2A, B, 3, and 4A**).

3-4) *It is not clear what the UEV domain is doing to allow autophagosome closure to occur. That it is dispensable for EGFR degradation is interesting, but doesn't explain mechanistically what this domain is doing. In light of the dispensability for MVB sorting, is UBAP (which forms a MVB-specific ESCRT-I complex) also dispensable for autophagosome closure? Does CHMP2A get recruited directly by VPS37A, or are intervening components of ESCRT-I, -II and -III needed? As now a genome wide CRIPSR screen and a genome wide siRNA screen have failed to identify these intermediates, I think the authors should show the mechanistic link between VPS37's UEV and CHMP2A.*

Response: We thank the reviewer for this comment to strengthen our manuscript. As described in response *1-2*, we now show that TSG101 and VPS28, but not UBAP-1, are required for autophagosome completion and that VPS37A loss suppresses the phagophore accumulation of GFP-VPS28 induced by CHMP2A depletion (**Figure 8A, D**). Since VPS37A forms a complex with TSG101 and VPS28 (**Figure 5C**) and the loss of TSG101 and VPS28 destabilizes VPS37A (**Figure 8C**), we propose that VPS37A functions together with other ESCRT components to induce the closure event. Moreover as the loss of the ESCRT-II component VPS25, which also results in the depletion of ESCRT-II component VPS22 (**Figure 8C**), shows minimal effect on autophagosome completion (**Figure 8A**), other complex subunits including Alix may function in parallel to ESCRT-II to recruit ESCRT-III for phagophore closure. We are in full agreement with the reviewer that it is important to clarify the mechanism by which VPS37A recruits ESCRT components during autophagy. However, we feel that this is work to be addressed by future studies and is beyond the scope of the current manuscript.

3-5) *Regarding point 4, the authors speculate ubiquitin may be recruiting VPS37A via its UEV domain. However, residues required for ubiquitin binding are thought to be absent in VPS37A's UEV domain (Stuchell, JBC, 2004). The authors should show whether this domain binds ubiquitin if they want to propose something in contrast to the literature.*

Response: As the reviewer 1 mentioned in his/her comment *1-1*, the homology between the TSG101 UEV domain and the VPS37A putative UEV domain are very low, and a previous report describes that they cannot detect the interaction between VPS37A and ubiquitin (PMID: 15240819). Therefore, while the current study identifies the functional importance of this domain, we fully agree that further studies are required to characterize

the identity of the region. To avoid being misleading, we have modified the manuscript to refer to this region as a "putative UEV" (PUEV) domain and have clearly addressed such in the discussion.

3-6) *Recently, ESCRT-III has been proposed to localise to damaged endomembranes. As the HT-LC3 assay employs a membrane permeabilization and cytoplasmic extraction to allow illumination of membrane-bound LC3 with MIL, it would be good to demonstrate ESCRT recruitment to APs was distinct from ESCRT recruitment to damaged membranes.*

Response: As the reviewer mentioned, recent studies have shown that ESCRT components localize to LLOME-induced damaged lysosomes for the membrane repair in a lysophagy-independent manner (PMID: 29622626; 30314966). As phosphatidylinositol 3-phosphate generation is required for lysophagy, but not ESCRT recruitment to damaged lysosomes (PMID: 29622626), we have examined the localization of VPS37A upon LLOME exposure in the presence or absence of the phosphatidylinositol 3-kinase inhibitor wortmannin (WM). As shown in **Figure S3**, we observe that LLOME strongly induces GFP-VPS37A signal accumulation on MIL^+ immature autophagosomal membranes that enwrap mCherry-GAL3-positive ruptured lysosomes, indicating the induction of lysophagy. Interestingly, these results suggest that ESCRT-mediated phagophore closure is utilized under conditions beyond starvation; a notion that has yet to be investigated by our group but is an area of future interest. While LLOME-induced accumulation of GFP-VPS37A signals is significantly decreased by WM, GFP-VPS37A recruitment to MIL-negative, mCherry-GAL3-positive damaged lysosomes is still observed, indicating VPS37A recruitment during lysophagy-independent membrane repair. Notably, no MIL-positive, GFP-VPS37A or mCherry-GAL3 foci are observed when the HT-LC3 assay is performed under starvation in the presence of WM to indicate that VPS37A recruitment to autophagosomal membranes is distinct from ESCRT recruitment to damaged membranes.

3-7) *In general, many of the data were n = 2 or not stated. I think the authors should ensure n = 3 for as many studies as possible.*

Response: To ensure the robustness and reproducibility, we have indicated the number of replicates in the following key experiments and performed additional replicates where necessary: GFP-ULK1 and GFP-ATG5 foci formation assay combined with LC3 and p62 immunofluorescence (**Figure 3A, C**; $n = 3$); mRFP-GFP-LC3 assay (**Figure 4A**; $n = 3$); immunoblotting-based autophagic flux assay (**Figure 4C**; $n = 3$); immunoprecipitation/GFP-Trap assay (**Figure 5C**; $n = 3$); EGFR degradation assay in the presence of BafA1 (**Figure 5F**; $n = 2$); HT-LC3 assay in GFP-FL/ΔPUEV-expressing VPS37A KO cells (**Figure 6A**; $n = 3$); VPS37A localization analysis under starvation conditions (**Figure 7A**; $n = 3$); mRFP-GFP-VPS37A FL/ΔPUEV assays (**Figure S2**; $n = 3$); VPS37A localization analysis under LLOME exposure (**Figure S3A**; $n = 2$); HT-LC3 assay in ESCRT (VPS37A, TSG101, VPS28, UBAP1, and VPS25) KO cells (**Figure 8A**; $n = 3$); VPS28 localization analysis (**Figure 8D**; $n = 2$); CHMP2A localization analysis (**Figure 9A**; $n = 3$).

July 16, 2019

RE: JCB Manuscript #201902170R

Prof. Hong-Gang Wang
Penn State College of Medicine
500 University Dr.
Hershey, PA 17033

Dear Prof. Wang,

Thank you for submitting your revised manuscript entitled "VPS37A directs ESCRT recruitment for phagophore closure". You will see that the reviewers are supportive of publication pending final, minor text and figure changes. Please address all of Reviewer #3's comments, adapt the model figure according to Rev#2 and tone down the text accordingly. Further experimentation is not needed. We would be happy to publish your paper in JCB pending changes to address the reviewers' points and final revisions necessary to meet our formatting guidelines (see details below).

1) Text limits: Character count for Articles and 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

2) 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 provide an eTOC statement on the title page that starts with "Takahashi, Liang, et al...." to meet our style guide.

3) Figure formatting:
Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please add molecular weight with unit labels on the following panel: 4F (please add unit labels)

4) 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.
Please indicate n/sample size/how many experiments the data are representative of: 1D, 2C, 4BDE, 5E

5) 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.
- Please provide the species for all antibodies.
- 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.).

6) A summary paragraph of all supplemental material should appear at the end of the Materials and methods section.
- Please include ~1 descriptive sentence per item.

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Sincerely,

Richard Youle, PhD
Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

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

In this revised manuscript Takahashi et al., address most of my comments but one still remains outstanding and that is the evidence that some VPS37A is degraded in the autophagosome. The evidence that is presented to further support this (as requested in the first review) is mostly the same as was originally present in the manuscript (Figures 4C was 3E, 4E is a new quantification of 4C), in addition the experiment to look at flux of mRFP-GFP-VPS37A is Figure S2. As Fig. S2 shows the conclusion that VPS37A is degraded by autophagy and that this targeting requires the PUEV domain is not robust. As the author's themselves acknowledge in the rebuttal to point 2-2. In addition, the existing evidence previously provided is not convincing. There is no change in the levels of VPS37A in starvation (no decrease see Fig. 4C and E), no increase with BafA alone (see Fig. 4C and E). Furthermore in Fig. 5B in control cells the levels of VPS37A actually increase in starvation (although my conclusion is from this single western blot). This is not a major point in the manuscript (mentioned on page 10) but more importantly, (perhaps because visual images remain in readers mind) in Fig. 9E the authors have drawn a model in which the VPS37A complex is inside the autophagosome. I feel this is not supported by the data and the model figure should be corrected, along with conclusions in the text about the degradation of VPS37A by autophagy.

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

Takahashi et al., have revised their manuscript and I think it makes a strong and convincing case for the pUEV domain of VPS37A in coordinating ESCRT-III-dependent autophagosome closure in their assay. The majority of my concerns have been addressed and I think publication is appropriate.

Regarding my 1st specific concern, the 'hit validation' and description of how they got from 11% of the genome to just 4 new genes (50% of which didn't validate) is still quite poor. Table S1 was absent from the revised submission and from the figure legends, so it really isn't clear how the authors chose to focus on VPS37A, EGP5, MYOC and SCAP. Moreover, the validation (Fig 1D) is still only from n = 1. I think when performing a genome wide screen, readers should be able to assess the robustness of the hits reported for follow up analysis; if half of the top 4 genes selected were false positives and others (TSG101 (ranked 18755/19114), VPS28 (ranked 10178/19114)) were false negatives then it greatly reduces the value of the dataset. I also appreciate that there is not much you can do about Fig 1 of a MS at this stage, but it would be great if a clearer picture of

the true positives could be obtained from the hits. Legends for tables S1 and S2 are missing.

Minor
P7: I think the call outs to Figure 5D and 5E should be to 2D and 2E
Figure 1B: the text states that 'starvation increased MIL and MPL intensities', however, you haven't presented/examined the fed condition on Figure 1B, so readers can't tell the extent of this increase upon starvation.
Figure 4B : is the colocalization analysis performed in the fed or starved state?
Figure 4E: It would be nice to include the blots demonstrating changes in VPS37A levels.

2nd Revision - Authors' Response to Reviewers: July 23, 2019

Point-By-Point Response (Manuscript # 201902170R)

We thank the referees for their thoughtful comments and constructive criticisms to further improve our manuscript. We have endeavored to attend to each of the issues raised through revisions to the text and figures. A point-by-point description follows:

Reviewer #2

2-1) *In this revised manuscript Takahashi et al., address most of my comments but one still remains outstanding and that is the evidence that some VPS37A is degraded in the autophagosome. The evidence that is presented to further support this (as requested in the first review) is mostly the same as was originally present in the manuscript (Figures 4C was 3E, 4E is a new quantification of 4C), in addition the experiment to look at flux of mRFP-GFP-VPS37A is Figure S2. As Fig. S2 shows the conclusion that VPS37A is degraded by autophagy and that this targeting requires the PUEV domain is not robust. As the author's themselves acknowledge in the rebuttal to point 2-2. In addition, the existing evidence previously provided is not convincing. There is no change in the levels of VPS37A in starvation (no decrease see Fig. 4C and E), no increase with BafA alone (see Fig. 4C and E). Furthermore in Fig. 5B in control cells the levels of VPS37A actually increase in starvation (although my conclusion is from this single western blot). This is not a major point in the manuscript (mentioned on page 10) but more importantly, (perhaps because visual images remain in readers mind) in Fig. 9E the authors have drawn a model in which the VPS37A complex is inside the autophagosome. I feel this is not supported by the data and the model figure should be corrected, along with conclusions in the text about the degradation of VPS37A by autophagy.*

Response: We agree with the reviewer's assessment and suggestions. In the revised manuscript, we deleted the sentence "suggesting simultaneous upregulation and degradation of VPS37A during autophagy" on page 8, toned down the interpretation on page 10 to read as "suggest a possibility that at least a portion of VPS37A located on the phagophore remains on the luminal side of the membrane upon closure and is delivered to lysosomes", and modified Fig. 9E by removing ESCRT complex from the lumen of autophagosome.

Reviewer #3

3-1) *Takahashi et al., have revised their manuscript and I think it makes a strong and convincing case for the pUEV domain of VPS37A in coordinating ESCRT-III-dependent autophagosome closure in their assay. The majority of my concerns have been addressed and I think publication is appropriate.*

Response: We thank the reviewer's support.

3-2) *Regarding my 1st specific concern, the 'hit validation' and description of how they got from 11% of the genome to just 4 new genes (50% of which didn't validate) is still quite poor. Table S1 was absent from the revised submission and from the figure legends, so it really isn't clear how the authors chose to focus on VPS37A, EGP5, MYOC and SCAP. Moreover, the validation (Fig 1D) is still only from n = 1. I think when performing a genome wide screen, readers should be able to assess the robustness of the hits reported for follow up analysis; if half of the top 4 genes selected were false positives and others (TSG101 (ranked 18755/19114), VPS28 (ranked 10178/19114)) were false negatives then it greatly reduces the value of the dataset. I also appreciate that there is not much you can do about Fig 1 of a MS at this stage, but it would be great if a clearer picture of the true positives could be obtained from the hits. Legends for tables S1 and S2 are missing.*

Response: We apologize for not uploading Table S1 (and S2) in the initial submission of the revised manuscript. As described in the text (page 6), the 5 genes chosen for secondary screening were based on their highest probability scores and functional implications in autophagy (PMID: 30093494 (VPS37A); 20550938 (EPG5); 24732711 (MYOC); 30462530 (SCAP); 30030437 (CHMP2A)). In this manuscript, we focused on characterizing the role of VPS37A in autophagy since our previous study has identified a role for the ESCRT-III component CHMP2A in phagophore closure (PMID: 30030437). Due to the nature of the assay, we cannot sort and expand the 'positive' population for the subsequent next-generation sequencing procedure. Moreover, while we optimized the condition to minimize sample loss, we still experienced a partial cell loss during the staining and washing procedures after plasma membrane permeabilization. Therefore, to recover sufficient amount of genomic DNAs for sequencing, a relatively wide FACS gate setting was applied to the $MIL^{high}MPL^{low}$ population (10.9% of total samples). We think that this may attribute to a relatively high noise ratio in our screening. Nonetheless, our screenings identified additional 141 genes whose averaged logP values (from 4 independent screenings) are below -1.4 ($p<0.005$) (Table S1). It would be of interest to validate these candidates in the future. Tables S1 and S2

shows data from the primary screen and sgRNA sequences used for the experiments, respectively. These information are included in Methods and Supplemental Material.

3-3) *P7: I think the call outs to Figure 5D and 5E should be to 2D and 2E*
Response: We thank the reviewer for the careful assessment of our work. We have corrected the figure numbers accordingly.

3-4) *Figure 1B: the text states that 'starvation increased MIL and MPL intensities', however, you haven't presented/examined the fed condition on Figure 1B, so readers can't tell the extent of this increase upon starvation.*
Response: We have edited the text accordingly.

3-5) *Figure 4B : is the colocalization analysis performed in the fed or starved state.*
Response: The experiment was performed under starvation conditions. We have edited the figure legend accordingly.

3-6) *Figure 4E: It would be nice to include the blots demonstrating changes in VPS37A levels.*
Response: We have included additional blots in Fig. S2.