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# TOR-mediated Ypt1 phosphorylation regulates autophagy initiation complex assembly

Cong Yi, Wei Yao, Yu Chen, Ying Chen, pengwei zhao, Jing Liu, Yi Zhang, Qiang Jiang, Yu Xie, Yuyao Feng, Chou Wu, Xue Bai, Mingzhu Fan, Shan Feng, Juan Wang, Yi Cui, Hongguang Xia, Liqin Zhang, Qiming Sun, Wei Liu, Siyu Fan, Miao Ye, Yigang Wang, Cheng Ma, and Zhiping Xie
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| Editorial Decision: | 4th Nov 22 |
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## Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

**1st Editorial Decision** **4th Nov 2022**

Dear Prof. Yi,

Thank you again for the submission of your manuscript entitled "mTOR-mediated Ypt1 as an autophagy determinant regulates the stepwise assembly of ATG proteins". I have now received the referees' reports, which are copied to the bottom of this message.

At its heart, all referees agree that the work is based on a technically accomplished and well-described collection of experiments. They also state unambiguously that the manuscript is timely and the topic is important. However, the feedback was not unambiguously positive. The data that you present will need to be given a wider mechanistic context if they are to be published in EMBO Journal.

I would like to invite you to address the comments of all referees in a revised version of the manuscript. All of the referees' reports contain clear and explicit recommendations for your revised version (many of which overlap). Our usual revision time of three months is used as a guideline, not a deadline; manuscripts frequently take longer to revise. I will be available and happy to talk next week if you have any questions, I recommend that we go over our next steps and discuss the referees' comments further over Zoom.

I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve these concerns at this stage. I believe the concerns of the referees are reasonable and addressable, but please contact me if you have any questions, need further input on the referee comments or if you anticipate any problems in addressing any of their points. Please, follow the instructions below when preparing your manuscript for resubmission.

I would also like to point out that as a matter of policy, competing manuscripts published during this period will not be taken into consideration in our assessment of the novelty presented by your study ("scooping" protection). We have extended this 'scooping protection policy' beyond the usual 3 month revision timeline to cover the period required for a full revision to address the essential experimental issues. Please contact me if you see a paper with related content published elsewhere to discuss the appropriate course of action.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Again, please contact me at any time during revision if you need any help or have further questions.

Thank you very much again for the opportunity to consider your work for publication. I look forward to your revision.

Best regards,

William Teale

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William Teale, Ph.D.
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1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point response to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/Author Checklist%20-%20EMBO%20J-1561436015657.xlsx). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised

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6) We require a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition). If no data deposition in external databases is needed for this paper, please then state in this section: This study includes no data deposited in external repositories. Note that the Data Availability Section is restricted to new primary data that are part of this study.

Note - All links should resolve to a page where the data can be accessed.

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8) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.).

9) We would also encourage you to include the source data for figure panels that show essential data. Numerical data can be provided as individual .xls or .csv files (including a tab describing the data). For 'blots' or microscopy, uncropped images should be submitted (using a zip archive or a single pdf per main figure if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at .

10) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc. in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: .

- Additional Tables/Datasets should be labelled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

11) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

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Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

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- individual production quality figure files (one file per figure)
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- Expanded View files (replacing Supplementary Information)
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We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (11th Jul 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

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Yours sincerely,

William Teale, PhD
Editor
The EMBO Journal
w.teale@embojournal.org

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- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines
(https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
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We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (2nd Feb 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

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Referee #1:

In the initial step of autophagy in yeast, Atg9 vesicles must be recruited to the PAS, which contains Atg11, Atg13, Atg17-Atg29-Atg31 as a scaffold. Previous studies identified Ypt1 as a component of Atg9 vesicles and binds Atg11 and Atg1. However, the molecular roles of Ypt1 in the initial steps of autophagy and the mechanism of Atg9 vesicle recruitment to the PAS are not sufficiently understood. In this manuscript, the authors studied the interaction of Ypt1 with Atg proteins by combination of in vitro pulldown assay, co-IP, and BiFC assays and identified Atg23, Atg17 and Atg1 as Ypt1 binding proteins. The authors confirmed that binding of Ypt1 to Atg23 and Atg17 is important for the recruitment of Atg9 vesicles to the PAS by mutational analysis. Moreover, the authors identified S174 in Ypt1 as a phosphorylation site by TOR and showed that phosphorylation at S174 impaired the recruit of Atg1 to the PAS and its activation. Finally, the authors also identified T75 in Rab1 as a phosphorylation site by mTOR and found that phosphorylation at T75 impaired the interaction of Rab1 with ULK1 and autophagy activity in mammalian cells.
Binding assays were well performed using three different methods and identification of direct interaction between Ypt1 and core Atg proteins is valuable because such experiments were scarcely reported and would contribute to the understanding of the initial steps of autophagy. On the other hand, this manuscript contains lots of shortcomings listed below and it is unclear whether the obtained model summarized in Fig 9 is correct or not at the present form. The authors must strengthen their model by resolving the concerns listed below.

Major points

1) Ypt1/Rab1 becomes active when bound to GTP and interacts with their effectors whereas becomes inactive when bound to GDP. Switch I and II in Ypt1/Rab1 have a conformation quite different between GTP-bound and GDP-bound forms. Throughout the manuscript, the authors did not study which form of Ypt1 interacts with Atg factors at all. Prepare both GTP-bound and GDP-bound forms of Ypt1 and compare the binding affinity between these two forms against Atg23, Atg17, Atg1, and Hrr25.

2) The authors identified S174 in Ypt1 as a phosphorylation site by mTORC1 and showed that phosphorylation at S174 negatively regulates autophagy by impairing interaction with Atg1. S174 is located at the C-terminal region of Ypt1 and is far from Switch I and II. On the other hand, the authors identified T75 in Rab1 as a phosphorylation site by mTORC1, which is located at Switch I. Discuss this point in detail.

3) The authors proposed a model in Fig 9, in which Ypt1 recruits Atg9 vesicles to the PAS via interaction with both Atg17 and Atg23. If this model is true, Ypt1 should be able to bind Ag17 and Atg23 simultaneously. Moreover, Ypt1 could also bind Atg1 together with Atg17 and Atg23 if the model in Fig 9 is true. Perform competitive binding assay using purified proteins and study whether Ypt1 can bind Atg17, Atg23, and Atg1 simultaneously or each binding is competitive. If the binding is competitive, the authors must correct the proposed model in Fig 9.

4) In Fig. 1C, the authors showed that the cytoplasmic regions of Atg9 did not interact with GST-Ypt1 by pulldown assay. In these assays, they used Atg9 as a fusion with Trigger factor (TF), but TF-fusion protein often behaves as a soluble protein even when the fused protein is denatured. Purify Atg9 proteins without TF tag and re-perform pulldown assay using them.

5) In Fig. 3A, the authors concluded that L170N-L171N-L173N or L188N-L189N mutation in Atg23 impaired the PAS recruitment of Atg9 vesicles. However, it may also be possible that these mutations impaired the generation of normal Atg9 vesicles (highly mobile vesicles in the cytoplasm) since Atg23 is important for Atg9 vesicle formation. Perform live imaging and confirm that formation of mobile Atg9 vesicles was not affected by these mutations by providing supplementary video (refer to PMID 22826123).

6) In Fig. 4, the authors identified the binding region in Atg17 for Ypt1 using deletion mutants. However, crystal structure of Atg17 suggests that such deletion would severely affect the folding of Atg17. Therefore identify the Ypt1 binding region in Atg17 by point mutations as performed for Atg23.

7) In Fig 7B,C, CBB staining suggests that Atg1 and Ypt1 S174D form a 1:1 complex whereas Atg1 binds large excess amounts of Ypt1 WT. Similarly, it is likely that Hrr25 and Ypt1 S174D form a 1:1 complex whereas Hrr25 binds large excess amounts of Ypt1 WT. These data seem to suggest that S174D mutation impaired the oligomerization of Ypt1 rather than the interaction of Ypt1 with Atg1 or Hrr25. If the authors want to claim that S174D mutation impaired the interaction with Atg1 or Hrr25, study the binding affinity using quantitative methods such as SPR or ITC.

8) In Fig 5D-J, only S174D mutant of Ypt1 was studied. Study Ypt1 S174A mutant and compare the results with S174D and WT.

9) Mammalian cells have Rab1A and Rab1B. In Fig 8, which type of Rab1 was expressed? Did Rab1b shRNA1 affect the expression level of Rab1A? Does the anti-Rab1 antibody recognize Rab1A and Rab1B equally?

Minor points
1) In page 5, lines 14-17, the sentence is difficult to read and should be revised.

2) In page 6, lines 6-7, "As it is known that Ypt1 regulates the PAS recruitment of Atg9 vesicles and PAS localization of Ypt1 and Atg9 vesicles," this sentence is difficult to read. Ypt1 regulates PAS localization of Ypt1?

3) In Fig. 1B, 7A-C, S5, The results of pulldown assays stained with CBB should be shown as a single gel image or provide a single gel as Supplementary.

4) In page 11, line 14, W65A should be W62A.

5) In Fig 4E, provide WB of Atg17 and confirm that the deletion mutants of Atg17 were expressed similarly with wild-type Atg17.

6) In Fig. 5C,G, provide WB of Ypt1 and confirm that the mutants of Ypt1 were expressed similarly with wild-type Ypt1.
7) In page 17, line 24, "These results further supported that mTOR-mediated Ypt1 phosphorylation is required for PAS recruitment of Atg1", the data show that Ypt1 phosphorylation impairs PAS recruitment of Atg1 and thus revise this sentence.

8) In page 19, lines 17-19, "We found significant accumulation of p62 in Rab1 KD cells, indicating that Rab1 negatively regulates autophagy in mammals", negatively should be positively.

9) Discussion section is largely redundant with Results section. Delete the redundant description of the results and fucus on writing "discussion" in the Discussion section.

10) Throughout the manuscript, "mTOR" should be written as "TOR" when referring to yeast protein.

Referee #2:

The authors examine the molecular mechanisms underlying autophagosome biogenesis in yeast. They focus on the small GTPaseYpt1/Rab1 previously linked to autophagosome biogenesis. The authors identify two new interactors of Ypt1, Atg23 and Atg9. Atg9 is an essential autophagy protein, which marks Atg9 vesicles that are incorporated into the phagophore where Atg9 functions as a scramblase. Previous work has shown that Atg9 forms a complex with Atg23 and Atg27 in yeast, which are important for the biogenesis of Atg9 vesicles and their transport to a site of autophagosome formation (PAS). The authors use a number of biochemical and cell biological approaches to show that Ypt1 physically interacts with Atg23. Mutations that disrupt this interaction result in impaired assembly of the core autophagy machinery and formation of autophagosomes. In addition, the authors detect physical interactions of Ypt1 with Atg17. Atg17 forms a complex with Atg29 and Atg31 and marks sites of autophagosome biogenesis providing a platform for the recruitment of downstream Atg proteins. The authors identify Atg17 variants that are impaired in Ypt1 binding and display defects in autophagy. Finally, the authors identify phosphorylation sites within Ypt1 and mammalian Rab1 for TORC1/mTOR, a central regulator of cell growth and inhibitor of autophagy. Interestingly, non-phosphorylatable or phospho-mimetic variants of Ypt1 result in elevated or delayed autophagy, suggesting a regulatory role for TORC1-mediated phosphorylation of Ypt1 I autophagy.

The study provides a solid set of biochemical and cell biological approaches that identify novel connections for Ypt1 with the autophagy protein machinery with mechanistic insights. Based on the presented data, the authors propose in principle an interesting link from TORC1 siganling to Ypt1 and new regulatory interactions. However, the manuscript requires additional experimentation to fully support the proposed model:

Critical points:

(1) Are in vitro interactions of Ypt1 dependent on its GTP/GDP bound state?
The authors do not describe the experimental conditions of their in vitro work in the manuscript in terms of protein concentrations, or presence of GTP/GDP (s. below). To test the specificity of the Ypt1 interactions in vitro, it would be important to test constitutive GTP or GDP bound variants of Ypt1 for interaction with Atg23 and Atg17
(2) Characterization of the Atg9-23-27 complex in dependence of Ypt1
The authors propose that the Atg23 L170,171,173N or L188,189N variants fail to interact with Ypt1, which they link to the phenotypical outcome. However, it is equally possible that these variants show impaired Atg9 binding. Since Atg23 is required for the biogenesis of peripheral Atg9 vesicles and anterograde transport of Atg9, a defect in Atg9 binding could explain the downstream effects on Atg9 and Atg11, Atg13, and Atg17 binding. Thus, it will be important to assess the physical interaction of Atg9 and the Atg23 variants. To examine potential defects in the biogenesis of peripheral Atg9 vesicles, the authors need to quantify the number of Atg9 puncta in cells and their intensities in the presence of Atg23 L170,171,173N or L188,189N variants. In addition, in Figure 1C, the authors should test the interaction of Ypt1 and full-length Atg9.
(3) Characterization of the Atg17 complex in dependence of Ypt1
It is possible that the Atg17 variants impaired for Ypt1 interaction are also defective in forming a proper Atg17 complex. A defect in Atg17 complex formation may explain the changes that authors observe and assign to the impaired recruitment of Ypt1 and Atg1. Thus, do Atg17 variants that are impaired in Ypt1 binding still form a proper Atg17-29-31 complex and localize to the PAS? The authors should also test Ypt1-W62A or other Ypt1 variants for Atg17 binding.
(4) Examining the link between TORC1 and Ypt1
Figure 6C-H should include a proper time course analysis (0, 1, 2, 4, 8, 12, 24h) to assess the overall effect of ypt1S174A or D on autophagy not just one timepoint.
(5) Materials and Methods
All methods should be described not just referenced. The description of protein purification and the in vitro interaction assays are completely missing and thus I could not evaluate the validity of the approaches.

Referee #3:

Yao et al. present numerous novel findings regarding Ypt1's role in Autophagy including several previously unknown interactions, key phosphorylated residues, and mechanistic conservation across distant eukaryotes. While these results could add much to the field's understanding of autophagy protein recruitment to the PAS, several critical issues remain with the manuscript. Perhaps the biggest issue is the authors' failure to interpret their findings through the lens of the recent, and highly related, paper from Hawkins et al., Cell Reports 2022. Knowledge of this paper's findings raises several key issues with Yao and colleagues' interpretation of their data regarding Atg23. Other studies that contradict some of the findings in the present paper have also been ignored.
1. Line 19-21, page 3: Needs to be re-written as it currently does not make sense.
2. Introduction: The authors mention the role of Ypt1 in regulating membrane tethering events. This should be discussed in light of Atg23-mediated membrane tethering as revealed in the 2022 paper by Hawkins et al., "Dimerization-dependent membrane tethering by Atg23 is essential for yeast autophagy".
3. Similarly, the findings of this paper will be more convincing if the researchers discuss the potential reasons for the contradictory results between other previous studies and the present paper. For example, the 2013 paper by Wang et al. titled "Ypt1 recruits the Atg1 kinase to the preautophagosomal structure" shows that Ypt1 does not recruit Atg17 to the PAS (The PAS localization of Atg17-GFP is not disrupted in the ypt1-2 mutant). Also, the conclusion in this paper that Ypt1-Atg23 binding is required for the PAS recruitment of Atg9 vesicles is not consistent with a previous 2012 study by Kakuta et al., "Atg9 vesicles recruit vesicle-tethering proteins Trs85 and Ypt1 to the autophagosome formation site", which showed that Ypt1 is localized to the preautophagosomal structure in an Atg9-dependent manner. It will be important for the authors to summarize all the novel findings in this paper that are not in line with previous studies and discuss the reasons for the inconsistencies.
4. Along these lines, it might be inappropriate to claim that Atg23 is a previously unrecognized binding partner of Ypt1. A paper published in 2013 by Graef et al., "ER exit sites are physical and functional core autophagosome biogenesis components" reported the potential binding of Atg23 with Ypt1. This manuscript at least needs to be cited in the present paper.
5. Line 11, page 6: I do not follow the logic behind this conclusion. I do not think that this finding necessarily implies that Atg23 is recruited before Atg9.
6. Figure 1A: A full list of mass spec hits should be presented, perhaps as a supplemental figure.
7. Figure 1B: No input was shown for His-Atg23 alone. I recommend cutting that lane from the pulldown.
8. Figure 1C: Explain the meaning of "TF". Abbreviations such as "TF" and "TAP" need to be explained upon first usage.
9. Figure 1C: Why didn't the authors test full-length Atg9? If they cannot purify the full-length protein, they should repeat the experiment using an atg23Δ strain. Explain the meaning of the asterisks in the figure legend. It would be nice to see a reverse pulldown of Ypt1 with anti-GST beads. I suggest changing the conclusion to "we think Atg9 is not likely to directly interact with Ypt1".
10. Figure 1D: Atg23 has been shown to form many puncta, the authors should repeat this with a PAS marker to see if this one colocalization event is at the PAS or elsewhere.
11. Figure 2B, S1C; Line 6, page 8: The authors need to explain how these amino acid residues were selected. The same

applies to Ypt1 W62.
12. Figure S2B: The authors should more thoroughly demonstrate their process for determining the Ypt1 residues required for Atg23 binding and move this to a main figure.
13. Figure 2: The authors completely fail to mention the recent findings of Hawkins et al. Based on the findings of this previously published paper, mutation of the hydrophobic face of the CC1 domain would render Atg23 monomeric. The effects of Atg23 mutation reported by Yao et al. may thus be a result of Atg23 monomerization and not direct mutation of the Atg23-Ypt1 binding interface.
14. Figure 2E,F: Please provide quantification from multiple repeats.
15. Figure 3: Again, the authors fail to incorporate the findings of Hawkins et al. and thus potentially misinterpret their results.
16. Figure 3A: Deletion of ATG23 has been shown to result in fewer and less intense Atg9 puncta intensity (2012, Yamamoto et al., "Atg9 vesicles are an important membrane source during early steps of autophagosome formation"), but this is not seen here. Have the authors standardized exposure settings across all images?
17. When the authors investigate PAS recruitment, they always use one protein to label the PAS. For example, they used Atg17 to label the PAS in the experiment shown in Figure 3A-B. The result of this experiment will be more convincing if they can show the colocalization of Atg9 with more than one PAS marker, such as Trs85 or precursor Ape1. Considering that some conclusions in this paper (such as Atg9 recruitment to the PAS depending on Ypt1-Atg23 binding) are apparently different from other studies, the researchers might want to show this result by more than one assay.
18. Figure 4: While it is acceptable to begin with amino acid deletions, the authors must be able to show disrupted Ypt1 binding with a small number of amino acid conversions. Deleting residues from a critical helix in Atg17 could be completely disrupting the structure, and this could be the actual cause of all the observed defects. Are the deletion mutants still forming puncta on their own? Are they still able to interact with other known Atg17 binding partners? It is impossible to know if the defects observed in the Atg17 amino acid deletion mutants have any direct relation to Ypt1 binding.
19. Figure 4: Since the authors studied interaction between Ypt1 and Atg17, they should also include Atg17 in the MS data presentation shown in Figure 1. In fact, I recommend replacing Figure 1A with a panel showing the enrichments of known Ypt1 interactors in the MS data.
20. Figure 4: Since Ypt1 is a newly identified interactor of Atg17 in this research, I recommend the authors examine which of these proteins is recruited to the PAS first before they use the colocalization between Ypt1 and Atg17 to infer the recruitment of Ypt1 to the PAS.
21. Figure 4E to 4H: The authors need to show the expression level of exogenous proteins.
22. Figures 2 to 4: Although the mutations in Atg23 and Ypt1 affects their binding to each other, these data do not indicate if the mutated region is the direct binding region. Rather, these data indicate that these regions/amino acids are required for binding. Thus, I recommend that the authors change their wording. For example, in the sentence "To delineate which of the regions of Atg23 were responsible for its binding with Ypt1", I recommend replacing "responsible" with "required"; similarly, in the sentence "the binding region of Atg23-Ypt1 on Ypt1 was also delineated using same approach.", I recommend changing the wording, as the experiment cannot indicate if the region is the direct binding region. The same applies for the wording with regard to Atg17.
23. Although the complementary data from Atg23 and Ypt1 mutations reduce the likelihood that these mutations might be affecting some other functions of the protein that lead to defects in Atg9 recruitment to the PAS, the current data did not exclude such possibilities. Essentially, from the current data, where defects were seen in Atg23-Ypt1 interaction, in Atg9 recruitment to the PAS, and in autophagy, there are insufficient data to determine the causal relationship between these events (e.g., if defects in Atg9 recruitment are caused by defective Atg23-Ypt1 interaction). It is also not possible to exclude a compromised upstream event (e.g., Atg23 dimerization) that leads to all these defects. Accordingly, I recommend the authors check if the interaction between Atg9 and mutant Atg23 is similar to wild-type Atg23 using in vivo and in vitro pulldown. The authors can also try a yeast two-hybrid assay.
24. Figure 4: The authors concluded "Collectively, this data demonstrated Ypt1-Atg17 interactions to be clearly required for autophagy due to their function in the PAS recruitment of Ypt1 and Atg1". However, I think it is risky to conclude the causal relationship between defective autophagy and defective Ypt1 localization to the PAS without an experiment where the authors try to restore the localization of Ypt1 to the PAS in the atg17Δ strain. Similarly, the authors should demonstrate that the Atg17 mutants in Figure 5 do not affect the recruitment of Atg1 in a Ypt1-independent manner.
25. Figure 5A: This should be moved to the supplement. What is the upper band?
26. Figure 5B: Move to the supplement. I suggest that the authors replace these data with a representation of mass spec results that is more easily interpreted by cell biologists.
27. Figure S5G: The authors may want to include an HA-only control.
28. Figure 6: The authors need to show the expression level of exogenous proteins.
29. Figure 6A: Later results seem to show completely disrupted PAS formation in the presence of Ypt1 S174A. Yet, Atg8 is clearly still forming puncta. The authors should explain their thinking concerning this observation.
30. Figures 6A, B: The authors must show results for S174A and S174D at same timepoint or provide a good explanation as to why they tested 1 h starvation for cells expressing Ypt1 S174A but 4 h for the cells expressing Ypt1 S174D.
31. Figure 7J: The interpretation is unclear. The authors should show quantification of multiple repeats.
32. Figure 7D to J: The authors need to show the expression level of exogenous proteins.
33. Line 1, page 18: It cannot be said that Atg1 phosphorylation was "completely abolished".
34. Line 10, page 19: The authors' data suggests that Rab1 is a positive, not a negative, regulator of autophagy.
35. Figure 8B: As with Figure 5B, I suggest that the authors consider not showing raw mass spec results in the main figure and replace with a more intuitive representation.
36. Figure 8C: The authors should show if T75 is in a similar location to S174 in terms of primary and tertiary sequence.

37. Figure S8C: I do not see how this demonstrates the specificity of the antibody. There is no evidence provided by the authors to demonstrate that this antibody is specific to Atg1 T226 phosphorylation. The authors need to examine a T226 mutant to determine specificity.
38. Figure 8E: Quantification is required.
39. Figure 9: Atg23 should be bound directly to membrane. Are the authors implying that Tor and Ypt1 form a persistent complex? I suggest that the Atg9 representation should be changed to match the other proteins.
40. In general, the authors should include more details in the figure legends and/or description of the figures such as providing strain information. In figure 1C, for example, the authors should indicate if they are using Coomassie stain. The bottom label "Ypt1" in figure 2B has a scale bar erroneously placed over it.

**Dear Dr. Teale,**

**We have performed extensive follow-up experiments to address the concerns of the reviewers. Please find below our point-by-point responses to each of the reviewer's points below. Many thanks for the opportunity to address these issues.**

**Point-by-point responses to the reviewers:**

Referee #1:

In the initial step of autophagy in yeast, Atg9 vesicles must be recruited to the PAS, which contains Atg11, Atg13, Atg17-Atg29-Atg31 as a scaffold. Previous studies identified Ypt1 as a component of Atg9 vesicles and binds Atg11 and Atg1. However, the molecular roles of Ypt1 in the initial steps of autophagy and the mechanism of Atg9 vesicle recruitment to the PAS are not sufficiently understood. In this manuscript, the authors studied the interaction of Ypt1 with Atg proteins by combination of in vitro pulldown assay, co-IP, and BiFC assays and identified Atg23, Atg17 and Atg1 as Ypt1 binding proteins. The authors confirmed that binding of Ypt1 to Atg23 and Atg17 is important for the recruitment of Atg9 vesicles to the PAS by mutational analysis. Moreover, the authors identified S174 in Ypt1 as a phosphorylation site by TOR and showed that phosphorylation at S174 impaired the recruit of Atg1 to the PAS and its activation. Finally, the authors also identified T75 in Rab1 as a phosphorylation site by mTOR and found that phosphorylation at T75 impaired the interaction of Rab1 with ULK1 and autophagy activity in mammalian

cells.

Binding assays were well performed using three different methods and identification of direct interaction between Ypt1 and core Atg proteins is valuable because such experiments were scarcely reported and would contribute to the understanding of the initial steps of autophagy. On the other hand, this manuscript contains lots of shortcomings listed below and it is unclear whether the obtained model summarized in Fig 9 is correct or not at the present form. The authors must strengthen their model by resolving the concerns listed below.

Major points

1) Ypt1/Rab1 becomes active when bound to GTP and interacts with their effectors whereas becomes inactive when bound to GDP. Switch I and II in Ypt1/Rab1 have a conformation quite different between GTP-bound and GDP-bound forms. Throughout the manuscript, the authors did not study which form of Ypt1 interacts with Atg factors at all. Prepare both GTP-bound and GDP-bound forms of Ypt1 and compare the binding affinity between these two forms against Atg23, Atg17, Atg1, and Hrr25.

**Response: Because Atg1 and Hrr25 have been reported to be associated with the GTP-bound form of Ypt1(PMID:23716696; 26195667), here we only tested whether the association of Atg17 or Atg23 with Ypt1 depends on its GTP-bound or GDP-bound forms. To this end, we generated Ypt1 variants with disrupted ability to bind GTP or GDP. As shown in Figure R1(now Figure 1G and S8C),**

**GST pulldown results indicated that Atg17 and Atg23 preferentially pulldown with the GTP-bound form of Ypt1 (Ypt1$^{Q67L}$), not its GDP-bound form (Ypt1$^{S22N}$), suggesting that the binding of Ypt1 with Atg17 and Atg23 depends on its GTP-bound form.**

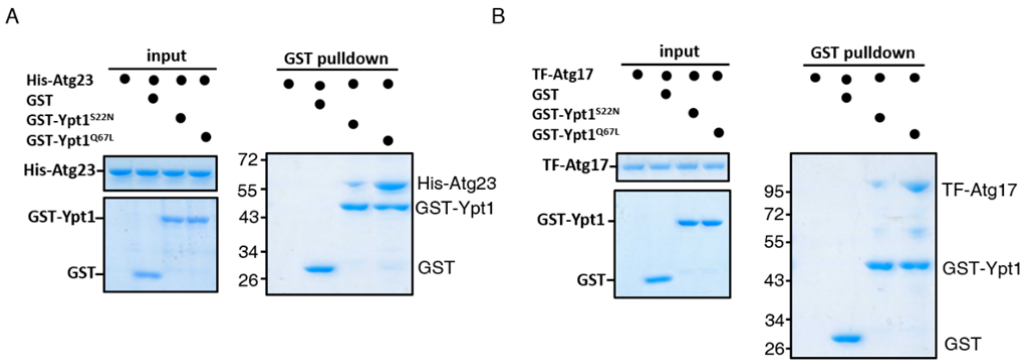


**Figure R1: The binding of Ypt1 with Atg17 and Atg23 depends on its GTP-bound form. (A)** GST pulldowns were performed using purified $His_6$-tagged Atg23 with GST, GST-Ypt1, GST-Ypt1$^{Q67L}$ (GTP-bound form), or GST-Ypt1$^{S22N}$ (GDP-bound form) from *E. Coli*. **(B)** GST pulldowns were performed using purified His-tagged TF-Atg17 with GST, GST-Ypt1$^{Q67L}$, or GST-Ypt1$^{S22N}$ from *E. Coli*. Protein samples were separated by SDS-PAGE, and then detected using Coomassie blue staining.

2) The authors identified S174 in Ypt1 as a phosphorylation site by mTORC1 and showed that phosphorylation at S174 negatively regulates autophagy by impairing interaction with Atg1. S174 is located at the C-terminal region of Ypt1 and is far from Switch I and II. On the other hand, the authors identified T75 in Rab1 as a phosphorylation site by mTORC1, which is located at Switch I. Discuss this point in detail.

**Response: We thank the reviewer for these insightful comments. In fact, we also**

**observed that the phosphorylation site of Ypt1 and Rab1 by TOR/mTOR are not conservative in yeast and mammals. We think that the reason for this discrepancy may be caused by the different three-dimensional structure of the mTORC1 complex in mammalian and yeast cells, or the different microenvironment where Ypt1 and Rab1 bind with the mTORC1 complex. In fact, some studies found that mTOR phosphorylates the same substrate, despite the sites being different, in yeast and mammals. For example, Atg13 is reported to be phosphorylated by TOR/mTOR in both yeast and mammals. However, Atg13 is phosphorylated by mTOR at Ser 258 residue in mammals (PMID: 26801615), while in yeast cells, Atg13 S348, S437, S438, S496, S535, S541, S646, and S649 residues were found to be phosphorylated by TOR (PMID: 19995911). Although the phosphorylation sites of Atg13 by TOR are different between yeast and mammals, the phosphorylation of Atg13 caused by TOR/mTOR functions similarly to block autophagy. Dephosphorylation of these sites on Atg13 enhances the formation of Atg1 puncta and the activation of Atg1. Similarly, in this study, although the phosphorylation sites of Ypt1 and Rab1 by TOR/mTOR are different, the phosphorylation of Ypt1 and Rab1 by mTOR inhibits the binding of Ypt1/Rab1 with ULK1, thus also inhibiting subsequent autophagy. We discuss this point in the revised discussion section.**

3) The authors proposed a model in Fig 9, in which Ypt1 recruits Atg9 vesicles to the PAS via interaction with both Atg17 and Atg23. If this model is true, Ypt1 should be

able to bind Ag17 and Atg23 simultaneously. Moreover, Ypt1 could also bind Atg1 together with Atg17 and Atg23 if the model in Fig 9 is true. Perform competitive binding assay using purified proteins and study whether Ypt1 can bind Atg17, Atg23, and Atg1 simultaneously or each binding is competitive. If the binding is competitive, the authors must correct the proposed model in Fig 9.

**Response: We thank the reviewer for this valuable comment. As suggested, we performed competitive GST pulldowns using purified TF-Atg1, TF-Atg17, and His-Atg23 to test whether Ypt1 could bind Atg1, Atg17, and Atg23 simultaneously. As shown in Figure R2 (now Figure S15B), TF-Atg1, TF-Atg17 and His-Atg23 could bind with Ypt1 simultaneously, indicating that their binding with Ypt1 is not competitive.**

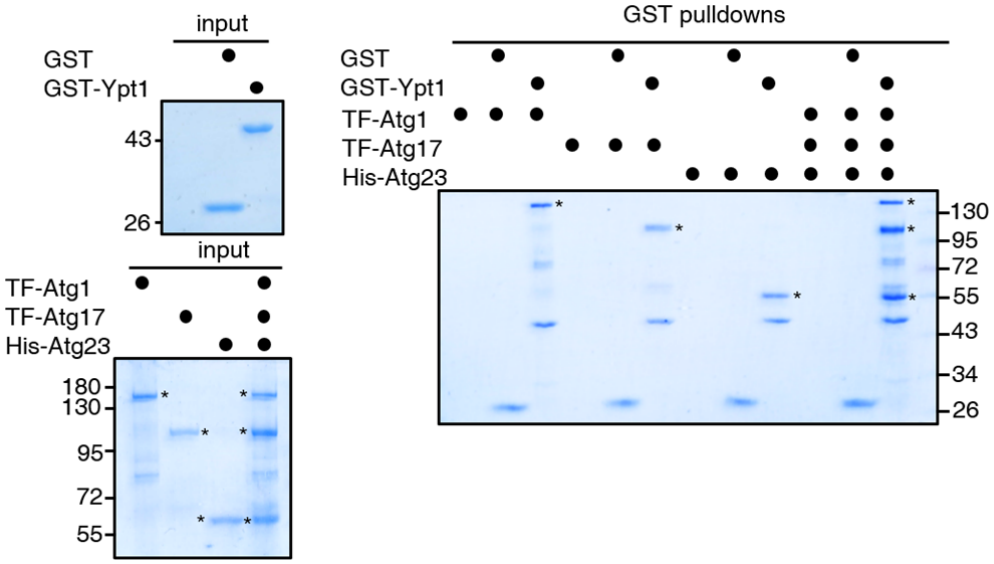


**Figure R2: The binding of Atg1, Atg17, or Atg23 with Ypt1 is not competitive.** GST pulldowns were performed using purified GST or GST- Ypt1 with TF-Atg1, TF-Atg17, and $His_6$-Atg23 from *E. Coli*. Protein samples were separated by SDS-PAGE, and then detected using Coomassie blue staining. The asterisk represents the target

protein.

4) In Fig. 1C, the authors showed that the cytoplasmic regions of Atg9 did not interact with GST-Ypt1 by pulldown assay. In these assays, they used Atg9 as a fusion with Trigger factor (TF), but TF-fusion protein often behaves as a soluble protein even when the fused protein is denatured. Purify Atg9 proteins without TF tag and re-perform pulldown assay using them.

**Response: We thank the reviewer for this rigorous comment. As suggested, we used thrombin to cleave TF-Atg9-N, -M or -C fusion proteins. Unfortunately, as cleaved Atg9-N, -M or -C proteins are very unstable without TF tags, we did not obtain Atg9-N, -M or -C proteins without TF tags. In fact, the pCold TF DNA Vector uses cold shock technology for high yield protein expression combined with Trigger Factor (chaperone) expression to facilitate correct protein folding, thus enabling efficient soluble protein production for otherwise intractable target proteins. pCold TF DNA Vector is a fusion cold shock expression vector that expresses Trigger Factor (TF) chaperone as a soluble tag. Trigger Factor is a prokaryotic ribosome-associated chaperone protein (48 kDa) which facilitates co-translational folding of newly expressed polypeptides (www.takarabio.com). Therefore, pCold TF DNA vector is often used to express proteins with large molecular weight or proteins that cannot be expressed in other *E. coli* expression vectors such as Pet and pGEX (PMID: 19995911).**

**In this study, we not only expressed and purified Atg9-N, -M and -C proteins with TF tags, but also purified TF-Hrr25, TF-Atg11 CC2, TF-Atg11 CC3, and TF-Atg1 proteins. Atg11-CC2, Atg11-CC3, Atg1, and Hrr25 proteins have been reported to be directly associated with Ypt1. Our Ni-NTA pulldown results also found that TF-Hrr25, TF-Atg1, TF-Atg11-CC2 and CC3 proteins could directly bind with Ypt1 (now Figure S1A-C), indicating that TF tags do not affect the binding of these proteins with Ypt1 and that these fused proteins have normal biological activity.**

**However, as the reviewer said, we cannot completely rule out whether TF-Atg9-N, M or C protein is denatured or has no biological function. Because Atg9 directly bound to Atg23, we tested whether TF-Atg9-N, M or C proteins have natural biological activity by detecting their association with Atg23. We first detected which segments of the Atg9 cytoplasmic region binds to Atg23 using a Y2H assay. The results showed that there was a direct interaction of Atg23-AD with Atg9-N-BD and Atg9-C-BD (Figure R3A, and now Figure S2B). We then used purified TF-Atg9-N, M, or C proteins to perform GST pulldown experiments with GST-Atg23 protein from *E.Coli*. Consistent with the results of Y2H assays, TF-Atg9-N and C could, but TF-Atg9-M could not bind to Atg23 (Figure R3B, and now Figure S2A). This result shows that TF-Atg9-N, C and M have natural biological activity. Concurrently, GST pulldown results indicated that GST-Ypt1 does not bind with TF-Atg9-N, M or C (Figure R3C, and now**

**Figure 1C). The Co-IP experiment also indicated that the deletion of *ATG23* significantly decrease the association of Ypt1 with Atg9 (Figure R3D, and now Figure 1D). Finally, to express our conclusion more rigorously, we revised our conclusion as "Atg9 is not likely to directly interact with Ypt1".**

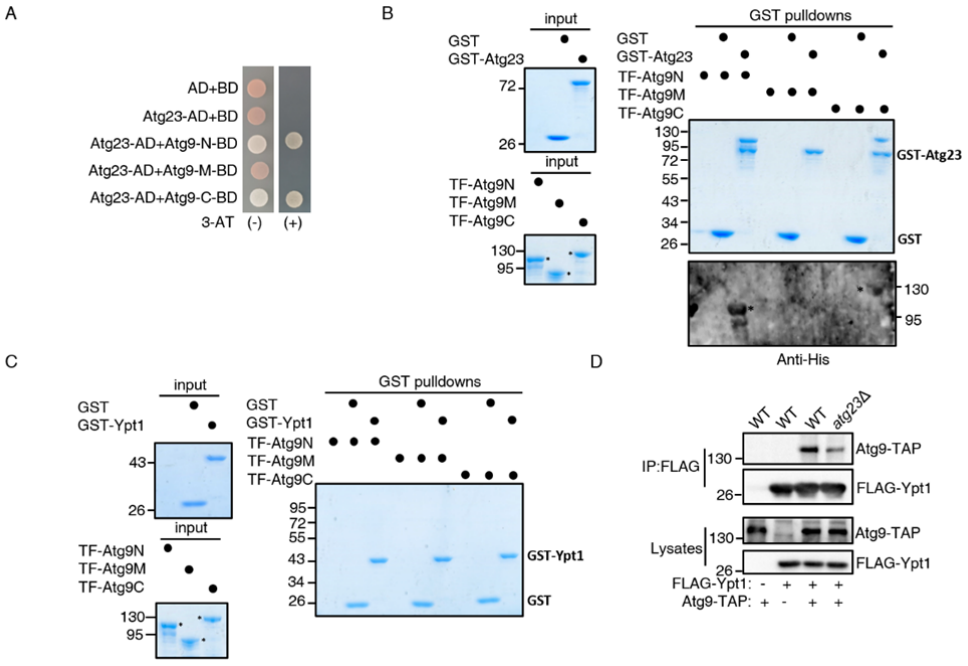


**Figure R3: Atg9 is not likely to directly interact with Ypt1. (A)** The AH109 strain was transformed with plasmids expressing Atg23-AD and plasmids expressing Atg9-N-BD (2-318aa), Atg9-M-BD (395-534aa), or Atg9-C-BD (747-997aa). These strains were grown on SD-Leu-Trp or SD-His-Leu-Trp+3-AT agar plates at 30°C for 3 d. **(B)** GST pulldowns were performed using purified His-tagged TF-Atg9 N (2-318aa), TF-Atg9 M (395-534aa), or TF-Atg9 C (747-997aa) with GST or GST-Attg23 from *E. Coli*. Protein samples were separated by SDS-PAGE, and then detected using Coomassie blue staining or western-blot with anti-His antibody. The asterisk represents the target protein. **(C)** GST pulldowns were performed using purified His-tagged TF-Atg9 N, TF-Atg9 M, or TF-Atg9 C with GST or GST-Ypt1 from *E. Coli*.

Protein samples were separated by SDS-PAGE, and then detected using Coomassie blue staining. The asterisk represents the target protein. **(D)** Atg9-TAP (tandem affinity purification tag, CBP-TEV-PA) and FLAG-Ypt1 were co-expressed in wild type or *atg23*Δ yeast strains. Cells were grown to the log-growth phase. Cell lysates were then immunoprecipitated with anti-FLAG agarose beads and analyzed with anti-Protein A antibody.

5) In Fig. 3A, the authors concluded that L170N-L171N-L173N or L188N-L189N mutation in Atg23 impaired the PAS recruitment of Atg9 vesicles. However, it may also be possible that these mutations impaired the generation of normal Atg9 vesicles (highly mobile vesicles in the cytoplasm) since Atg23 is important for Atg9 vesicle formation. Perform live imaging and confirm that formation of mobile Atg9 vesicles was not affected by these mutations by providing supplementary video (refer to PMID 22826123).

**Response: We thank the reviewer for the insightful suggestions. Reviewer 2# and 3# also had the same concerns and suggested that we test whether these two mutants affect the dimerization of Atg23 and the binding of Atg23 to Atg9. Atg23 has been previously reported to be able to form dimers with Atg23 and its dimerization is required for the binding of Atg23 with Atg9 (PMID: 35443167). Both Co-IP and *in vitro* pulldown assays were performed and showed that Atg23$^{L170N-L171N-L173N}$ or Atg23$^{L188N-L189N}$ mutants blocked the dimerization of Atg23 and subsequently also blocked the association of Atg23 with Atg9 (now Figure 2H-I and S3F-G).**

**Next, we wanted to identify the amino acid residues on the Atg23 that impair the binding of Atg23-Ypt1 but do not affect the dimerization of Atg23. BiFC assays indicated that Atg23 CC1 domain is also required for the dimerization of Atg23 (Figure S3H). We then conducted the Ni-NTA pulldown experiments on a series of indicated deletion mutants (only 5aa deletion) in the Atg23 CC1 domain, and found that the deletion mutants on the CC1 domain that impair the binding of Ypt1 to Atg23 also block the formation of Atg23 dimerization (now Figure S4). Taken together with these data, we conclude that the dimerization of Atg23 is a prerequisite for the binding of Atg23 to Ypt1.**

**Ypt1 W62 residue was identified as a key amino acid required for the binding of Ypt1 to Atg23 in our original manuscript. To test whether Ypt1$^{W62A}$ affects the binding of Ypt1 with Atg17, Atg1, Hrr25, or Atg11, as well as its dimerization, GST or Ni-NTA pulldown assays were performed. Results demonstrated that Ypt1$^{W62A}$ does not show impaired either their interactions or its dimerization (now Figure S5B-G). These data indicated that Ypt1 W62 residue specifically regulates its binding to Atg23. We then performed live imaging and confirm that formation of mobile Atg9 vesicles was not affected by Ypt1 $^{W62A}$ mutant (now Figure 4C, S7E and Movie EV1).**

6) In Fig. 4, the authors identified the binding region in Atg17 for Ypt1 using deletion

mutants. However, crystal structure of Atg17 suggests that such deletion would severely affect the folding of Atg17. Therefore identify the Ypt1 binding region in Atg17 by point mutations as performed for Atg23.

**Response: We performed further point mutations in these two regions to identify the key amino acids responsible for the binding of Atg17-Ypt1. To this end, we generated a series of random amino acid mutation combinations in these two regions for BiFC screening. The results showed that the vYFP signal was absent in the Atg17 $^{L286N-V290N}$ or Atg17 $^{I293E-I294E}$ mutants fused with VN, indicating that their binding was dependent on these residues on Atg17(now Figure 5C). Atg17 $^{L286N-V290N}$ and Atg17 $^{I293E-I294E}$ mutants' capacity to bind with Ypt1 were subsequently tested by using Ni-NTA pulldown assays. The results further confirmed that Atg17-Ypt1 binding was almost entirely lost in Atg17 $^{L286N-V290N}$ or Atg17 $^{I293E-I294E}$ mutants (now Figure 5D). These data indicated that Atg17 L286-V290 or I293-I294 residues are crucial for the binding of Ypt1-Atg17.**

**We then used these mutants in a GFP-Atg8 cleavage assay to investigate whether Atg17-Ypt1 binding was required for autophagic activity. Empty vector, Atg17 WT, Atg17$^{L286N-V290N}$, or Atg17$^{I293E-I294E}$ plasmids were separately transformed into an *atg17*Δ yeast strain co-expressing GFP-Atg8 and Vph1-Cherry. The cleavage of the GFP-Atg8 processing assay showed that Atg17 $^{L286N-V290N}$ or Atg17$^{I293E-I294E}$ mutants all led to free GFP levels in nitrogen starvation conditions becoming decreased to similar levels to those observed in *atg17*Δ yeast cells, suggesting that Ypt1-Atg17 binding is required for autophagy (now Figure**

**5E).**

**Since Atg17 forms a complex with Atg29 and Atg31, we next wanted to explore whether deficiency in the binding of Ypt1-Atg17 impairs the formation of the Atg17-Atg29-Atg31 complex by transforming Atg17 WT-2×GFP, Atg17$^{L286N-V290N}$-2×GFP, Atg17$^{I293E-I294E}$-2×GFP, or the empty vector plasmids into an *atg17*Δ yeast strain co-expressing Atg29-TAP and Atg31-GFP. Subsequent Co-IP assays revealed no significant change in the association of Atg17 with Atg29 and Atg31 in the absence of the binding of Ypt1-Atg17 binding (Figure S8F). Atg17 and Ape1 are two proteins widely used as PAS markers. We therefore detected whether deficiency in the binding of Ypt1-Atg17 affects the PAS formation by transforming Atg17 WT-2×GFP, Atg17 $^{L286N-V290N}$-2×GFP, Atg17 $^{I293E-I294E}$-2×GFP, or the empty vector plasmids into an *atg17*Δ yeast strain expressing RFP-Ape1. As shown in Figure S8G, Atg17-2×GFP, as the puncta form, was well co-localized with RFP-Ape1 in cells expressing the Atg17$^{L286N-V290N}$ or Atg17 $^{I293E-I294E}$ mutants under nutrient-rich medium. These data suggested that the Atg17$^{L286N-V290N}$ or Atg17$^{I293E-I294E}$ mutations do not affect the formation of the Atg17-Atg29-Atg31 complex or the PAS recruitment of Atg17.**

**Mechanistically, we then examined whether disruption of Ypt1-Atg17 interaction impaired the PAS recruitment of Ypt1. Transformation of 3×FLAG-tagged Atg17 WT, L286N-V290N, or I293E-I294E plasmids into an *atg17*Δ yeast strain co-expressing RFP-Ape1(PAS marker) and GFP-Ypt1, under nitrogen starvation conditions showed that recruitment of Ypt1 to the PAS was significantly**

**decreased in cells expressing Atg17 $^{L286N-V290N}$ or Atg17 $^{I293E-I294E}$ mutants (now Figure 5F-G). These findings suggested that the binding of Ypt1-Atg17 had provided a substantial contribution to the PAS recruitment of Ypt1 in response to nitrogen starvation. Given that Ypt1 is required for Atg1 puncta formation and that the absence of Ypt1-Atg17 binding adversely affects PAS recruitment of Ypt1, we next examined whether the Ypt1-Atg17 interaction was required for Atg1 puncta formation. To test this hypothesis, 3×FLAG-tagged plasmids carrying Atg17 WT, L286N-V290N, or I293E-I294E were transformed into an *atg17*Δ yeast cells expressing Atg1-GFP. As shown in Figure 5H-I, the formation of Atg1 puncta was significantly impaired in cells expressing any of two Atg17 FLAG-tagged mutants under nitrogen starvation conditions. Collectively, this data demonstrated Ypt1-Atg17 binding is required for autophagy by regulating the PAS recruitment of Ypt1 and Atg1.**

7) In Fig 7B, C, CBB staining suggests that Atg1 and Ypt1 S174D form a 1:1 complex whereas Atg1 binds large excess amounts of Ypt1 WT. Similarly, it is likely that Hrr25 and Ypt1 S174D form a 1:1 complex whereas Hrr25 binds large excess amounts of Ypt1 WT. These data seem to suggest that S174D mutation impaired the oligomerization of Ypt1 rather than the interaction of Ypt1 with Atg1 or Hrr25. If the authors want to claim that S174D mutation impaired the interaction with Atg1 or Hrr25, study the binding affinity using quantitative methods such as SPR or ITC.

**Response: We thank the reviewer for these insightful comments. In the light of this suggestion, we tested whether Ypt1$^{S174D}$ impaired the oligomerization of**

**Ypt1. Both Co-IP and Ni-NTA pulldown experiments showed that Ypt1$^{S174D}$ does not impair the dimerization of Ypt1 (Figure R4, now Figure S12C). Regarding the issue of "Hrr25 and Ypt1 S174D form a 1:1 complex whereas Hrr25 binds large excess amounts of Ypt1 WT". We suggest that the reason for this phenomenon may be that previously purified TF-Atg17, TF-Atg1, or TF-Hrr25 have some nonspecific bands or that this is due to some other unknown reason. Before repeating this experiment, we conducted further purification by molecular-sieve chromatography on these proteins after they had been purified by Ni column. We then repeated the Ni-NTA pulldown assays and found that TF-Atg1, TF-Atg17, TF-Hrr25 formed a 1:1 complex with Ypt1 WT, while the binding of Ypt1$^{S174D}$ to Atg1, Atg17, or Hrr25 was significantly decreased (now Figure 8A-C).**

**Furthermore, we measured the binding affinities of Ypt1 WT or S174D with further purified TF-Atg1, Atg17, or Hrr25 by SPR assay using Biacore. $K_d$ values showed that Ypt1 $^{S174D}$ did significantly decrease its binding to Atg1, Atg17, or Hrr25 (now Figure S13). Taken together, these data showed that the phosphorylation of Ypt1 by TOR inhibits the binding of Ypt1 with Atg1, Atg17, and Hrr25.**

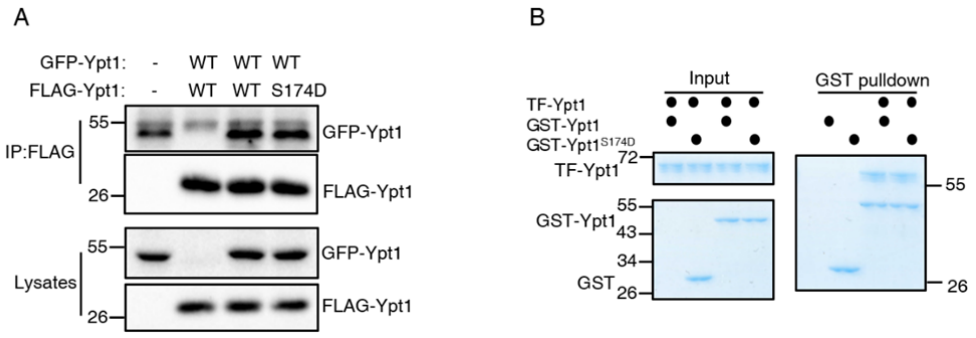


**Figure R4: Ypt1$^{S174D}$ mutation does not impair the dimerization of Ypt1. (A)** GFP-Ypt1 and FLAG-Ypt1 or FLAG-Ypt1$^{S174D}$ were co-expressed in wild type yeast strains. Cells were grown to the log-growth phase. Cell lysates were immunoprecipitated with anti-FLAG agarose beads and then analyzed with anti-GFP antibody. **(B)** GST pulldowns were performed using purified GST-Ypt1 or GST-Ypt1$^{S174D}$ with TF-Ypt1 from *E. Coli*. Protein samples were separated by SDS-PAGE, and then detected using Coomassie blue staining.

8) In Fig 5D-J, only S174D mutant of Ypt1 was studied. Study Ypt1 S174A mutant and compare the results with S174D and WT.

**Response: We added Ypt1$^{S174A}$-related data to the original Figure 5. As shown in Figure 8D-I, S14A-B and S14E-H, Ypt1$^{S174A}$ promotes the PAS recruitment of Ypt1, Atg1 and Hrr25 by enhancing the association of Ypt1 with Atg17, Atg1 and Hrr25. In addition, the Ypt1$^{S174A}$ mutant promotes the kinase activity of Atg1 under nitrogen starvation conditions (Figure 8J and S14D).**

9) Mammalian cells have Rab1A and Rab1B. In Fig 8, which type of Rab1 was expressed? Did Rab1b shRNA1 affect the expression level of Rab1A? Does the anti-Rab1 antibody recognize Rab1A and Rab1B equally?

**Response: In mammalian cells, Rab1 has two isoforms, Rab1A and Rab1B, which sharing 92% amino-acid sequence homology and are thought to be functionally redundant. Because Rab1a is 4 amino acids longer than Rab1b, we selected to express Rab1A in this study. Rab1b shRNA1 and shRNA2 sequences are "UGCCAGCGAGAACGUCAAUAA" and "CACGUACACAGAGAGCUACAU", respectively, these two shRNA sequences exist in both Rab1A and Rab1B. Regarding whether the anti-Rab1 antibody recognizes Rab1A and Rab1B equally, we purchased antibodies that only recognize Rab1A (11671-1-AP, Proteintech) or Rab1B (17824-1-AP, Proteintech) separately. As shown in Figure R5 (now Figure 9C), the data showed that both Rab1b shRNA1 and shRNA2 could efficiently knockdown Rab1A or Rab1B. We have added these data to the revised Figure.**

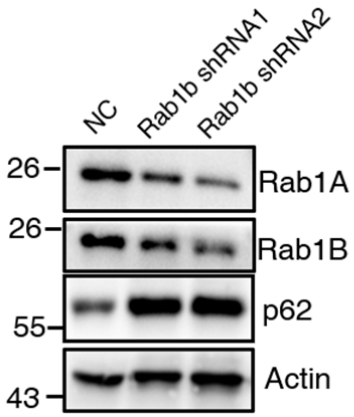


**Figure R5: The detection of knockdown efficiency of Rab1b shRNA1 and shRNA2 in HEK293T cells.** The expression levels of Rab1A and Rab1B in NC (non-specific control), and Rab1 stable knockdown HEK293T cells were analyzed using western-blot with anti-Rab1A antibody and anti-Rab1B antibody, respectively. The

expression levels of p62 were detected using anti-p62 antibody. β-Actin served as a loading control.

Minor points

1) In page 5, lines 14-17, the sentence is difficult to read and should be revised.

**Response: We have now rewritten this sentence in the revised manuscript.**

2) In page 6, lines 6-7, "As it is known that Ypt1 regulates the PAS recruitment of Atg9 vesicles and PAS localization of Ypt1 and Atg9 vesicles," this sentence is difficult to read. Ypt1 regulates PAS localization of Ypt1?

**Response: We thank the reviewer for pointing these out and now have changed it to "As it is known that Ypt1 regulates the PAS recruitment of Atg9 vesicles" in the revised manuscript.**

3) In Fig. 1B, 7A-C, S5, The results of pulldown assays stained with CBB should be shown as a single gel image or provide a single gel as Supplementary.

**Response: As suggested, we have now put a single gel image into the corresponding Figure. Since Atg13 and Atg14 were not associated with FLAG-Ypt1 in our immunoprecipitation experiment (Figure 1A), we did not include the related pulldown data in the revised manuscript. However, we performed the experiment again, the results showed that Atg13 and Atg14 did not bind to Ypt1(Figure R6).**

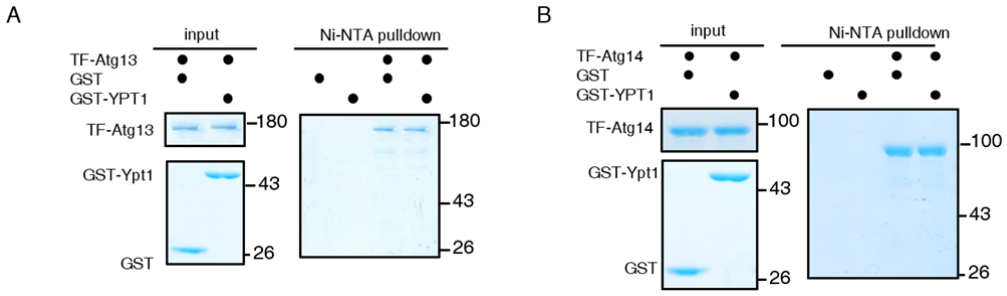


**Figure R6: Atg13 and Atg14 did not bind with Ypt1. (A-B)** Ni-NTA pulldowns were performed using purified GST or GST-Ypt1 with TF-Atg13(A) or TF-Atg14(B) from *E. Coli*. Protein samples were separated by SDS-PAGE, and then detected using Coomassie blue staining.

4) In page 11, line 14, W65A should be W62A.

**Response: We have now revised "W65A" as "W62A" in the revised manuscript.**

5) In Fig 4E, provide WB of Atg17 and confirm that the deletion mutants of Atg17 were expressed similarly with wild-type Atg17.

**Response: Because we further identified Atg17 L286-V290 or I293-V290 residues responsible for mediating the binding of Atg17 to Ypt1, this original Figure 4E was replaced by the new Figure 5E.**

6) In Fig. 5C, G, provide WB of Ypt1 and confirm that the mutants of Ypt1 were expressed similarly with wild-type Ypt1.

**Response: We added this data to the revised Figure 7C and 7G.**

7) In page 17, line 24, "These results further supported that mTOR-mediated Ypt1

phosphorylation is required for PAS recruitment of Atg1", the data show that Ypt1 phosphorylation impairs PAS recruitment of Atg1 and thus revise this sentence.

**Response: We have now changed this to "Ypt1 phosphorylation impairs PAS recruitment of Atg1" in the revised manuscript.**

8) In page 19, lines 17-19, "We found significant accumulation of p62 in Rab1 KD cells, indicating that Rab1 negatively regulates autophagy in mammals", negatively should be positively.

**Response: We have now changed this in the revised manuscript.**

9) Discussion section is largely redundant with Results section. Delete the redundant description of the results and fucus on writing "discussion" in the Discussion section.

**Response: Many thanks for your comments. We have now rewritten the discussion section.**

10) Throughout the manuscript, "mTOR" should be written as "TOR" when referring to yeast protein.

**Response: We have now modified mTOR as TOR when referring to yeast in the revised manuscript.**

**Referee #2:**

The authors examine the molecular mechanisms underlying autophagosome biogenesis in yeast. They focus on the small GTPaseYpt1/Rab1 previously linked to autophagosome biogenesis. The authors identify two new interactors of Ypt1, Atg23 and Atg9. Atg9 is an essential autophagy protein, which marks Atg9 vesicles that are incorporated into the phagophore where Atg9 functions as a scramblase. Previous work has shown that Atg9 forms a complex with Atg23 and Atg27 in yeast, which are important for the biogenesis of Atg9 vesicles and their transport to a site of autophagosome formation (PAS). The authors use a number of biochemical and cell biological approaches to show that Ypt1 physically interacts with Atg23. Mutations that disrupt this interaction result in impaired assembly of the core autophagy machinery and formation of autophagosomes. In addition, the authors detect physical interactions of Ypt1 with Atg17. Atg17 forms a complex with Atg29 and Atg31 and marks sites of autophagosome biogenesis providing a platform for the recruitment of downstream Atg proteins. The authors identify Atg17 variants that are impaired in Ypt1 binding and display defects in autophagy. Finally, the authors identify phosphorylation sites within Ypt1 and mammalian Rab1 for TORC1/mTOR, a central regulator of cell growth and inhibitor of autophagy. Interestingly, non-phosphorylatable or phospho-mimetic variants of Ypt1 result in elevated or delayed autophagy, suggesting a regulatory role for TORC1-mediated phosphorylation of Ypt1 I autophagy.

The study provides a solid set of biochemical and cell biological approaches that

identify novel connections for Ypt1 with the autophagy protein machinery with mechanistic insights. Based on the presented data, the authors propose in principle an interesting link from TORC1 siganling to Ypt1 and new regulatory interactions. However, the manuscript requires additional experimentation to fully support the proposed model:

Critical points:

(1) Are in vitro interactions of Ypt1 dependent on its GTP/GDP bound state? The authors do not describe the experimental conditions of their in vitro work in the manuscript in terms of protein concentrations, or presence of GTP/GDP (s. below). To test the specificity of the Ypt1 interactions in vitro, it would be important to test constitutive GTP or GDP bound variants of Ypt1 for interaction with Atg23 and Atg17

**Response: As suggested, we generated Ypt1 variants with disrupted ability to bind to GTP or GDP. As shown in Figure R1 (now Figure 1G and S8C), GST pulldown results indicated that Atg17 and Atg23 preferentially pulldown with GTP-bound form of Ypt1 ($Ypt1^{Q67L}$), not its GDP-bound form ($Ypt1^{S22N}$), suggesting that the binding of Ypt1 with Atg17 and Atg23 depends on its GTP-bound form.**

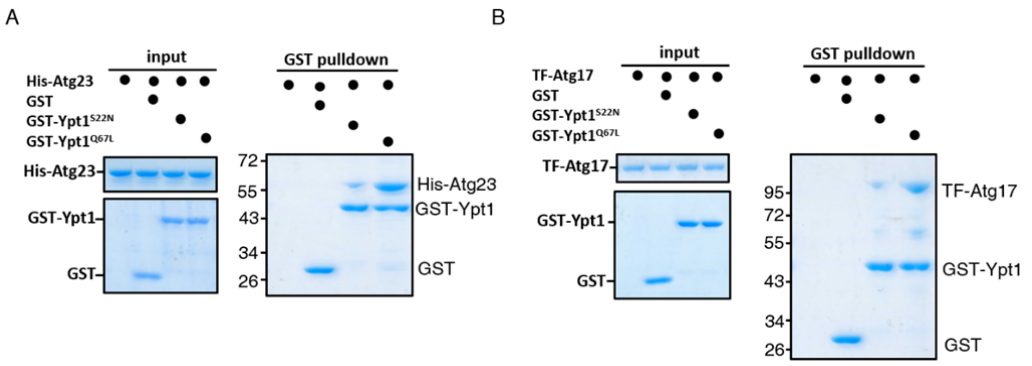


**Figure R1: The binding of Ypt1 with Atg17 and Atg23 depends on its GTP-bound form. (A)** GST pulldowns were performed using purified $His_6$-tagged Atg23 and GST, GST-Ypt1, GST-Ypt1$^{Q67L}$ (GTP-bound form), or GST-Ypt1$^{S22N}$ (GDP -bound form) from *E. Coli*. **(B)** GST pulldowns were performed using purified $His_6$-tagged TF-Atg17 and GST, GST-Ypt1$^{Q67L}$, or GST-Ypt1$^{S22N}$ from *E. Coli*. Protein samples were separated by SDS-PAGE, and then detected using Coomassie blue staining.

(2) Characterization of the Atg9-23-27 complex in dependence of Ypt1

The authors propose that the Atg23 L170,171,173N or L188,189N variants fail to interact with Ypt1, which they link to the phenotypical outcome. However, it is equally possible that these variants show impaired Atg9 binding. Since Atg23 is required for the biogenesis of peripheral Atg9 vesicles and anterograde transport of Atg9, a defect in Atg9 binding could explain the downstream effects on Atg9 and Atg11, Atg13, and Atg17 binding. Thus, it will be important to assess the physical interaction of Atg9 and the Atg23 variants. To examine potential defects in the biogenesis of peripheral Atg9 vesicles, the authors need to quantify the number of Atg9 puncta in cells and their intensities in the presence of Atg23 L170,171,173N or L188,189N variants. In addition, in Figure 1C, the authors should test the interaction

of Ypt1 and full-length Atg9.

**Response: We thank the reviewer for the insightful comment. Reviewer 1# and 3# also had same concerns and suggested that we test whether these two mutants impair the dimerization of Atg23 and the binding of Atg23 to Atg9. Atg23 has been reported to be able to form dimers with Atg23 and its dimerization is required for the binding of Atg23 to Atg9 (PMID: 35443167). Both Co-IP and *in vitro* pulldown assays were performed and showed that Atg23$^{L170N-L171N-L173N}$ or Atg23$^{L188N-L189N}$ mutants blocked the dimerization of Atg23 and the association of Atg23 with Atg9 (now Figure 2H-I and S3F-G).**

**Next, we wanted to identify the amino acid residues on the Atg23 that impair the binding of Atg23-Ypt1 but do not affect the dimerization of Atg23. BiFC assays indicated that the Atg23 CC1 domain is also required for the dimerization of Atg23 (Figure S3H). Next, we conducted the Ni-NTA pulldown experiments on a series of indicated deletion mutants (5aa deletion mutants) in the Atg23 CC1 domain, and found that the deletion mutants on the Atg23 CC1 domain that impair the binding of Ypt1 to Atg23 also block the formation of Atg23 dimerization (now Figure S4). Taken together with these data, we conclude that dimerization of Atg23 is a prerequisite for the binding of Ypt1 to Atg23.**

**In our original manuscript, the Ypt1 W62 residue was identified as a key amino acid required for the binding of Ypt1 to Atg23. To test whether Ypt1$^{W62A}$ also**

**affects the binding of Ypt1 to Atg17, Atg1, Hrr25, or Atg11, as well as its dimerization, GST or Ni-NTA pulldown assays were performed and found that Ypt1$^{W62A}$ does not result in impairments to either their interactions or its dimerization (Figure S5B-G). These data indicated that Ypt1 W62 residue specifically regulates its binding to Atg23. Subsequently, we found that Ypt1 W62 is required for autophagy and the PAS recruitment of Atg9 vesicles under nitrogen starvation conditions (now Figure 3E-F, 4A-B and S7A-B). As suggested, we quantified the number of Atg9 puncta in cells and their intensities in the presence of the Ypt1$^{W62A}$ variant. Quantitative results showed that there was no significant difference in the density of Atg9 puncta between Ypt1$^{W62A}$ and wild type cells (now Figure 4C), indicating that the deletion of the binding of Ypt1-Atg23 did not affect the biogenesis of peripheral Atg9 vesicles.**

**Regarding the reviewer's issue of "the authors should test the interaction of Ypt1 and full-length Atg9". Atg9 is about a 115kDa transmembrane protein, which has six transmembrane helices. As a multi-transmembrane protein, it is very difficult for our lab to express and purify full length Atg9 protein from *E. Coli* (In fact, we used almost all *E. coli* expression vectors to express Atg9 protein and tried many purification methods). To better address the issue, we performed Co-IP assay to test whether the association of Atg9 with Ypt1 depends on Atg23. As shown in Figure R7A (now Figure 1D), the deletion of *ATG23* significantly decreased the binding of Ypt1 with Atg9, suggesting that Atg23 positively**

**regulates the association Atg9 with Ypt1. Concurrently, a GST pulldown assay was performed and the result showed that Ypt1 does not bind to the Atg9-N, M or C fragment (Figure R7B, now Figure 1C). Finally, to express our conclusion more rigorously, we revised our conclusion as "Atg9 is not likely to directly interact with Ypt1".**

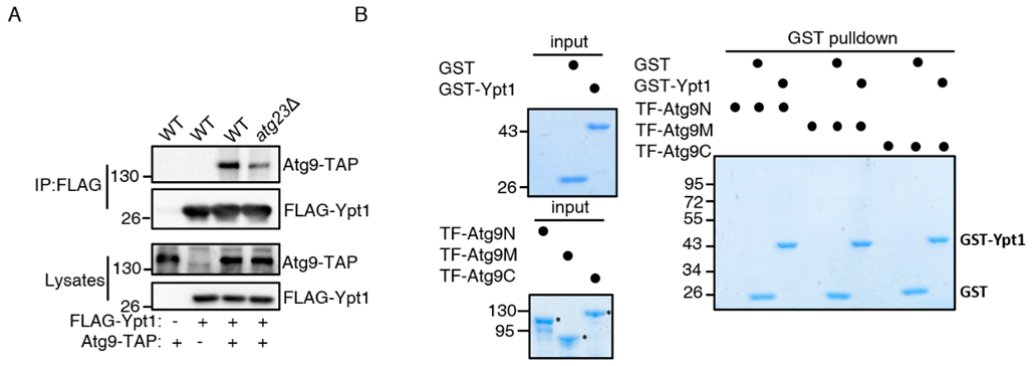


**Figure R7: Atg9 is not likely to directly interact with Ypt1. (A)** Atg9-TAP (tandem affinity purification tag, CBP-TEV-PA) and FLAG-Ypt1 were co-expressed in wild type or *atg23*Δ yeast strains. Cells were grown to the log-growth phase. Cell lysates were immunoprecipitated with anti-FLAG agarose beads and then analyzed with anti-Protein A antibody. **(B)** GST pulldowns were performed using purified $His_6$-tagged TF-Atg9 N (2-318aa), TF-Atg9 M (395-534aa), or TF-Atg9 C (747-997aa) with GST or GST-Ypt1 from *E. Coli*. Protein samples were separated by SDS-PAGE, and then detected using Coomassie blue staining. The asterisk represents the target protein.

(3) Characterization of the Atg17 complex in dependence of Ypt1

It is possible that the Atg17 variants impaired for Ypt1 interaction are also defective in forming a proper Atg17 complex. A defect in Atg17 complex formation may explain the changes that authors observe and assign to the impaired recruitment of Ypt1 and Atg1. Thus, do Atg17 variants that are impaired in Ypt1 binding still form a proper

Atg17-29-31 complex and localize to the PAS? The authors should also test Ypt1-W62A or other Ypt1 variants for Atg17 binding.

**Response: We thank the reviewer for the insightful suggestions. Reviewer1# and 3# also had the same concerns. Reviewer 1# suggested that we should identify the Ypt1 binding region in Atg17 by point mutations as performed for Atg23, so we performed further point mutations in these two regions (Atg17 286-290aa and 291-295aa) to identify the key amino acids responsible for the binding of Atg17-Ypt1. To this end, we generated a series of random amino acid mutation combinations in these two regions for BiFC screening. The results showed that vYFP signal was absent in the Atg17 $^{L286N-V290N}$ or Atg17 $^{I293E-I294E}$ mutants fused with VN, indicating that their binding was dependent on these residues in Atg17 (now Figure 5C). Atg17 $^{L286N-V290N}$ and Atg17 $^{I293E-I294E}$ mutants were subsequently tested their Ypt1 binding using Ni-NTA pulldown assays. The results further confirmed that Atg17-Ypt1 binding was almost entirely lost in Atg17 $^{L286N-V290N}$ or Atg17 $^{I293E-I294E}$ mutants (now Figure 5D). These data indicated that Atg17 L286-V290 or I293-I294 residues are crucial for the binding of Ypt1-Atg17.**

**We then used these mutants in a GFP-Atg8 cleavage assay to investigate whether Atg17-Ypt1 binding was required for autophagic activity. Empty vector, Atg17 WT, Atg17$^{L286N-V290N}$, or Atg17$^{I293E-I294E}$ plasmids were separately transformed into an *atg17*Δ yeast strain co-expressing GFP-Atg8 and Vph1-Cherry. As shown in Figure 5E, the cleavage of the GFP-Atg8 processing assay showed that Atg17 $^{L286N-V290N}$ or Atg17$^{I293E-I294E}$ mutants all led to free GFP levels in nitrogen**

**starvation conditions becoming decreased to similar levels to those observed in *atg17*Δ yeast cells. This suggests that Ypt1-Atg17 binding is required for autophagy.**

**Since Atg17 forms a complex with Atg29 and Atg31, we next wanted to explore whether deficiency in the binding of Ypt1-Atg17 impairs the formation of the Atg17-Atg29-Atg31 complex by transforming Atg17 WT-2×GFP, Atg17$^{L286N-V290N}$-2×GFP, Atg17$^{I293E-I294E}$-2×GFP, or the empty vector plasmids into an *atg17*Δ yeast strain co-expressing Atg29-TAP and Atg31-GFP. Subsequent Co-IP assays revealed no significant change in the association of Atg17 with Atg29 and Atg31 in the absence of Ypt1-Atg17 binding (now Figure S8F). Atg17 and Ape1 are two proteins widely used as PAS markers. We therefore detected whether deficiency in the binding of Ypt1-Atg17 affects PAS formation by transforming Atg17 WT-2×GFP, Atg17 $^{L286N-V290N}$-2×GFP, Atg17 $^{I293E-I294E}$-2×GFP, or the empty vector plasmids into an *atg17*Δ yeast strain expressing RFP-Ape1. As shown in Figure S8G, Atg17-2×GFP, as the puncta form, was well co-localized with RFP-Ape1 in cells expressing the Atg17$^{L286N-V290N}$ or Atg17$^{I293E-I294E}$ mutants under nutrient-rich medium. These data suggested that the Atg17$^{L286N-V290N}$ or Atg17$^{I293E-I294E}$ mutations does not affect the formation of the Atg17-Atg29-Atg31 complex or the PAS recruitment of Atg17.**

**Mechanistically, we then examined whether disruption of Ypt1-Atg17 interaction impaired the PAS recruitment of Ypt1. Transformation of 3×FLAG-tagged Atg17 WT, L286N-V290N, or I293E-I294E plasmids into an *atg17*Δ yeast strain**

**co-expressing RFP-Ape1(PAS marker) and GFP-Ypt1, under nitrogen starvation conditions showing that recruitment of Ypt1 to the PAS was significantly decreased in cells expressing Atg17 $^{L286N-V290N}$ or Atg17 $^{I293E-I294E}$ mutants (now Figure 5F-G). These findings suggested that the binding of Ypt1-Atg17 provides a substantial contribution to the PAS recruitment of Ypt1 in response to nitrogen starvation. Given that Ypt1 is required for Atg1 puncta formation, and that the absence of Ypt1-Atg17 binding adversely affects PAS recruitment of Ypt1, we next examined whether the Ypt1-Atg17 interaction was required for Atg1 puncta formation. To test this hypothesis, 3×FLAG-tagged plasmids carrying Atg17 WT, L286N-V290N, or I293E-I294E plasmids were transformed into an *atg17*Δ yeast cells expressing Atg1-GFP. As shown in Figure 5H-I, the formation of Atg1 puncta was significantly impaired in cells expressing any of two Atg17 FLAG-tagged mutants under nitrogen starvation conditions. Collectively, this data demonstrated that Ypt1-Atg17 binding is required for autophagy by regulating the PAS recruitment of Ypt1 and Atg1.**

**As suggested, we also tested whether Ypt1$^{W62A}$ affects the binding of Ypt1 with Atg17. As shown in Figure R8 (now Figure S5C), GST pulldown assays revealed that, compared with Ypt1 WT, Ypt1$^{W62A}$ does not impair the association of Ypt1 with Atg17.**

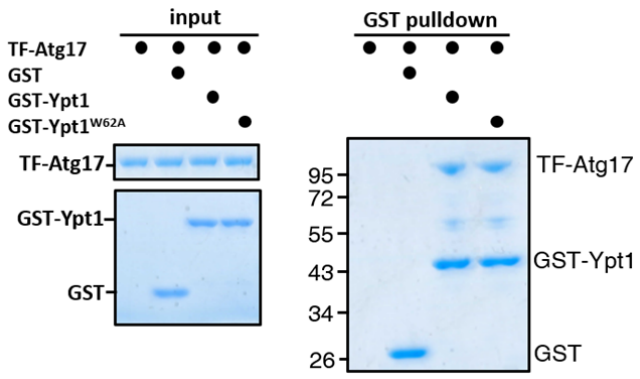


**Figure R8: Ypt1$^{W62A}$ does not impair the binding of Ypt1 with Atg17.** GST pulldowns were performed using purified TF-Atg17 with GST, GST-Ypt1, or GST-Ypt1$^{W62A}$ from *E. Coli*. Protein samples were separated by SDS-PAGE, and then detected using Coomassie blue staining.

(4) Examining the link between TORC1 and Ypt1

Figure 6C-H should include a proper time course analysis (0, 1, 2, 4, 8, 12, 24h) to assess the overall effect of ypt1S174A or D on autophagy not just one timepoint.

**Response: To answer the reviewer's question, we performed ALP assay to examine autophagic activity of Ypt1$^{S174A}$ and Ypt1$^{S174D}$ mutants at multiple starvation time points. As shown in Figure R9 (now Figure S11E), compared with the wild type Ypt1, the Ypt1$^{S174D}$ mutant displayed significantly decreased autophagic activity, while Ypt1$^{S174A}$ mutant showed accelerated autophagy at different time points under nitrogen starvation conditions. These data further indicated that Ypt1 is an autophagic determinant that is controlled by TOR.**

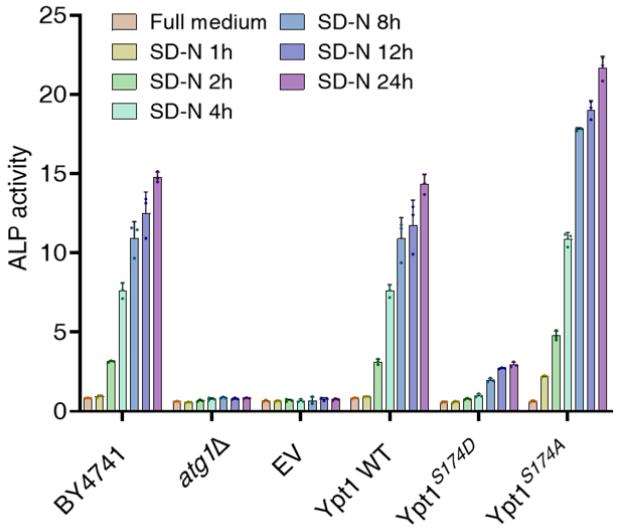


**Figure R9: The phosphorylation of Ypt1 by TOR negatively regulates autophagy.** AID-3×HA-Ypt1 ALP yeast strains expressing empty vector, FLAG-Ypt1, FLAG-Ypt1$^{S174A}$, or FLAG-Ypt1$^{S174D}$ plasmids were grown to early log-growth phase, IAA was added to induce the degradation of AID-3×HA-Ypt1 protein. These yeast strains were then subject to nitrogen starvation for the indicated timepoints in the presence of IAA. ALP activity was tested from n=3 independent experiments. Error bars indicate standard deviation (s.d.).

(5) Materials and Methods

All methods should be described not just referenced. The description of protein purification and the in vitro interaction assays are completely missing and thus I could not evaluate the validity of the approaches.

**Response: We have re-written related parts adding this into the revised Materials and Methods.**

Referee #3:

Yao et al. present numerous novel findings regarding Ypt1's role in Autophagy including several previously unknown interactions, key phosphorylated residues, and mechanistic conservation across distant eukaryotes. While these results could add much to the field's understanding of autophagy protein recruitment to the PAS, several critical issues remain with the manuscript. Perhaps the biggest issue is the authors' failure to interpret their findings through the lens of the recent, and highly related, paper from Hawkins et al., Cell Reports 2022. Knowledge of this paper's findings raises several key issues with Yao and colleagues' interpretation of their data regarding Atg23. Other studies that contradict some of the findings in the present paper have also been ignored.

1. Line 19-21, page 3: Needs to be re-written as it currently does not make sense.

**Response: We thank the reviewer for pointing this out and we have rewritten this sentence in the revised manuscript.**

2. Introduction: The authors mention the role of Ypt1 in regulating membrane tethering events. This should be discussed in light of Atg23-mediated membrane tethering as revealed in the 2022 paper by Hawkins et al., "Dimerization-dependent membrane tethering by Atg23 is essential for yeast autophagy".

**Response: Many thanks for the reviewer's comments. We have now cited this paper in the revised introduction section.**

3. Similarly, the findings of this paper will be more convincing if the researchers discuss the potential reasons for the contradictory results between other previous studies and the present paper. For example, the 2013 paper by Wang et al. titled "Ypt1 recruits the Atg1 kinase to the preautophagosomal structure" shows that Ypt1 does not recruit Atg17 to the PAS (The PAS localization of Atg17-GFP is not disrupted in the ypt1-2 mutant). Also, the conclusion in this paper that Ypt1-Atg23 binding is required for the PAS recruitment of Atg9 vesicles is not consistent with a previous 2012 study by Kakuta et al., "Atg9 vesicles recruit vesicle-tethering proteins Trs85 and Ypt1 to the autophagosome formation site", which showed that Ypt1 is localized to the preautophagosomal structure in an Atg9-dependent manner. It will be important for the authors to summarize all the novel findings in this paper that are not in line with previous studies and discuss the reasons for the inconsistencies.

**Response: We appreciate the reviewer for the valuable comments and have discussed this in the revised discussion section.**

4. Along these lines, it might be inappropriate to claim that Atg23 is a previously unrecognized binding partner of Ypt1. A paper published in 2013 by Graef et al., "ER exit sites are physical and functional core autophagosome biogenesis components" reported the potential binding of Atg23 with Ypt1. This manuscript at least needs to be cited in the present paper.

**Response: We have now cited this paper in the revised manuscript.**

5. Line 11, page 6: I do not follow the logic behind this conclusion. I do not think that this finding necessarily implies that Atg23 is recruited before Atg9.

**Response: We totally agree with the reviewer's comment and have deleted this sentence in the revised manuscript.**

6. Figure 1A: A full list of mass spec hits should be presented, perhaps as a supplemental figure.

**Response: We thank the reviewer for the comment. We have put a full list of mass spec hits into the Dataset EV1 in the revised version.**

7. Figure 1B: No input was shown for His-Atg23 alone. I recommend cutting that lane from the pulldown.

**Response: We have added input for His-Atg23 alone in the corresponding figure.**

8. Figure 1C: Explain the meaning of "TF". Abbreviations such as "TF" and "TAP" need to be explained upon first usage.

**Response: TF and TAP tags are abbreviations of "trigger factor tag" and "tandem affinity purification tag" respectively. We have explained the meaning of TF and TAP in the revised Figure 1C and 3C.**

9. Figure 1C: Why didn't the authors test full-length Atg9? If they cannot purify the full-length protein, they should repeat the experiment using an atg23Δ strain. Explain

the meaning of the asterisks in the figure legend. It would be nice to see a reverse pulldown of Ypt1 with anti-GST beads. I suggest changing the conclusion to "we think Atg9 is not likely to directly interact with Ypt1".

**Response: We thank the reviewer for the valuable comments. Atg9 is about a 115kDa transmembrane protein, which have six transmembrane helices. As a multi-transmembrane protein, it is very difficult for our lab to express and purify full length Atg9 protein from *E. Coli* (In fact, we used almost all *E. coli* expression vectors to express Atg9 protein and tried many purification methods). To better address the issue, we performed Co-IP assay to test whether the association of Atg9 with Ypt1 depends on Atg23. As shown in Figure R7A (now Figure 1D), the deletion of *ATG23* significantly decreased the binding Ypt1 with Atg9, suggesting that Atg23 is required for the association Atg9 with Ypt1. Concurrently, as suggested, a GST pulldown assay was performed and the result showed that Ypt1 does not bind to the Atg9-N, M or C fragment (Figure R7B, now Figure 1C). Finally, to express our conclusion more rigorously, we revised our conclusion as "Atg9 is not likely to directly interact with Ypt1".**

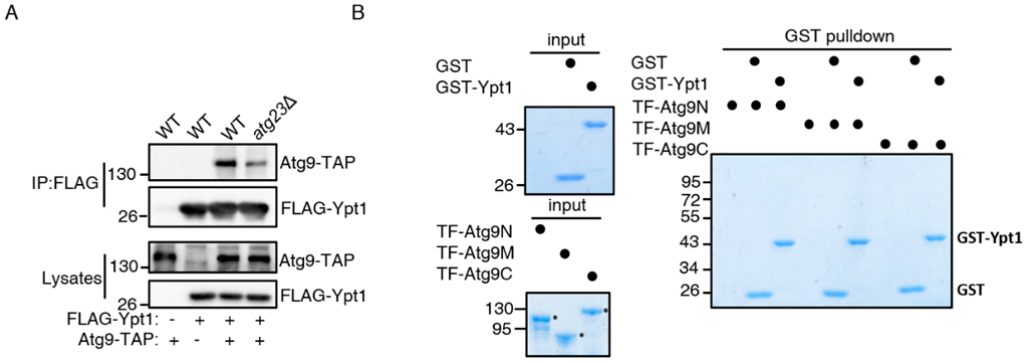


**Figure R7: Atg9 is not likely to directly interact with Ypt1. (A)** Atg9-TAP (tandem

affinity purification tag, CBP-TEV-PA) and FLAG-Ypt1 were co-expressed in wild type (WT) or *atg23*Δ yeast strains. Cells were grown to the log-growth phase. Cell lysates were then immunoprecipitated with anti-FLAG agarose beads and then analyzed with anti-TAP antibody. **(B)** GST pulldowns were performed using purified $His_6$-tagged TF-Atg9 N (2-318aa), TF-Atg9 M (395-534aa), or TF-Atg9 C (747-997aa) with GST or GST-Ypt1 from *E. Coli*. Protein samples were separated by SDS-PAGE, and then detected using Coomassie blue staining. The asterisk represents the target protein.

10. Figure 1D: Atg23 has been shown to form many puncta, the authors should repeat this with a PAS marker to see if this one colocalization event is at the PAS or elsewhere.

**Response: We thank the reviewer for this insightful comment. In the light of this suggestion, we observed whether PAS marker Ape1 colocalizes with Atg23-vYFP-Ypt1, As shown in Figure R10 (now Figure S2C), the image data showed that the PAS marker Ape1 highly colocalizes with the puncta of Atg23-vYFP-Ypt1 under full medium or nitrogen starvation conditions, suggesting that this one colocalization event is at the PAS.**

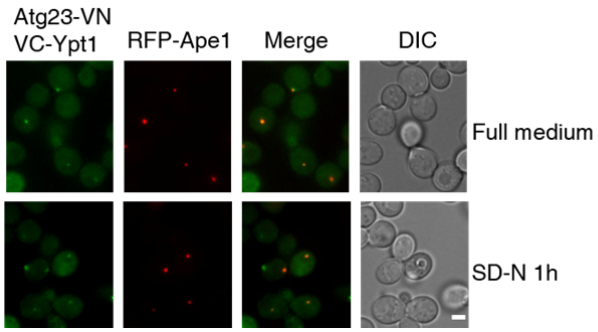


**Figure R10: RFP-Ape1 colocalizes with Atg23-vYFP-Ypt1 under full medium or nitrogen starvation conditions.** Yeast cells expressing the BiFC constructs Atg23-

VN, VC-Ypt1, and RFP-Ape1 cultured in nutrient-rich medium and shifted to nitrogen starvation medium for 1 h. Images were obtained by fluorescence microscopy. Scale bar: 2 μm.

11. Figure 2B, S1C; Line 6, page 8: The authors need to explain how these amino acid residues were selected. The same applies to Ypt1 W62.

**Response: We have explained how Atg23$^{L170\text{-}L171\text{-}L173}$, Atg23$^{L188\text{-}L1189}$ and Ypt1$^{W62}$ were selected in the revised manuscript.**

12. Figure S2B: The authors should more thoroughly demonstrate their process for determining the Ypt1 residues required for Atg23 binding and move this to a main figure.

**Response: We have now moved the original Figure S2B to a main Figure 3 in the revised manuscript.**

13. Figure 2: The authors completely fail to mention the recent findings of Hawkins et al. Based on the findings of this previously published paper, mutation of the hydrophobic face of the CC1 domain would render Atg23 monomeric. The effects of Atg23 mutation reported by Yao et al. may thus be a result of Atg23 monomerization and not direct mutation of the Atg23-Ypt1 binding interface.

**Response: We thank the reviewer for the insightful suggestions. Reviewer 2# also had same concerns and suggest that we test whether these two mutants affect the binding of Atg23 with Atg9. As the reviewer said, Atg23 was reported to be**

**formed dimers with Atg23 and its dimerization was required for the binding of Atg23 to Atg9(PMID: 35443167). We then performed both Ni-NTA pulldowns and Co-IP assays, and found that Atg23$^{L170N\text{-}L171N\text{-}L173N}$ or Atg23$^{L188N\text{-}L189N}$ mutants blocked the dimerization of Atg23 and its subsequent association with Atg9 (Figure 2H-I, S3F-G).**

**Next, we wanted to identify the amino acid residues on the Atg23 that impair the binding of Atg23-Ypt1 but do not affect the dimerization of Atg23. BiFC assays indicated that Atg23 CC1 domain is also required for the dimerization of Atg23 (Figure S3H). We then conducted the Ni-NTA pulldown experiments on a series of indicated deletion mutants (5aa deletion variants) in the Atg23 CC1 domain, and found that the deletion mutants on the Atg23 CC1 domain that impair the binding of Ypt1 to Atg23 also block the formation of Atg23 dimerization (Figure S4). Taken together with these data, we conclude that the dimerization of Atg23 is a prerequisite for its binding to Ypt1.**

**In our original manuscript, the Ypt1 W62 residue was identified as a key amino acid required for the binding of Ypt1-Atg23. To test whether Ypt1$^{W62A}$ also affects its binding to Atg17, Atg1, Hrr25, and Atg11, as well as its dimerization, GST or Ni-NTA pulldown assays were performed. Results showed Ypt1$^{W62A}$ does not display impaired either their associations or its dimerization (Figure S5B-G). These data indicated that the Ypt1 W62 residue specifically regulates its binding**

**to Atg23. Subsequently, we found that Ypt1 W62 is required for autophagy and the PAS recruitment of Atg9 vesicles under nitrogen starvation conditions (now Figure 3E-F, 4A-B and S7A-B). Furthermore, we used Ypt1$^{W62A}$ to study the effect of the deficiency of Ypt1-Atg23 interaction on the PAS recruitment of Atg9 vesicles and its corresponding molecular mechanisms. Once again, we thank the reviewer for pointing out our inappropriate conclusions.**

14. Figure 2E, F: Please provide quantification from multiple repeats.

**Response: We have now quantified in the original Figure 2E and F (now Figure 2E and G).**

15. Figure 3: Again, the authors fail to incorporate the findings of Hawkins et al. and thus potentially misinterpret their results.

**Response: We have now cited this paper in our revised reference.**

16. Figure 3A: Deletion of ATG23 has been shown to result in fewer and less intense Atg9 puncta intensity (2012, Yamamoto et al., "Atg9 vesicles are an important membrane source during early steps of autophagosome formation"), but this is not seen here. Have the authors standardized exposure settings across all images?

**Response: We thank the reviewer for the valuable comments. Because Atg23 $^{L170N-L171N-L173N}$ or Atg23 $^{L188N-L189N}$ mutants blocked the dimerization of Atg23 and its subsequent association with Atg9, we deleted these image data in the**

**revised manuscript. Instead, we used Ypt1$^{W62A}$ to study the effect the deficiency of Ypt1-Atg23 interaction on the PAS recruitment of Atg9 vesicles. We repeated the fluorescence microscope experiment by standardized exposure settings across all images and quantifying the number of Atg9 puncta in cells and their intensities in the presence of the Ypt1$^{W62A}$ variant. Quantitative results showed that there was no significant difference in the density of Atg9 puncta between Ypt1 $^{W62A}$ and wild type (Figure 4C), indicating that the deletion of the binding of Ypt1-Atg23 did not affect the biogenesis of peripheral Atg9 vesicles. Concurrently, we also tested the number of Atg9 puncta in *atg23*Δ yeast cells where imaging data and quantitative results showed that the number of Atg9 puncta in *atg23*Δ cells was significantly lower than that in wild type cells (Figure R11).**

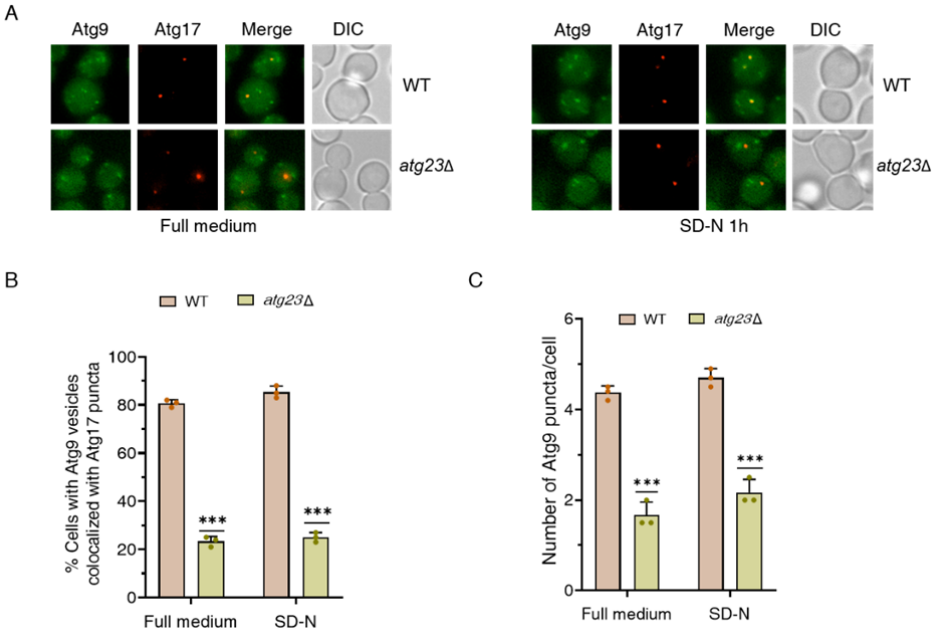


**Figure R11. Atg23 is required for the PAS recruitment of Atg9 vesicles and the biogenesis of Atg9 vesicles. (A)** Co-expressing Atg17-2×mCherry and Atg9-

2×GFP in wild type or *atg23*Δ yeast strains were grown to log-growth phase and then subjected to SD-N for 0h or 1h. Images of cells were obtained using the inverted fluorescence microscope. Scale bar, 2 μm. **(B)** Cells from (A) were quantified for the number of cells in which Atg9-2×GFP colocalized with Atg17-2×mCherry. n=300 cells were pooled from three independent experiments. Data are shown as mean ± SD. ***p < 0.001; two-tailed Student's t tests were used. **(C)** Cells from (A) were quantified for the number of Atg9-2×GFP. n=300 cells were pooled from three independent experiments. Data are shown as mean ± SD. ***p < 0.001; two-tailed Student's t tests were used.

17. When the authors investigate PAS recruitment, they always use one protein to label the PAS. For example, they used Atg17 to label the PAS in the experiment shown in Figure 3A-B. The result of this experiment will be more convincing if they can show the colocalization of Atg9 with more than one PAS marker, such as Trs85 or precursor Ape1. Considering that some conclusions in this paper (such as Atg9 recruitment to the PAS depending on Ypt1-Atg23 binding) are apparently different from other studies, the researchers might want to show this result by more than one assay.

**Response: We thank the reviewer for these comments. Because the dimerization of Atg23 is a prerequisite for its binding to Ypt1, we used Ypt1$^{W62A}$ to study the effect of the deficiency of Ypt1-Atg23 interaction on the PAS recruitment of Atg9 vesicles. As suggested, we used two PAS markers Atg17 and Ape1 to detect whether Ypt1$^{W62A}$ would affect the PAS recruitment of Atg9 vesicles. Imaging data and quantitative results showed that the PAS recruitment of Atg9 puncta**

**had significantly decreased in Ypt1$^{W62A}$ mutants under full medium or nitrogen starvation conditions (Figure 4A-B and S7A-B).**

18. Figure 4: While it is acceptable to begin with amino acid deletions, the authors must be able to show disrupted Ypt1 binding with a small number of amino acid conversions. Deleting residues from a critical helix in Atg17 could be completely disrupting the structure, and this could be the actual cause of all the observed defects. Are the deletion mutants still forming puncta on their own? Are they still able to interact with other known Atg17 binding partners? It is impossible to know if the defects observed in the Atg17 amino acid deletion mutants have any direct relation to Ypt1 binding.

**Response: We thank the reviewer for the insightful suggestions. Reviewer 1# and 2# also had the same concerns. As suggested, we performed further point mutations in these two regions (Atg17 286-290aa and 291-295aa) to identify the key amino acids responsible for the binding of Atg17-Ypt1. To this end, we generated a series of random amino acid mutation combinations in these two regions for BiFC screening. The results showed that the vYFP signal was absent in the Atg17 $^{L286N-V290N}$ or Atg17 $^{I293E-I294E}$ mutants fused with VN, indicating that their binding was dependent on these residues in Atg17(now Figure 5C). Atg17 $^{L286N-V290N}$ and Atg17 $^{I293E-I294E}$ mutants were subsequently tested for their Ypt1 binding using Ni-NTA pulldown assays. The results further confirmed that Atg17-Ypt1 binding was almost entirely lost in Atg17 $^{L286N-V290N}$ or Atg17 $^{I293E-}$**

**$^{I294E}$ mutants (now Figure 5D). These data indicated that Atg17 L286-V290 or I293-I294 residues are crucial for the binding of Ypt1-Atg17.**

**We then used these mutants in a GFP-Atg8 cleavage assay to investigate whether Atg17-Ypt1 binding was required for autophagic activity. Empty vector, Atg17 WT, Atg17$^{L286N-V290N}$, or Atg17$^{I293E-I294E}$ plasmids were separately transformed into an *atg17*Δ yeast strain co-expressing GFP-Atg8 and Vph1-Cherry. As shown in Figure 5E, under nitrogen starvation conditions, the cleavage of the GFP-Atg8 processing assay showed that Atg17$^{L286N-V290N}$ or Atg17$^{I293E-I294E}$ mutants all led to free GFP levels becoming decreased to similar levels to those observed in *atg17*Δ yeast cells, suggesting that Ypt1-Atg17 binding is required for autophagy.**

**Since Atg17 forms a complex with Atg29 and Atg31, we next wanted to explore whether deficiency in the binding of Ypt1-Atg17 impairs the formation of the Atg17-Atg29-Atg31 complex by transforming Atg17 WT-2×GFP, Atg17$^{L286N-V290N}$-2×GFP, Atg17$^{I293E-I294E}$-2×GFP, or the empty vector plasmids into an *atg17*Δ yeast strain co-expressing Atg29-TAP and Atg31-GFP. Subsequent Co-IP assays revealed no significant change in the association of Atg17 with Atg29 and Atg31 in the absence of Ypt1-Atg17 binding (Figure S8F). Atg17 and Ape1 are two proteins widely used as PAS markers, we then detected whether deficiency in the binding of Ypt1-Atg17 affects the PAS formation by transforming Atg17 WT-2×GFP, Atg17 $^{L286N-V290N}$-2×GFP, Atg17 $^{I293E-I294E}$-2×GFP, or the empty vector plasmids into an *atg17*Δ yeast strain expressing RFP-Ape1. As shown in Figure S8G, Atg17-2×GFP, as the puncta form, was well co-localized with RFP-Ape1 in**

**cells expressing the Atg17$^{L286N-V290N}$ or Atg17$^{I293E-I294E}$ mutants under nutrient-rich medium. These data suggested that the Atg17$^{L286N-V290N}$ or Atg17$^{I293E-I294E}$ mutants does not affect the formation of Atg17-Atg29-Atg31 complex and the PAS recruitment of Atg17.**

**Mechanistically, we then examined whether the disruption of Ypt1-Atg17 interaction impaired the PAS recruitment of Ypt1. Transformation of 3×FLAG-tagged Atg17 WT, L286N-V290N, or I293E-I294E plasmids into an *atg17*Δ yeast strain co-expressing RFP-Ape1(PAS marker) and GFP-Ypt1, under nitrogen starvation conditions showed that recruitment of Ypt1 to the PAS was significantly decreased in cells expressing Atg17 $^{L286N-V290N}$ or Atg17 $^{I293E-I294E}$ mutants (Figure 5F-G). These findings suggested that the binding of Ypt1-Atg17 provided a substantial contribution to the PAS recruitment of Ypt1 in response to nitrogen starvation. Given that Ypt1 is required for Atg1 puncta formation and that the absence of Ypt1-Atg17 binding adversely affects PAS recruitment of Ypt1, we next examined whether the Ypt1-Atg17 interaction was required for Atg1 puncta formation. To test this hypothesis, 3×FLAG-tagged plasmids carrying Atg17 WT, L286N-V290N, or I293E-I294E plasmids were transformed into an *atg17*Δ yeast cells expressing Atg1-GFP. As shown in Figure 5H-I, the formation of Atg1 puncta was significantly impaired in cells expressing any of two Atg17 FLAG-tagged mutants under nitrogen starvation conditions. Collectively, this data demonstrated that Ypt1-Atg17 binding is required for autophagy by regulating the PAS recruitment of Ypt1 and Atg1.**

19. Figure 4: Since the authors studied interaction between Ypt1 and Atg17, they should also include Atg17 in the MS data presentation shown in Figure 1. In fact, I recommend replacing Figure 1A with a panel showing the enrichments of known Ypt1 interactors in the MS data.

**Response: We thank the reviewer for the suggestions. As suggested, we replaced Figure 1A with a panel showing the enrichments of known Ypt1 interactors from the MS data in the revised manuscript.**

20. Figure 4: Since Ypt1 is a newly identified interactor of Atg17 in this research, I recommend the authors examine which of these proteins is recruited to the PAS first before they use the colocalization between Ypt1 and Atg17 to infer the recruitment of Ypt1 to the PAS.

**Response: We thank the reviewer for the insightful comment. In the light of this suggestion, we constructed the yeast strains that co-expressed Atg17-2×mCherry and the indicated ATG proteins with GFP tag in wild type or AID-3×HA-Ypt1 cells. IAA was added to induce the degradation of endogenous AID-3×HA-Ypt1 protein. Image data analysis found that IAA-mediated Ypt1 degradation had severely affected the PAS recruitment of Atg9 vesicles and the puncta formation of Atg1, Atg2, Atg5, Atg8, and Atg14. However, IAA-mediated Ypt1 degradation had not impaired the puncta formation and PAS recruitment of Atg11 and Atg13 (now Figure S9C). Taken together, these results indicated that Ypt1 regulates the PAS recruitment of Atg9 vesicles, PI3K complex I, Atg2-Atg18 complex, and two**

**ubiquitin-like systems under nitrogen starvation conditions.**

21. Figure 4E to 4H: The authors need to show the expression level of exogenous proteins.

**Response: We have showed the expression level of exogenous of proteins in the corresponding Figure.**

22. Figures 2 to 4: Although the mutations in Atg23 and Ypt1 affects their binding to each other, these data do not indicate if the mutated region is the direct binding region. Rather, these data indicate that these regions/amino acids are required for binding. Thus, I recommend that the authors change their wording. For example, in the sentence "To delineate which of the regions of Atg23 were responsible for its binding with Ypt1", I recommend replacing "responsible" with "required"; similarly, in the sentence "the binding region of Atg23-Ypt1 on Ypt1 was also delineated using same approach.", I recommend changing the wording, as the experiment cannot indicate if the region is the direct binding region. The same applies for the wording with regard to Atg17.

**Response: We totally agree with the reviewer's rigorous comments. As suggested, we have revised and highlighted this in the revised manuscript.**

23. Although the complementary data from Atg23 and Ypt1 mutations reduce the likelihood that these mutations might be affecting some other functions of the protein that lead to defects in Atg9 recruitment to the PAS, the current data did not exclude

such possibilities. Essentially, from the current data, where defects were seen in Atg23-Ypt1 interaction, in Atg9 recruitment to the PAS, and in autophagy, there are insufficient data to determine the causal relationship between these events (e.g., if defects in Atg9 recruitment are caused by defective Atg23-Ypt1 interaction). It is also not possible to exclude a compromised upstream event (e.g., Atg23 dimerization) that leads to all these defects. Accordingly, I recommend the authors check if the interaction between Atg9 and mutant Atg23 is similar to wild-type Atg23 using in vivo and in vitro pulldown. The authors can also try a yeast two-hybrid assay.

**Response: We thank the reviewer for the insightful comments. As mentioned above, Atg23$^{L170N\text{-}L171N\text{-}L173N}$ or Atg23$^{L188N\text{-}L189N}$ mutants blocked the dimerization of Atg23 and its subsequent association with Atg9. We used Ypt1$^{W62A}$ to study the effect the deficiency of Ypt1-Atg23 interaction on the PAS recruitment of Atg9 vesicles and the corresponding molecular mechanism.**

24. Figure 4: The authors concluded "Collectively, this data demonstrated Ypt1-Atg17 interactions to be clearly required for autophagy due to their function in the PAS recruitment of Ypt1 and Atg1". However, I think it is risky to conclude the causal relationship between defective autophagy and defective Ypt1 localization to the PAS without an experiment where the authors try to restore the localization of Ypt1 to the PAS in the atg17Δ strain. Similarly, the authors should demonstrate that the Atg17 mutants in Figure 5 do not affect the recruitment of Atg1 in a Ypt1-independent manner.

**Response: We thank the reviewer for pointing these out. As suggested, we modified this sentence as "Collectively, this data demonstrated Ypt1-Atg17 interaction is required for autophagy by regulating the PAS recruitment of Ypt1 and Atg1."**

25. Figure 5A: This should be moved to the supplement. What is the upper band?

**Response: We have moved original Figure 5A to the revised Figure S10A. Regarding the upper band, MS identified the protein as GDI1, which is a GDP dissociation inhibitor that regulates vesicle traffic in secretory pathways by regulating the dissociation of GDP from the Sec4/Ypt/Rab family of GTP binding proteins.**

26. Figure 5B: Move to the supplement. I suggest that the authors replace these data with a representation of mass spec results that is more easily interpreted by cell biologists.

**Response: Point well taken. We moved the original Figure 5B to the supplemental Figure 10B (Figure S10B) and replaced it with a more intuitive figure (Figure 6A).**

27. Figure S5G: The authors may want to include an HA-only control.

**Response: We have now added an HA-only control in the corresponding figure.**

28. Figure 6: The authors need to show the expression level of exogenous proteins.

**Response: We now show the expression level of exogenous proteins in the revised Figure 6 (now Figure 7).**

29. Figure 6A: Later results seem to show completely disrupted PAS formation in the presence of Ypt1 S174A. Yet, Atg8 is clearly still forming puncta. The authors should explain their thinking concerning this observation.

**Response: We thank the reviewer for the careful observation. As the reviewer said, quantitative analysis showed that GFP-Atg8 was clearly still forming puncta in Ypt1$^{S174D}$ mutant under nitrogen starvation conditions, while there were almost no GFP-Atg8 puncta caused by IAA-induced degradation of Ypt1 (Figure R12). We think that the reasons for this discrepancy may be as follows: the Ypt1$^{S174D}$ mutant impairs the association of Ypt1 with Atg1, Atg17 and Hrr25, but it does not affect the binding of Ypt1 with Atg23 and Atg11, which indicates that Ypt1 can also be recruited to the PAS by interacting with Atg11 and recruit Atg9 vesicles to the PAS by associating of Atg23 with Ypt1. Concurrently, we observed that although GFP-Atg8 puncta was formed on the Ypt1 $^{S174D}$ mutant, its puncta size was smaller than that of wild-type Ypt1. Based on these data, coupled with the observation that *atg1*Δ does not affect the formation of GFP-Atg8 puncta, we think that the formation of GFP-Atg8 puncta in Ypt1 $^{S174D}$ mutant is reasonable.**

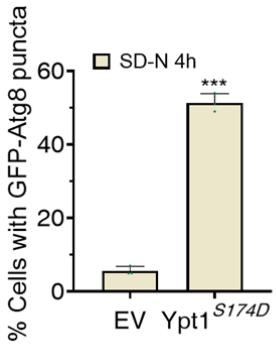


**Figure R12: GFP-Atg8 puncta can be formed in Ypt1$^{S174D}$ mutant under nitrogen starvation conditions.** AID-3×HA-Ypt1 yeast strains co-expressing GFP-Atg8 and Vph1-mCherry were transformed into empty vector or FLAG-Ypt1$^{S174D}$ plasmids. These yeast strains were grown to early log-growth phase. IAA was added to induce the degradation of endogenous AID-3×HA-Ypt1 for 2h. These yeast strains were then subjected to nitrogen starvation for 4h. Images of cells were obtained using an inverted fluorescence microscope. The number of cells with GFP-Atg8 puncta were assessed. n=300 cells were pooled from three independent experiments. Data are presented as mean ± SD. ***$p < 0.001$; two-tailed Student's t tests were used.

30. Figures 6A, B: The authors must show results for S174A and S174D at same timepoint or provide a good explanation as to why they tested 1 h starvation for cells expressing Ypt1 S174A but 4 h for the cells expressing Ypt1 S174D.

**Response: As suggested, we tested the autophagic activity of Ypt1$^{S174A}$ and Ypt1$^{S174D}$ mutants under nitrogen starvation for 1h and 4h respectively. Western-blot and quantitative analysis results showed that Ypt1$^{S174D}$ mutant inhibited autophagy while Ypt1$^{S174A}$ mutant accelerated autophagy under nitrogen starvation conditions (now Figure 7).**

31. Figure 7J: The interpretation is unclear. The authors should show quantification of multiple repeats.

**Response: We have now quantified the original Figure 7J. Because Reviewer1# suggested that we should add the results of Ypt1$^{S174A}$ mutant as a comparison, we performed that experiment again. Western blot and quantitative analysis results indicated that, compared with the results of wild-type Ypt1, the phosphorylation of Atg1 T226 in the Ypt1$^{S174D}$ mutant was significantly reduced, while increased in the Ypt1$^{S174A}$ mutant under nitrogen starvation (Figure 8J and S14D), implying that dephosphorylation of Ypt1 was required for the activation of Atg1 during autophagy induction.**

32. Figure 7D to J: The authors need to show the expression level of exogenous proteins.

**Response: We tested the expression level of exogenous proteins in the revised corresponding Figure (now Figure S14A, S14B, and S14G).**

33. Line 1, page 18: It cannot be said that Atg1 phosphorylation was "completely abolished".

**Response: We totally agree with the reviewer's comment. As suggested, we revised it as "Western blot and quantitative analysis results indicated that compared with the results of wild-type Ypt1, the phosphorylation of Atg1 T226 in the Ypt1$^{S174D}$ mutant was significantly reduced, while it was increased in the**

**Ypt1$^{S174A}$ mutant under nitrogen starvation. This implies that the dephosphorylation of Ypt1 was required for the activation of Atg1 during autophagy induction." We have revised this sentence and highlighted it with a yellow highlight in the revised manuscript.**

34. Line 10, page 19: The authors' data suggests that Rab1 is a positive, not a negative, regulator of autophagy.

**Response: We thank the reviewer for pointing out this error. We revised it as "indicating that Rab1 positively regulates autophagy in mammals."**

35. Figure 8B: As with Figure 5B, I suggest that the authors consider not showing raw mass spec results in the main figure and replace with a more intuitive representation.

**Response: Point well taken. We have put raw mass spec results into the supplementary data (now Figure S15A).**

36. Figure 8C: The authors should show if T75 is in a similar location to S174 in terms of primary and tertiary sequence.

**Response: Many thanks for the reviewer's comment. Reviewer 1# also had the same concerns. According to the prediction of the tertiary structures of Ypt1 and Rab1 by Alphafold2 software, the S174 amino acid of Ypt1 and the T75 amino acid of Rab1 are not in the same location (data not shown). In fact, we also observed that the phosphorylation site of Ypt1 and Rab1 by TOR/mTOR are not**

**conservative in yeast and mammals. We suspect that this discrepancy may be caused by the different three-dimensional structure of mTORC1 complex in mammalian and yeast cells, or the different microenvironment where Ypt1 and Rab1 bind with the mTORC1 complex. In fact, some studies found that mTOR phosphorylates the same substrate, despite the sites being different in yeast and mammals. For example, Atg13 is reported to be phosphorylated by mTOR in both yeast and mammals. However, Atg13 is phosphorylated by mTOR at Ser 258 in mammals (PMID: 26801615), while in yeast cells, Atg13 S348, S437, S438, S496, S535, S541, S646, and S649 residues was found to be phosphorylated by TOR (PMID: 19995911). Although the phosphorylation sites of Atg13 by mTOR in yeast and mammalian cells are different, the phosphorylation of Atg13 caused by mTOR functions similarly to block autophagy. Dephosphorylation of these sites on Atg13 enhances the formation of Atg1 puncta and the activation of Atg1. Similarly, in this study, although the phosphorylation sites of Ypt1 and Rab1 by mTOR are different, the phosphorylation of Ypt1 and Rab1 by mTOR inhibits the binding of Ypt1/Rab1 to Ulk1 and subsequent autophagy. We have discussed this point in the revised discussion section.**

37. Figure S8C: I do not see how this demonstrates the specificity of the antibody. There is no evidence provided by the authors to demonstrate that this antibody is specific to Atg1 T226 phosphorylation. The authors need to examine a T226 mutant to determine specificity.

**Response: We thank the reviewer for pointing these out. As suggested, we constructed a FLAG-Atg1$^{T226A}$ mutant to test the specificity of this antibody. As shown in Figure S14C, anti-p-T226-Atg1 antibody is a specific antibody that recognizes the phosphorylation level of the Atg1 T226 residue.**

38. Figure 8E: Quantification is required.

**Response: We have now quantified this in the revised manuscript.**

39. Figure 9: Atg23 should be bound directly to membrane. Are the authors implying that Tor and Ypt1 form a persistent complex? I suggest that the Atg9 representation should be changed to match the other proteins.

**Response: We thank the reviewer for this comment. As suggested, we adjusted Atg23 protein to match previous study and Atg9 to match the configuration of other proteins. Regarding whether the TOR and Ypt1 form a persistent complex, we need to perform more experiments such as such as Gel-filtration chromatography or TAP purification to elaborate this. Our Co-IP experiment indicated that Tor1 can associate with Ypt1 under nutrient-rich medium (Figure 10).**

40. In general, the authors should include more details in the figure legends and/or description of the figures such as providing strain information. In figure 1C, for example, the authors should indicate if they are using Coomassie stain. The bottom

label "Ypt1" in figure 2B has a scale bar erroneously placed over it.

**Response: We thank the reviewer for pointing these out. We have now added more details in the revised figure legends and description of the figures and highlighted them in the revised manuscript.**

**Overall, we thank the reviewers for these insightful and constructive comments, which have helped us strengthen the rigor of our study and clarified the conclusions of our manuscript. We hope that, following their guidance, our paper is sufficiently improved to meet the appropriately high standards necessary for publication in the *EMBO* journal.**

**Best wishes,**

**Cong**

Dear Prof. Yi,

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen again by the three original referees. Their second round of comments are shown below.

As you will see, significant issues remain. These centre upon (but are not limited to) the robustness and specificity of the binding assays you perform and the phosphorylation status of Ypt1 S174 under stress conditions. The referees have also asked for a more comprehensive submission of Source Data.

In exceptional circumstances, we allow a second round of revision, and I have decided to follow this course here. I would therefore invite you to submit a revised version of your manuscript that addresses all of the referees' comments. Please also submit unprocessed photographs of all blots shown. Whether or not this manuscript is accepted for publication will depend on the referees' reaction to this next version.

It may also be useful to have a Zoom call at this stage. Please let me know when you would be available.

Thank you once again for the opportunity to consider your work for publication. I look forward to your revision.

Best wishes,

William

William Teale, PhD
Editor
The EMBO Journal
w.teale@embojournal.org

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Referee #1:

The authors have performed lots of additional experiments and addressed many of my concerns.
However, there remain some critical concerns. Most of the binding data obtained by in vitro pulldown experiments and all SPR data using purified proteins are too strong. Most of the pulldown data showed 1:1 binding (judged by CBB stained bands), indicating that Ypt1 binds to Atg1, Atg11, Atg17, Atg23, Hrr25, and Ypt1 itself with strong affinity. SPR data showed that Ypt1 binds to TF-Atg1, TF-Atg17, and TF-Hrr25 with the KD value of 2-17 nM, which mean very strong interaction typically observed for stable, constitutive complexes. Even Ypt1 S174D mutant showed high affinity with TF-Atg1, TF-Atg17, and TF-Hrr25 with the KD value of 48, 4.8, 20 nM, which seems to be sufficient to form a complex in cells. This reviewer is concerned that there might be some technical problems for binding assays.
In Figure S15B, all of the nonspecific bands seen in Input are seen in the pulldown one as well (see TF-Atg1 and TF-Atg17). Is this system able to detect specific binding? In vitro pulldown assays showed that the GTP-bound form, but not the GDP-bound form, of Ypt1 binds with TF-Atg1, TF-Atg17, and His-Atg23. These data suggest that the switch I/II region of Ypt1 is involved in the binding with these three proteins. On the other hand, in vitro pulldown assays showed that Ypt1 binds to all these three proteins without competition (Figure S15B). Considering that Ypt1 is a small protein and that the switch I/II region is the common binding site, it is confusing that the binding of Ypt1 to large proteins (TF-Atg1, TF-Atg17, His-Atg23, TF-Ypt1) is not competitive.

Referee #2:

The authors addressed all of my questions.
However, the presentation of the source data needs to be changed. The authors need to show the whole gel including marker lanes. Not just slightly bigger cutouts of their images.

Referee #3:

The authors provided a broad investigation of the role of Ypt1 in autophagy. They used MS to identify and confirmed new autophagy-related interactors of Ypt1. Solid biochemical study was subsequently conducted, providing rich mechanistic insights into how these interactions affect the recruitment of autophagy proteins and the autophagy process. The identification of TOR as the kinase in both yeast and human, and the fact that disruption of the phosphorylation site affects autophagy in yeast greatly (but not so much in the mammalian system) is another highlight of the paper. The extensive amount of data presented in this

manuscript is no doubt of extreme value for the scientific community. A few minor experiments would help to fill the few remaining holes in the story. My primary concern at this point is with the writing of the manuscript. Writing in many places is difficult to understand. Some of the authors' conclusions stated in the discussion do not seem to fit with previous literature. And last, I am concerned that the references cited throughout the paper were not diligently verified to be supportive of the associated claim. These issues could be quickly remedied, however, justifying publication in the EMBO journal.

Page 3, line 3: The autophagosome does not engulf cargo. It is the resulting structure after cargo has been engulfed by the phagophore.

Page 3, line 16: The authors introduce the mammalian mTORC1 and then talk about yeast. Please alleviate this confusion.

Page 4, line 9: The meaning of "PAS" should be defined the first time it is used in the introduction.

Page 4, line 11: What is the perivacuolar membrane if not the PAS?

Page 4, line 16: Please mention the nutrient-specific roles of Atg11 and Atg17 to avoid confusion.

Page 4, line 21: Change "isolation membrane" to "phagophore".

Page 4, line 24: "In both nutrient-rich and nitrogen starvation conditions, Atg23 is crucial for Atg9 vesicle recruitment to the PAS (20)". Reference 20 does not test starvation conditions and thus does not support this statement. To my knowledge, no paper has directly tested the requirement of Atg23 for Atg9 recruitment to the PAS in starvation conditions. It is critical that the authors ensure that the papers they cite actually support the preceding claim. All references should be re-examined by the authors for validity.

Page 6, line 24: In Figure 1C, the pulldown gel should include regions around 130 kDa.

Page 7, line 8: The authors concluded that "the binding of Ypt1-Atg9 depends on Atg23" Yet in Figure 1D, Atg9 is still pulled down despite the absence of Atg23. Additionally, a technical issue prevents the direct test of interaction between full-length Atg9 and Ypt1 in vitro. I think the current data do not rule out the possibility that Ypt1 can interact with Atg9 in an Atg23-independent manner. It should be mentioned that the pulldown observed between Ypt1 and Atg9 in the absence of Atg23 could be the result of an indirect interaction.

Page 8, line 5: Since it's not a black-and-white difference, the authors should provide quantification for the replicates for Figure 1G.

Page 8, line 7: Please define what you mean by "effector".

Page 10, line 10: Sentence doesn't make sense. Please rewrite.

Page 11, line 2: Disruption of Atg23 dimerization has been shown to cause mislocalization of the protein (Hawkins 2022). The authors should mention that the failure of monomeric Atg23 to bind Ypt1 could be an indirect effect from Atg23 failing to colocalize with Ypt1.

Page 11, line 10: Can Ypt1W62A properly localize to the PAS? Is the localization of Ypt1 Atg23 dependent? I suggest, to rule out the possibility that Ypt1W62A affects autophagy via means other than its interaction with Atg23, the authors look at the localization of Ypt1 and Ypt1W62A in both WT and atg23Δ strains.

Page 18, line 1: The authors should show if the phosphorylation on Ypt1 S174 is reduced in nitrogen starvation, either by MS, or by means such as phos-tag gels. As TOR might not be the only kinase for this site, this piece of information is important.

Page 23, line 17: In Figures 9D-E, the authors used the level of LC3-II and p62 to demonstrate the defects of autophagy. However, as LC3-II is supplemented by LC3-I and degraded by autophagy, looking at the LC3-II does not really indicate the flux of autophagy in the cell. On top of the current setup in Figure 9D, the authors need to co-treat the cells with autophagy inhibitors such as Bafilomycin A1 to block the degradation of LC3-II to determine the autophagy flux.

Page 24, line 17: Several autophagy proteins are phosphorylated by Tor, including Atg1. Why do you selectively mention Atg13? This makes it seem as if it is the only one.

Page 24, line 25: Figure S15B alone is not enough to support the confirmation that "Ypt1 could bind Atg1, Atg17, and Atg23 simultaneously". If Ypt1 binds to them separately, or their binding to Ypt1 is competitive (i.e., these 3 interactors do not all appear in one complex), the gel in Figure S15B would still look the same in an in vitro assay where excess Ypt1 is provided.

Page 25, line 10: Sentence doesn't make sense.

Page 25, line 15: The authors word this section as if the primary role of Atg23 is to dock Atg9 to the PAS through interaction with Ypt1. When ATG23 is deleted, Atg9 vesicle formation is severely impaired (Yamamoto 2012). However, the authors do not see a defect in Atg9 vesicle formation when the Atg23-Ypt1 interaction is disrupted. This implies that Atg23 has another critical, Ypt1-independent role in Atg9 vesicle formation at the peripheral sites in addition to this newfound role at the PAS. The authors should reflect this in their discussion section.

Page 26, line 16: Your BiFC results with Atg23 and Ypt1 show that the pair exclusively colocalize at the PAS. Atg23 is thought to be present on Atg9 vesicles. Don't your results suggest that Ypt1 is not present on Atg9 vesicles? Please remedy this apparent contradiction.

Page 26, line 19: What do you mean "Atg9 vesicles provide a scaffold platform for PAS recruitment of Ypt1"? Please elaborate.

**Dear Dr. Teale,**

**We appreciate the editor giving us the opportunity for second revision and for the many valuable suggestions provided by the reviewers. As suggested, we have added extensive experiments to address the reviewers' questions and comments. As shown in our point-by-point response, we addressed every question asked by the reviewers.**

**Point-by-point responses to the reviewers:**

Referee #1:

The authors have performed lots of additional experiments and addressed many of my concerns. However, there remain some critical concerns. Most of the binding data obtained by in vitro pulldown experiments and all SPR data using purified proteins are too strong. Most of the pulldown data showed 1:1 binding (judged by CBB stained bands), indicating that Ypt1 binds to Atg1, Atg11, Atg17, Atg23, Hrr25, and Ypt1 itself with strong affinity. SPR data showed that Ypt1 binds to TF-Atg1, TF-Atg17, and TF-Hrr25 with the KD value of 2-17 nM, which mean very strong interaction typically observed for stable, constitutive complexes. Even Ypt1 S174D mutant showed high affinity with TF-Atg1, TF-Atg17, and TF-Hrr25 with the KD value of 48, 4.8, 20 nM, which seems to be sufficient to form a complex in cells. This reviewer is concerned that there might be some technical problems for binding assays. In Figure

S15B, all of the nonspecific bands seen in Input are seen in the pulldown one as well (see TF-Atg1 and TF-Atg17). Is this system able to detect specific binding? In vitro pulldown assays showed that the GTP-bound form, but not the GDP-bound form, of Ypt1 binds with TF-Atg1, TF-Atg17, and His-Atg23. These data suggest that the switch I/II region of Ypt1 is involved in the binding with these three proteins. On the other hand, in vitro pulldown assays showed that Ypt1 binds to all these three proteins without competition (Figure S15B). Considering that Ypt1 is a small protein and that the switch I/II region is the common binding site, it is confusing that the binding of Ypt1 to large proteins (TF-Atg1, TF-Atg17, His-Atg23, TF-Ypt1) is not competitive.

**Response: We thank the reviewer for these insightful suggestions and for helping us avoid inappropriate conclusions or over-interpretations. To better elaborate the questions raised by the reviewer, we divided it into four parts to answer:**

**1. Specificity**

**To address this issue, we tested whether Ypt1 can directly bind with other autophagy-related proteins. MS results indicated that Ypt1 can associate with Ykt6 (Figure 1A). However, *in vitro* Ni-NTA pulldown assay showed that Ypt1 did not directly bind to Ykt6 (Figure R1A). Concurrently, *in vitro* Ni-NTA Pulldown assays also found that Ypt1 does not interact with Atg13 and Atg14 (Figure R1B-C).**

**We next tested whether the autophagy-related proteins that direct bind to Ypt1**

**also interact with other Rab GTPases. *In vitro* GST or Ni-NTA pulldown assays indicated that Hrr25, Atg17, Atg1, or Atg23 does not bind with other two Rab GTPases Ypt6 and Ypt32 (Figure R2 and R3). Collectively, these data showed that the direct binding of Ypt1 with Hrr25, Atg17, Atg1, or Atg23 are specific.**

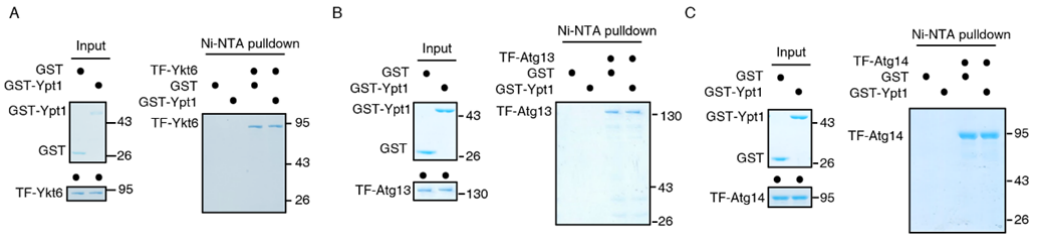


**Figure R1: Ykt6, Atg13, or Atg14 do not directly bind with Ypt1. (A-C)** Ni-NTA pulldowns were performed using purified TF-Ykt6(A), TF-Atg13(B), or TF-Atg14(C) with GST or GST-Ypt1 from *E. coli*. Protein samples were separated by SDS-PAGE, and then detected using Coomassie blue staining.

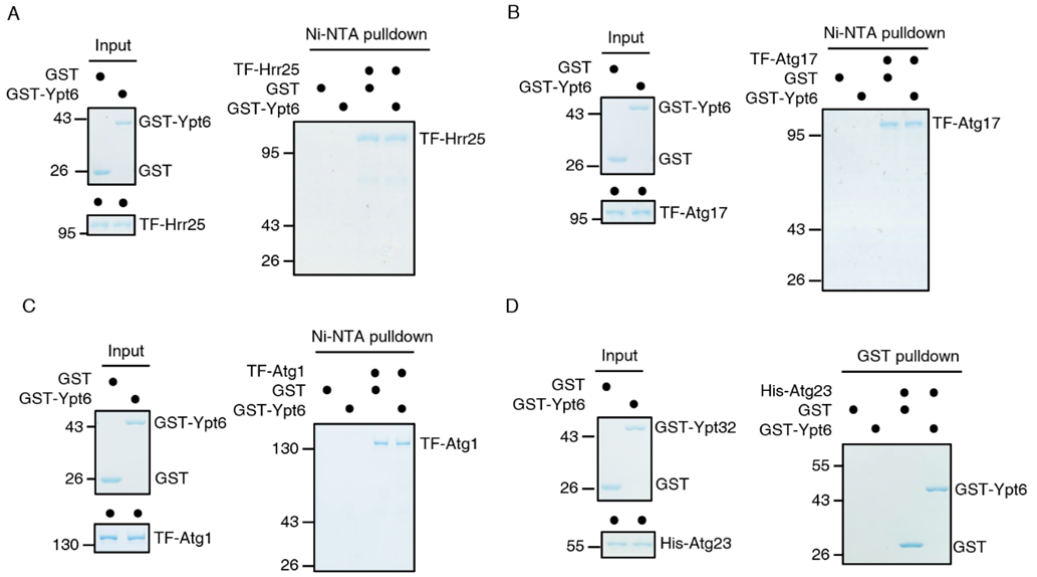


**Figure R2: Hrr25, Atg17, Atg1, or Atg23 do not directly bind to Ypt6. (A-C)** Ni-NTA pulldowns were performed using purified TF-Hrr25(A), TF-Atg17(B), or TF-Atg11(C) with GST or GST-Ypt6 from *E. coli*. Protein samples were separated by SDS-PAGE, and then detected using Coomassie blue staining. **(D)** GST pulldowns

were performed using purified His-Atg23 with GST or GST-Ypt6 from *E. coli*. Protein samples were separated by SDS-PAGE, and then detected using Coomassie blue staining.

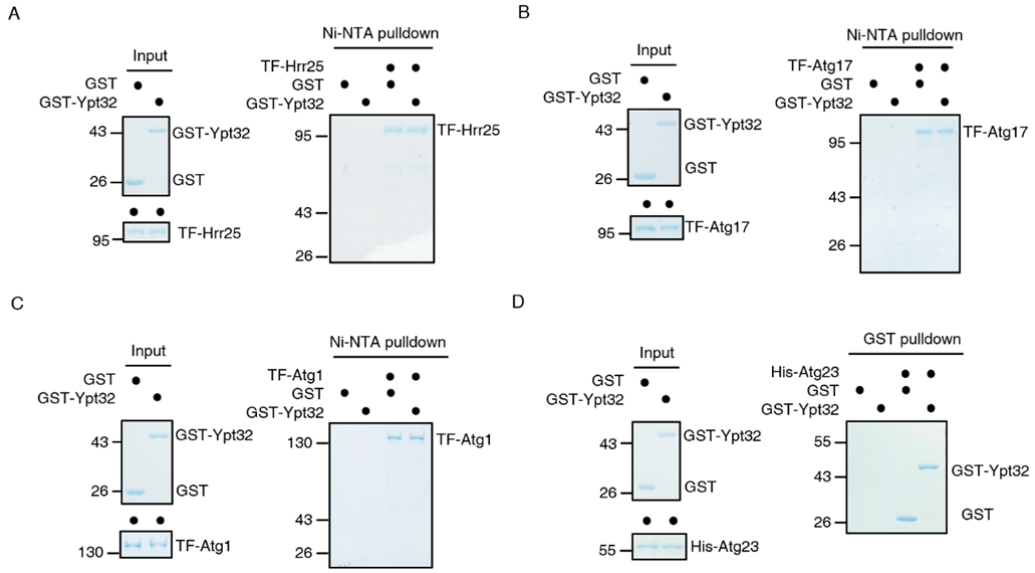


**Figure R3: Hrr25, Atg17, Atg1, or Atg23 do not directly bind to Ypt32. (A-C)** Ni-NTA pulldowns were performed using purified TF-Hrr25(A), TF-Atg17(B), or TF-Atg11(C) with GST or GST-Ypt32 from *E. coli*. Protein samples were separated by SDS-PAGE, and then detected using Coomassie blue staining. **(D)** GST pulldowns were performed using purified His-Atg23 with GST or GST-Ypt32 from *E. coli*. Protein samples were separated by SDS-PAGE, and then detected using Coomassie blue staining.

**2. Strength**

**To test the binding strength of Ypt1 with Hrr25, Atg1, Atg17, Atg23, or Atg11, we used washing buffer containing different concentrations of NaCl (100mM, 200mM, 300mM, or 400mM) to measure their binding efficiency. As shown in Figure R4, *in vitro* GST or Ni-NTA pulldown assays indicated that as the concentration of NaCl in the washing buffer increased, the binding strength of**

**Ypt1 with Hrr25, Atg1, Atg17, Atg23, or Atg11 CC2 decreased to varying degrees, especially with the significant decrease in Ypt1 binding with Hrr25 or Atg1. Our previous work found a 1:1 binding between the aggrephagy acceptor Cct2 and Atg8 (Cell, 2022, PMID:35366418). Cumulatively, these data suggested that Ypt1 has relatively strong binding with these proteins, especially Atg11, Atg17, and Atg23.**

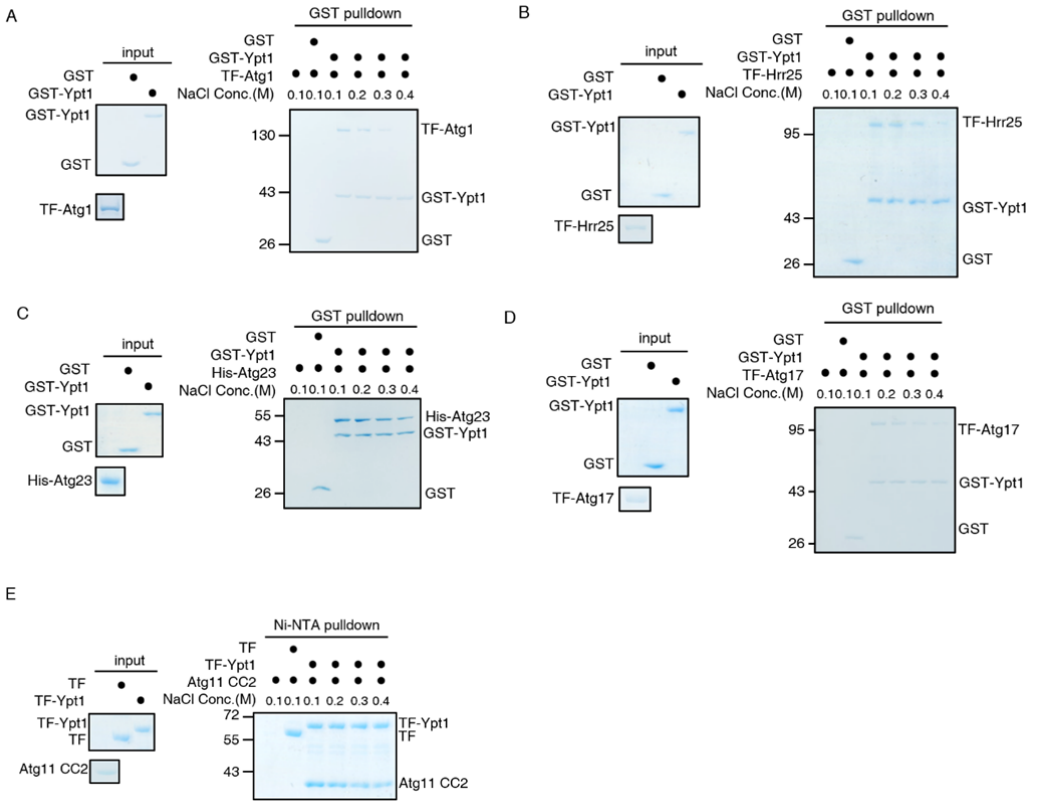


**Figure R4: *In vitro* detection of binding strength between Ypt1 and Atg1, Hrr25, Atg23, Atg17, or Atg11 CC2. (A-D)** GST pulldowns were performed using purified TF-Atg1(A), TF-Hrr25(B), His-Atg23(C), or TF-Atg17(D) with GST or GST-Ypt1 from *E. Coli* in washing buffer containing different concentrations of NaCl. Protein samples were separated by SDS-PAGE, and then detected using Coomassie blue staining. **(E)** Ni-NTA pulldowns were performed using purified Atg11 CC2 domain with TF or TF-Ypt1 from *E. Coli* in washing buffer containing different

concentrations of NaCl. Protein samples were separated by SDS-PAGE, and then detected using Coomassie blue staining.

**3. Competitiveness**

**About "In Figure S15B, all of the nonspecific bands seen in Input are seen in the pulldown one as well (see TF-Atg1 and TF-Atg17)", we also realize this point. To further eliminate non-specific proteins in TF-Atg1 and TF-Atg17 samples, we performed molecular sieves followed by anion exchange chromatography. As shown in the Figure R5A, we obtained TF-Atg1 and TF-Atg17 protein samples with high purity. Subsequently, we performed *in vitro* GST pulldown assays using the two purified proteins or His-Atg23 with GST-Ypt1 under different concentrations of GST-Ypt1 protein or washing buffers containing different concentrations of NaCl. The results showed that the bindings of Ypt1 with TF-Atg1, TF-Atg17, or His-Atg23 are competitive, with the priority order of their binding with Ypt1 being: Atg23>Atg17>Atg1 (Figure R5, now Appendix Fig S7B-D). We appreciate the reviewer again for helping us avoid inappropriate conclusions or over-interpretations.**

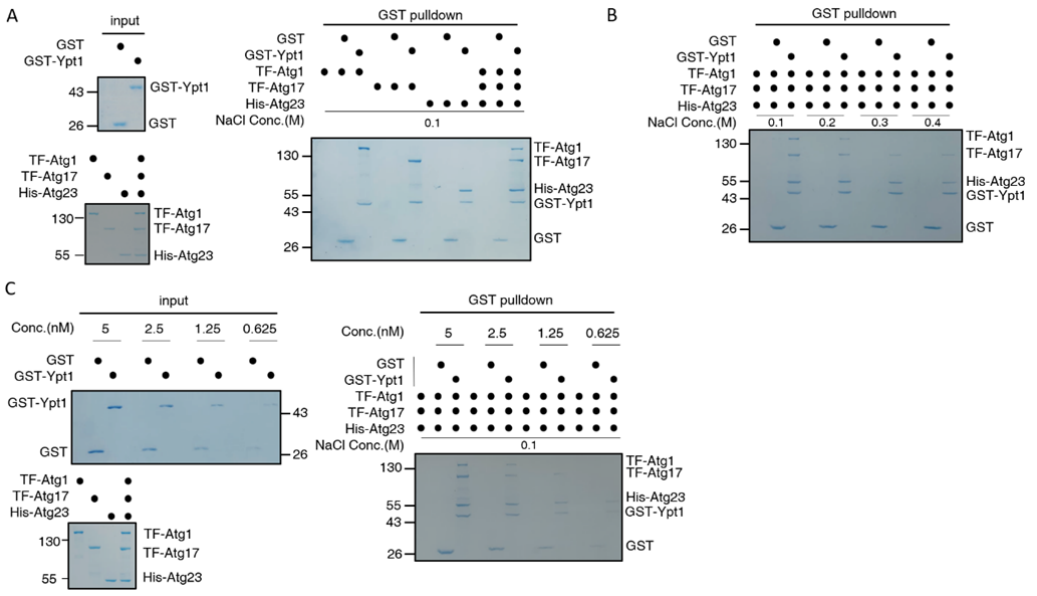


**Figure R5：The binding of Ypt1 with Atg1, Atg17, or Atg23 are competitive. (A)** GST pulldowns were performed by using GST or GST-Ypt1 with TF-Atg1, TF-Atg17, and $His_6$-Atg23 from *E. Coli* in the washing buffer containing 100mM NaCl. Protein samples were separated by SDS-PAGE, and then detected using Coomassie blue staining. **(B)** GST pulldowns were performed by using GST or GST-Ypt1 with TF-Atg1, TF-Atg17, and $His_6$-Atg23 from *E. Coli* in the washing buffer containing the indicated concentrations of NaCl. Protein samples were separated by SDS-PAGE, and then detected using Coomassie blue staining. **(C)** GST pulldowns were performed using TF-Atg1, TF-Atg17, and $His_6$-Atg23 with the indicated concentrations of GST or GST-Ypt1 proteins with from *E. Coli* in the washing buffer containing 100mM NaCl. Protein samples were separated by SDS-PAGE, and then detected using Coomassie blue staining.

**4. SPR assay**

**As suggested, we carefully examined the protocol about SPR assay, the assay procedure was standard and the washing buffer we used earlier was the default washing buffer (PBS) of the instrument. This time, to more accurately matches**

**the experimental conditions of the Ni-NTA pulldown assay, we changed the washing buffer to a washing buffer used for *in vitro* Ni-NTA pulldown assay. We then performed the SRP experiments using TF-Hrr25, newly purified TF-Atg1, or TF-Atg17 with GST Ypt1 WT or S174D in the washing buffer of Ni-NTA pulldown assay. Concurrently, we also invited the engineer of the instrument to guide us to do this assay. Results showed that Ypt1$^{S174D}$ did significantly impaired the binding of Ypt1 with Hrr25, Atg17, and Atg1 (Figure R6, now Appendix Fig S6).**

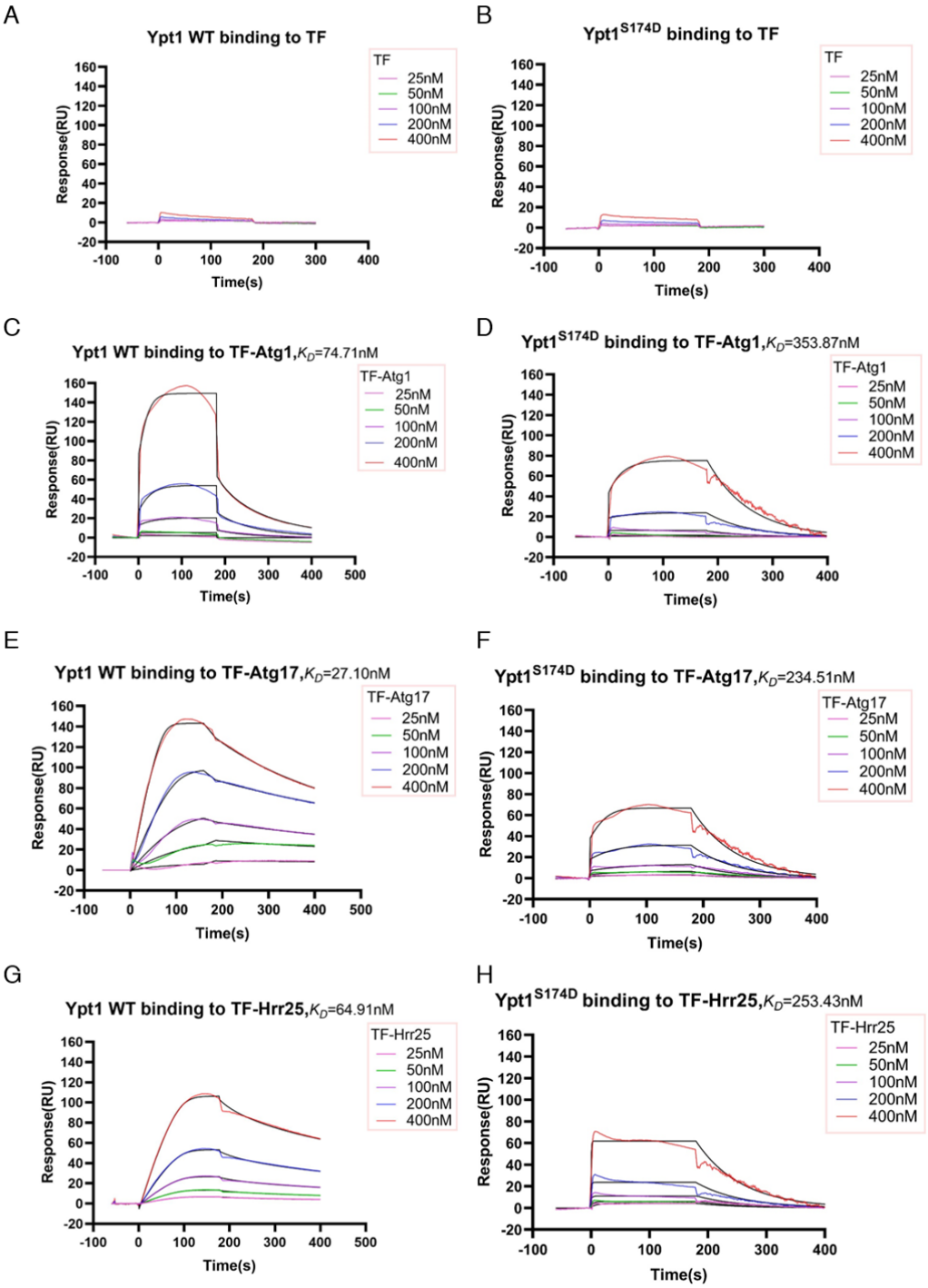


**Figure R6: Ypt1$^{S174D}$ impairs Ypt1 association with Atg1, Atg17, or Hrr25.** Purified recombinant proteins GST-Ypt1 WT or GST-Ypt1$^{S174D}$ were covalently immobilized on the sensor chip via their amine groups and purified recombinant proteins TF, TF-Atg1, TF-Atg17, or TF-Hrr25 that flowed over the GST-Ypt1 WT or

S174D. TF, TF-Atg1, TF-Atg17, or TF-Hrr25 proteins were diluted to the indicated concentrations (from 25nM to 400nM) before injection. The results were fit to a 1:1 binding model. Five different protein concentrations were used for calculating the $K_D$ values each time.

Referee #2:

The authors addressed all of my questions.

However, the presentation of the source data needs to be changed. The authors need to show the whole gel including marker lanes. Not just slightly bigger cutouts of their images.

**Response: We thank the reviewer for the comment and have now submitted unprocessed photographs of all blots with protein markers.**

Referee #3:

The authors provided a broad investigation of the role of Ypt1 in autophagy. They used MS to identify and confirmed new autophagy-related interactors of Ypt1. Solid biochemical study was subsequently conducted, providing rich mechanistic insights into how these interactions affect the recruitment of autophagy proteins and the autophagy process. The identification of TOR as the kinase in both yeast and human, and the fact that disruption of the phosphorylation site affects autophagy in yeast greatly (but not so much in the mammalian system) is another highlight of the paper. The extensive amount of data presented in this manuscript is no doubt of extreme value for the scientific community. A few minor experiments would help to fill the

few remaining holes in the story. My primary concern at this point is with the writing of the manuscript. Writing in many places is difficult to understand. Some of the authors' conclusions stated in the discussion do not seem to fit with previous literature. And last, I am concerned that the references cited throughout the paper were not diligently verified to be supportive of the associated claim. These issues could be quickly remedied, however, justifying publication in the EMBO journal.

Page 3, line 3: The autophagosome does not engulf cargo. It is the resulting structure after cargo has been engulfed by the phagophore.

**Response: We appreciate the reviewer for raising this point and have now rewritten this sentence in the revised manuscript (highlighted in yellow).**

Page 3, line 16: The authors introduce the mammalian mTORC1 and then talk about yeast. Please alleviate this confusion.

**Response: As suggested, we have now rewritten this sentence in the revised manuscript (highlighted in yellow).**

Page 4, line 9: The meaning of "PAS" should be defined the first time it is used in the introduction.

**Response: We have defined PAS (phagophore assembly site) in the revised manuscript (highlighted in yellow).**

Page 4, line 11: What is the perivacuolar membrane if not the PAS?

**Response: We appreciate the reviewer pointing out this issue and have now rewritten this sentence in the revised manuscript (highlighted in yellow).**

Page 4, line 16: Please mention the nutrient-specific roles of Atg11 and Atg17 to avoid confusion.

**Response: We have already clarified this point in the revised manuscript (highlighted in yellow).**

Page 4, line 21: Change "isolation membrane" to "phagophore".

**Response: As suggested, we have now revised "isolation membrane" as "phagophore" in the revised manuscript.**

Page 4, line 24: "In both nutrient-rich and nitrogen starvation conditions, Atg23 is crucial for Atg9 vesicle recruitment to the PAS (20)". Reference 20 does not test starvation conditions and thus does not support this statement. To my knowledge, no paper has directly tested the requirement of Atg23 for Atg9 recruitment to the PAS in starvation conditions. It is critical that the authors ensure that the papers they cite actually support the preceding claim. All references should be re-examined by the authors for validity.

**Response: We thank the reviewer for pointing these out and totally agree with the reviewer. We have now rewritten this sentence in the revised manuscript**

**(highlighted in yellow) and reexamined all references.**

Page 6, line 24: In Figure 1C, the pulldown gel should include regions around 130 kDa.

**Response: We have now added a marker of 130 kDa in revised Figure 1C.**

Page 7, line 8: The authors concluded that "the binding of Ypt1-Atg9 depends on Atg23" Yet in Figure 1D, Atg9 is still pulled down despite the absence of Atg23. Additionally, a technical issue prevents the direct test of interaction between full-length Atg9 and Ypt1 in vitro. I think the current data do not rule out the possibility that Ypt1 can interact with Atg9 in an Atg23-independent manner. It should be mentioned that the pulldown observed between Ypt1 and Atg9 in the absence of Atg23 could be the result of an indirect interaction.

**Response: We thank the reviewer for the valuable comments and totally agree with the reviewer. We have now added this sentence in the revised manuscript (highlighted in yellow).**

Page 8, line 5: Since it's not a black-and-white difference, the authors should provide quantification for the replicates for Figure 1G.

**Response: We have now provided quantification into the original Figure 1G.**

Page 8, line 7: Please define what you mean by "effector".

**Response: We thank the reviewer for the comments. The definition of effector originates from a review by Prof. Peter Novick on PNAS entitled "Rabs and their effectors: Achieving specificity in membrane traffic" (PMID: 16882731). "The term effector implies a protein that responds to a specific Rab and mediates at least one element of its downstream effects. They have been operationally defined through their ability to bind to a specific Rab, selectively in its GTP-bound state, and have been identified through a variety of approaches, such as the yeast two-hybrid system, genetic screens, and affinity purification." We have now added this reference into the revised manuscript.**

Page 10, line 10: Sentence doesn't make sense. Please rewrite.

**Response: We rewritten this sentence in the revised manuscript (highlighted in yellow).**

Page 11, line 2: Disruption of Atg23 dimerization has been shown to cause mislocalization of the protein (Hawkins 2022). The authors should mention that the failure of monomeric Atg23 to bind Ypt1 could be an indirect effect from Atg23 failing to colocalize with Ypt1.

**Response: We have now added this sentence to the revised manuscript (highlighted in yellow).**

Page 11, line 10: Can Ypt1W62A properly localize to the PAS? Is the localization of

Ypt1 Atg23 dependent? I suggest, to rule out the possibility that Ypt1W62A affects autophagy via means other than its interaction with Atg23, the authors look at the localization of Ypt1 and Ypt1W62A in both WT and atg23Δ strains.

**Response: We thank the reviewer for the insightful comment. As suggested, GFP-Ypt1 or GFP-Ypt1$^{W62A}$ plasmids were transformed into wild-type (WT) cells co-expressing Aid-3×HA-Ypt1 and Atg17-2×mCherry, or GFP-Ypt1 plasmids were transformed into *atg23*Δ cells co-expressing Aid-3×HA-Ypt1 and Atg17-2×mCherry. Fluorescence microscopy and image analysis showed that there was no significant difference in the number of cells co-located with Atg17 and Ypt1 between WT cells co-expressing GFP-Ypt1$^{W62A}$ and Atg17-2×mCherry and *atg23*Δ cells co-expressing GFP-Ypt1 and Atg17-2×mCherry, although their colocalization was moderately decreased compared to WT cells co-expressing GFP-Ypt1 and Atg17-2×mCherry (Figure R7, now Appendix Fig S3G-I). This result indicated that the PAS recruitment of Ypt1 does not rely on the binding of Ypt1-Atg23 to any great extent.**

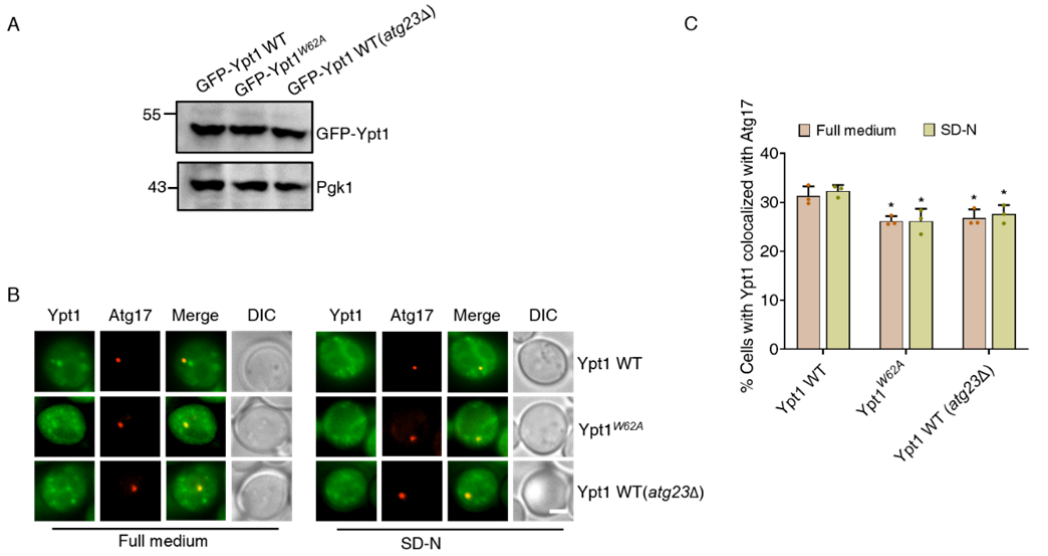


**Figure R7: The PAS recruitment of Ypt1 largely does not rely on the binding of Ypt1-Atg23. (A)** AID-3×HA-Ypt1 yeast cells co-expressing GFP-Ypt1 or GFP-Ypt1$^{W62A}$ with Atg17-2×mCherry or AID-3×HA-Ypt1 *atg23*Δ yeast cells co-expressing Atg17-2×mCherry with GFP-Ypt1 were grown to log phase, and then subjected to 0.5 mM IAA treatment for 2 h, the expression of GFP-Ypt1 or GFP-Ypt1$^{W62A}$ were detected by anti-GFP antibody. Pgk1 served as a loading control. **(B)** The yeast strains from (A) were treated with DMSO or IAA for 2h, and then subjected to SD-N in the presence or absence of IAA for 0h or 1h. Images of cells were obtained using the inverted fluorescence Microscope. Scale bar, 2 μm. **(C)** Cells from (B) were quantified for the number of cells in which GFP-Ypt1 or GFP-Ypt1$^{W62A}$ colocalized with Atg17-2×mCherry. n=300 cells were pooled from three independent experiments. Data are shown as mean ± SD. *p < 0.05; two-tailed Student's t tests were used.

Page 18, line 1: The authors should show if the phosphorylation on Ypt1 S174 is reduced in nitrogen starvation, either by MS, or by means such as phos-tag gels. As TOR might not be the only kinase for this site, this piece of information is important.

**Response: We thank the reviewer for the valuable suggestion. Fortunately, we**

**have just obtained a specific phosphorylation antibody targeting Ypt1 S174. Western-blot result showed that mutating S174 to alanine (S174A) completely abolished the phosphorylation signal (Figure R8A, now Figure 6E). As suggested, we subsequently tested whether the phosphorylation level of Ypt1 S174 residue dramatically reduced under nitrogen starvation conditions. The results showed that compared to nutrient-rich conditions, the phosphorylation level of Ypt1 S174 residue in cells was significantly decreased in response to nitrogen starvation (Figure R8B, now Figure 6F).**

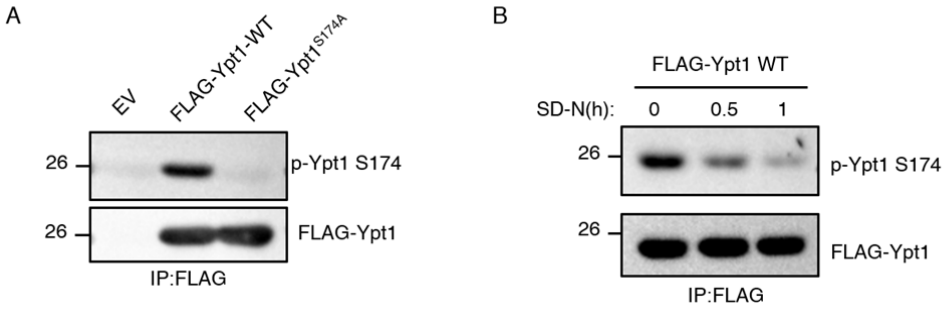


**Figure R8: The phosphorylation level of Ypt1 significantly decrease during nitrogen starvation. (A)** Yeast cells expressing empty vector, FLAG-Ypt1, or FLAG-Ypt1$^{S174A}$ were grown to early log phase. Cell lysates were immunoprecipitated with anti-FLAG agarose beads and then analyzed with anti-p-Ypt1 S174 antibody. **(B)** Yeast cells expressing FLAG-Ypt1 were subjected to nitrogen starvation for 0h, 0.5h, or 1h. Cell lysates were immunoprecipitated with anti-FLAG agarose beads and then analyzed with anti-p-Ypt1 S174 antibody.

Page 23, line 17: In Figures 9D-E, the authors used the level of LC3-II and p62 to

demonstrate the defects of autophagy. However, as LC3-II is supplemented by LC3-I and degraded by autophagy, looking at the LC3-II does not really indicate the flux of autophagy in the cell. On top of the current setup in Figure 9D, the authors need to co-treat the cells with autophagy inhibitors such as Bafilomycin A1 to block the degradation of LC3-II to determine the autophagy flux.

**Response: As suggested, we co-treated the cells with Bafilomycin A1 to block the degradation of LC3-II and to determine the autophagy flux. As shown in now Fig9F and G, Rab1$^{T75D}$ still reduced LC3 lipidation but did not alter p62 levels, indicating that an inhibition role for the phosphorylation of the Rab1 T75 residue on autophagosome formation.**

Page 24, line 17: Several autophagy proteins are phosphorylated by Tor, including Atg1. Why do you selectively mention Atg13? This makes it seem as if it is the only one.

**Response: We totally agree with the reviewer's comment. As the reviewer's said, several autophagy proteins can indeed be phosphorylated by TOR or mTOR in yeast and mammalian cells, such as Atg1, Atg13, etc. We have now added these references to the revised discussion. Here, the reason we chose Atg13 as an example is: In yeast and mammals, the function of the phosphorylation of Atg13 by TOR or mTOR are well studied and its phosphorylation sites are also well elucidated.**

Page 24, line 25: Figure S15B alone is not enough to support the confirmation that "Ypt1 could bind Atg1, Atg17, and Atg23 simultaneously". If Ypt1 binds to them separately, or their binding to Ypt1 is competitive (i.e., these 3 interactors do not all appear in one complex), the gel in Figure S15B would still look the same in an in vitro assay where excess Ypt1 is provided.

**Response: We totally agree with the reviewer's comment. To answer the question raised by the reviewer, we performed *in vitro* GST-pull down assays using TF-Atg1, TF-Atg17, or His-Atg23 with GST-Ypt1 under different concentrations of GST-Ypt1 protein or washing buffer containing different concentrations of NaCl. The results showed the binding of Ypt1 with TF-Atg1, TF-Atg17, or His-Atg23 to be competitive, with the priority order of their binding with Ypt1 as: Atg23>Atg17>Atg1 (Figure R5, now Appendix Fig S7B-D). We have changed this sentence in the revised manuscript and thank the reviewer again for the valuable suggestion.**

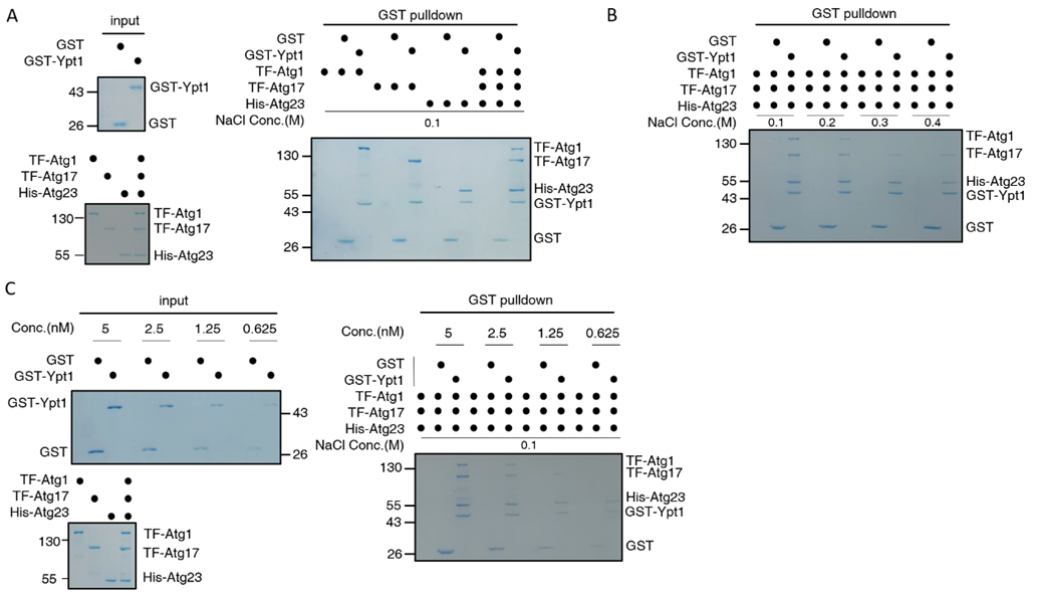


**Figure R5：The binding of Ypt1 with Atg1, Atg17, or Atg23 are competitive. (A)** GST pulldowns were performed by using GST or GST-Ypt1 with TF-Atg1, TF-Atg17, and $His_6$-Atg23 from *E. coli* in the washing buffer containing 100mM NaCl. Protein samples were separated by SDS-PAGE, and then detected using Coomassie blue staining. The asterisk represents the target protein. **(B)** GST pulldowns were performed by using GST or GST-Ypt1 with TF-Atg1, TF-Atg17, and $His_6$-Atg23 from *E. coli* in the washing buffer containing the indicated concentrations of NaCl. Protein samples were separated by SDS-PAGE, and then detected using Coomassie blue staining. The asterisk represents the target protein. **(C)** GST pulldowns were performed by using TF-Atg1, TF-Atg17, and $His_6$-Atg23 with the indicated concentrations of GST or GST-Ypt1 with from *E. coli* in the washing buffer containing 100mM NaCl. Protein samples were separated by SDS-PAGE, and then detected using Coomassie blue staining. The asterisk represents the target protein.

Page 25, line 10: Sentence doesn't make sense.

**Response: We rewritten this sentence in the revised manuscript (highlighted in yellow).**

Page 25, line 15: The authors word this section as if the primary role of Atg23 is to dock Atg9 to the PAS through interaction with Ypt1. When ATG23 is deleted, Atg9 vesicle formation is severely impaired (Yamamoto 2012). However, the authors do not see a defect in Atg9 vesicle formation when the Atg23-Ypt1 interaction is disrupted. This implies that Atg23 has another critical, Ypt1-independent role in Atg9 vesicle formation at the peripheral sites in addition to this newfound role at the PAS. The authors should reflect this in their discussion section.

**Response: We thank the reviewer for the valuable comment and have now added this sentence to the revised discussion section (highlighted in yellow).**

Page 26, line 16: Your BiFC results with Atg23 and Ypt1 show that the pair exclusively colocalize at the PAS. Atg23 is thought to be present on Atg9 vesicles. Don't your results suggest that Ypt1 is not present on Atg9 vesicles? Please remedy this apparent contradiction.

**Response: We have remedied this error in the revised manuscript (highlighted in yellow).**

Page 26, line 19: What do you mean "Atg9 vesicles provide a scaffold platform for PAS recruitment of Ypt1"? Please elaborate.

**Response: We appreciate the reviewer for pointing this out. Considering insufficient evidence to support this conclusion, so we have deleted this sentence**

**in the revised manuscript.**

**We thank the reviewers for their insightful and constructive comments, which have helped us to strengthen the rigor of our study and clarify our conclusions in this manuscript. We hope that, following their guidance, our paper is sufficiently improved to meet the appropriately high standards necessary for publication in *EMBO J.***

**Best wishes,**

**Cong**

Dear Cong,

I thought that is what had happened; I completely understand how these mistakes are made. Please replace the figures as you have indicated. In addition, now may be a good time for me to list some editorial points that also need addressing.
In this regard would you please:

- remove highlighted text from the manuscript,
- include grant number missing in eJP: National Natural Science Foundation of China 92254307 and Zhejiang Provincial Natural Science Foundation of China under Grant No.LR21C070001,
- include up to five keywords,
- references should be alphabetical, not numbered, with 10 authors + et al.,
- remove the author credit section,
- re-label figures as follows: Figure S1-S15 should be called out as Appendix Figure S1-S15; include callouts for Appendix Fig. S4A-D, S12A-C, S13A-H
remove EV legends from the ms file and include them as a separate sheet in Excel file, and
- include page numbers in the table of contents.

In addition,
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The data shown in figures should satisfy the following conditions:
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