Atg15 in *Saccharomyces cerevisiae* consists of two functionally distinct domains

Eri Hirata, Kyo Shirai, Tatsuya Kawaoka, Kosuke Sato, Fumito Kodama, and Kuninori Suzuki

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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. The original formatting of letters and referee reports may not be reflected in this compilation.)
Dear Dr. Suzuki,

Your manuscript has now been evaluated by two expert referees. As you will see from the two reviews, they are quite divergent in their opinions. Referee 1 is rather negative finding that much of the studies are replicates of others work, whereas referee 2 looks at this as a reanalysis of targeting. With two divergent evaluations on hand I went through your manuscript myself and for the most part I agree with referee 2. The analysis of the trafficking brings in new aspects and the characterisation of the residues required for lipase activity is also new. So I would be happy to receive a revised version of your manuscript. While I sided mostly with referee 2 this does not mean that you should ignore referee 1. I would appreciate to receive a point by point response to the criticisms raised by both referees which I will evaluate before reaching my decision on the revised manuscript.

Best regards,
Howard Riezman
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Suzuki,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): Link Not Available

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Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
Reviewer #1 (Remarks to the Author):

The authors have analyzed some aspects of structure-function relationships in the Atg15 lipase. The main advance made, in my opinion, is that they show that fusion of the Atg15 catalytic domain to the pho8 targeting region is sufficient for biological activity of the protein. However since Pho8 is at least partly dependent on the MVB pathway (as is Atg15 itself), I am not sure how important this finding is. On the negative side, large parts of this study are reproductions of the conclusions of earlier studies from the Klionsky and Thumm labs, and other segments do not seem to have sufficient experimental support for their conclusions.

Specific comments:

1) The impetus for the work described in Figure 1 and the associated text is unclear. Atg15 has been implicated in autophagic body breakdown and is known not to be defective in autophagosome formation. Why are the authors testing for effects of ATG15 deletion on IM expansion? Is there some underlying scientific rationale for this?

2) The authors need to prove that the structures which they label as 'IM' are indeed intermediates in the formation of autophagosomes. They could do this by making a movie showing that the intermediate ultimately closes and fuses with the vacuole, but as it stands there is no evidence that these are autophagic intermediates.

3) Regardless of the above, the outcome of the IM exercise is a negative result. This could be predicted from the data shown by Teter et al (Figure 1 therein). Therefore, it is unclear why this result is included in the manuscript.

4) The section dealing with trafficking of Atg15 through the MVB pathway is essentially a recap of the studies published by Ulrike Epple in Michael Thumm's lab and are not sufficiently novel.

5) The authors need to explain the appearance of free GFP from Atg15-GFP in pep4 cells. This is either experimental error or a complete paradigm shift in our understanding of Atg15 trafficking.

6) At the bottom of Page 6 the authors conclude that transport of Atg15 to the vacuole is essential for its function. However one page later at the bottom of page 7, they show that a completely mislocalized version of the protein, lacking the TMD, is partially functional. The authors must resolve this contradiction.

7) Page 8, 2nd para: fab1 mutants are not class E

8) The finding that the double apl5 vps4 mutant can fully suppress the autophagy defect of atg15 mutants requires more attention and a significant amount of work.

9) The heading "Residues S332, D387, and H435 are the putative catalytic triad" is nonsensical. Either they ARE or they ARE NOT. It is of little to no interest to the reader that they are putative. Have the authors scanned all the aspartate, serine, and histidine residues in the sequence? Have they carried out in vitro affinity labeling experiments with transition state analogs? It is mildly interesting that these residues are important for biological activity, but more stringent standards are required before they can link this with the catalytic mechanism.

Technical issues

10) In Figure 3A, 4 different blots were stitched together to generate the panel. This is problematic. First, there is no way to ensure that exposure and antibody incubations were identical. In addition there is no indication of how the authors normalized the protein extract loading. I am not a fan of loading controls, but how do the authors know that they loaded equal amounts of protein? The manuscript lacks loading controls/protein concentration determinations throughout.

11) Many of the blots and fluorescence panels (other than those which include statistical analysis) lack reproducibility statements.

Reviewer #2 (Remarks to the Author):
The manuscript of Hirata et al. focuses on the targeting and function of the Atg15 lipase. This protein is made at the ER with a N-terminal TMD, sorted via the Golgi and MVB pathway to the vacuole and then activated inside the vacuole by Pep4. To identify the exact function of Atg15, the authors reanalyzed the sorting of Atg15, and demonstrate a need for membrane anchoring. Sorting via the AP-3 pathway by replacing the N-terminal segment including the TMD with the Pho8-region still allows Atg15 to reach the vacuole lumen. Furthermore, the authors identify several residues that are involved in forming the catalytic triad, and map the region of Atg15 that may form the core of the enzyme.

The authors present an overall extensive and very well controlled analysis of Atg15. Their presentation contains all necessary controls, and they show clearly that sorting via the MVB pathway is not critical for Atg15 function. I have only a few minor issues that should be taken care of during the revision to complete the study.

1. The authors make fusions of Atg15 with Pho8, yet find that the protein is almost exclusively anchored to the membrane, whereas it is luminal when sorted via the MVB pathway. However, there is almost no change in activity. One difficulty in this analysis is that the authors always follow the GFP-tagged protein, or better the GFP-fluorescence when they do imaging. Is it possible to quantify the amount of Atg15 in the lumen by using an Atg15-specific antibody? This would be more informative as the amounts of Atg15 may differ.

2. The authors need to introduce their different parts with a hypothesis. I felt that this was lacking in some parts. For instance, why do they do C-terminal truncations? What do we learn from this? Why do they address the role of W466? Why do they make multiple truncations? I understand that they want to figure out how Atg15 is functioning and why it still requires activation by Pep4, but this only becomes clear when reading the discussion.

3. Minor issues:
   a. Title: "Constructs for..." - I find this title misleading. "Sorting of Atg15 via the AP-3 pathway maintains its hydrolytic activity" would be a more suitable title.
   b. Page 10...we think - I would say..."we conclude".
   c. W466 residue - not clear why this is mutated.
   d. Atg15 is a hydrolase - there should be a homology model that could be used to explain the function of each residue. Such a model would be useful as well as a model describing the biogenesis of Atg15.
Response to the reviewer #1:

We are grateful to the reviewer #1 for the useful suggestions, which helped us to improve our manuscript considerably. As indicated below, we took these suggestions into account in the revised version of our manuscript.

(General comment)
The authors have analyzed some aspects of structure-function relationships in the Atg15 lipase. The main advance made, in my opinion, is that they show that fusion of the Atg15 catalytic domain to the pho8 targeting region is sufficient for biological activity of the protein. However since Pho8 is at least partly dependent on the MVB pathway (as is Atg15 itself), I am not sure how important this finding is. On the negative side, large parts of this study are reproductions of the conclusions of earlier studies from the Klionsky and Thumm labs, and other segments do not seem to have sufficient experimental support for their conclusions.

(Answer to the general comment)
We appreciate the kind advice by the reviewer. From the viewpoint of the trafficking analysis, we show that membrane anchoring of Atg15 through its transmembrane domain is important for its delivery to the vacuole, which is necessary for normal autophagic activity (Figure 5). From the analysis of the lipase domain, we find at least two residues (H435 and W466) are important for its activity (Figures 7 and 9). Although we need stringent experiments as the reviewer 1 points out in the specific comment 9, we can propose that H435 is a candidate for the residues consisting of the catalytic triad from our analysis.

(Specific comment 1)
The impetus for the work described in Figure 1 and the associated text is unclear. Atg15 has been implicated in autophagic body breakdown and is known not to be defective in autophagosome formation. Why are the authors testing for effects of ATG15 deletion on IM expansion? Is there some underlying scientific rationale for this?

(Answer to the specific comment 1)
We thank the reviewer for the fundamental question. It is known that the completion of autophagy is required for protein synthesis to adapt to severe environmental conditions by supplying free amino acids (Onodera and Ohsumi, 2004; Suzuki SW et al., 2010). In Figure 1, we examine the activity of expansion of autophagic membranes, which correlates with the activity of autophagosome formation (Suzuki et al., 2013), in atg15Δ cells defective in supplying free amino acids, resulting in negative results. We have added the reason to the second and third paragraphs in page 5.

(Specific comment 2)
The authors need to prove that the structures which they label as 'IM' are indeed intermediates in the formation of autophagosomes. They could do this by making a movie showing that the intermediate ultimately closes and fuses with the vacuole, but as it stands there is no evidence that these are autophagic intermediates.

(Answer to the specific comment 2)

We appreciate the comment to our analysis. The reviewer suggests an experiment to take a movie showing that the ‘IM’s ultimately close to become mature autophagosomes. Previously, we have shown that the cup-shaped ‘IM’s visualized by Ape1 overexpression are long-lasting structures at the maximum size but cannot become mature autophagosomes (Suzuki et al., 2013). Moreover, we have took a movie the cup-shaped ‘IM’s physiologically disintegrate when the giant Ape1 complex is too large to be enclosed (Suzuki et al., 2013). As the reviewer points out, the structures which we label as ‘IM’ might not be true intermediates of autophagosomes. However, it is true that the lengths of the ‘IM’s reflect the magnitude of the activity of autophagosome formation (Suzuki et al., 2013). According to the reviewer’s advice, we have modified to use ‘autophagic membranes (AM)’ for the cup-shaped Atg8-labeled membranes instead of ‘IM’s in the manuscript.

(Specific comment 3)

Regardless of the above, the outcome of the IM exercise is a negative result. This could be predicted from the data shown by Teter et al (Figure 1 therein). Therefore, it is unclear why this result is included in the manuscript.

(Answer to the specific comment 3)

We thank the reviewer for the question about the significance of our analysis. As far as we know, there is no study estimating the activity of autophagosome membrane biogenesis in atg15Δ cells quantitatively (Epple et al., 2001; Teter et al., 2001). Here we show autophagosome forming activity is maintained in atg15Δ cells, which are defective in degradation of autophagic bodies (Figures 1 and S1). We think that this fact is worth mentioning.

(Specific comment 4)

The section dealing with trafficking of Atg15 through the MVB pathway is essentially a recap of the studies published by Ulrike Eppe in Michael Thumm's lab and are not sufficiently novel.

(Answer to the specific comment 4)

We appreciate the reviewer for the concern about the novelty of our trafficking analysis. We understand the reviewer on the point of novelty. In previous studies, immunofluorescence microscopy using haemagglutinin-tagged Atg15 has been used for localization analysis of Atg15 (Epple et al., 2001; Epple et al. 2003). Here we have shown that GFP-tagged Atg15 strains can be useful both for localization analysis by fluorescence microscopy (Figure 2) and for quantification of Atg15 trafficking to the
vacuolar lumen by immunoblot analysis (Figures 3). In these figures, we present these strains as new tools for future studies on Atg15. We think that this result would be worth showing.

(Specific comment 5)
The authors need to explain the appearance of free GFP from Atg15-GFP in pep4 cells. This is either experimental error or a complete paradigm shift in our understanding of Atg15 trafficking.

(Answer to the specific comment 5)
We thank the reviewer for the thoughtful opinion. We also recognize faint bands corresponding to the position of free GFP. However, the intensities of the faint bands after subtracting the background signals are estimated as almost zero. Thus, we cannot conclude that free GFP bands exist in pep4Δ cells. We performed the same immunoblot analysis again, which leads to the same conclusion (new Figure 3A).

(Specific comment 6)
At the bottom of Page 6 the authors conclude that transport of Atg15 to the vacuole is essential for its function. However one page later at the bottom of page 7, they show that a completely mislocalized version of the protein, lacking the TMD, is partially functional. The authors must resolve this contradiction.

(Answer to the specific comment 6)
We appreciate the reviewer for the question. As the reviewer points out, Atg15ΔTMD is mislocalized to the cytoplasm (Figure 5B). Immunoblot analysis shows that a small amount of mature Ape1 appears (Figure 5C). Since Ape1 maturation is a very sensitive assay, the activity of Atg15 would be very low. We discuss this phenotype as “However, a significant amount of mApe1 was still detected in GFP-Atg15ΔTMD cells (Figure 5C and D). This result implies a second MVB targeting site on Atg15 lacking TMD or alternative pathways of Atg15ΔTMD to the vacuolar lumen. These results suggest that the C-terminal domain of Atg15 is critical for its activity, and that transport of the C-terminal domain to the vacuolar lumen is important.” at the bottom of page 7.

(Specific comment 7)
Page 8, 2nd para: fab1 mutants are not class E

(Answer to the comment 7)
We thank the reviewer for pointing out our mistake. We modified the text according to the reviewer’s suggestion.

(Specific comment 8)
The finding that the double apl5 vps4 mutant can fully suppress the autophagy defect of atg15 mutants requires more attention and a significant amount of work.
We thank the reviewer for the interest in our results and for your advice. We are also very interested in this phenotype: Does the maturation of Ape1 depend on Pep4? and so on. However, we feel this research theme goes beyond this paper. As the reviewer suggests, this phenomenon is potentially very interesting and important. We will analyze this phenomenon in greater depth in future.

The heading "Residues S332, D387, and H435 are the putative catalytic triad" is nonsensical. Either they ARE or they ARE NOT. it is of little to no interest to the reader that they are putative. Have the authors scanned all the aspartate, serine, and histidine residues in the sequence? Have they carried out in vitro affinity labeling experiments with transition state analogs? It is mildly interesting that these residues are important for biological activity, but more stringent standards are required before they can link this with the catalytic mechanism. We thank the reviewer for the advice about the catalytic mechanism. As the reviewer points out, we might just analyze the phenotypes of D387A and D421 mutants (Figure 7). However, our experiments clearly show that D387 is an important residue of the two aspartic acid residues described in Teter et al. as candidates for the residues consisting of the catalytic triad (Figure 3B in Teter et al., 2001). We think this result is worth reporting. We understand that structural analysis is necessary to provide a stringent proof of the catalytic mechanism, and we are now in the process of the experiments using the minimum regions (residues 50–466) identified in this study. To show that we need more stringent results to prove that these residues correspond to the catalytic triad, we have added the statement that “Structural analysis will be necessary to prove whether these residues truly consist of the catalytic triad” at the end of this section.

In Figure 3A, 4 different blots were stitched together to generate the panel. This is problematic. First, there is no way to ensure that exposure and antibody incubations were identical. In addition there is no indication of how the authors normalized the protein extract loading. I am not a fan of loading controls, but how do the authors know that they loaded equal amounts of protein? The manuscript lacks loading controls/protein concentration determinations throughout.

I understand the reviewer’s concern about the immunoblot data in Figure 3A. The immunoblot data presented in previous Figure 3 were indeed obtained from the same membrane. Thus, the exposure time and the incubation time with antibodies were identical. However, the samples at the different time points were inserted between the presented data in the original immunoblot data. Thus, we removed extra data.
from the panel to save spaces. We performed the immunoblot for several times before submission but it failed by unknown reasons. After submission, we have successfully solved the problem. Now we present a new immunoblot image without stitching in new Figure 3A.

In Figure 3, the transport of Atg15-GFP is presented as ratios calculated by dividing the band intensities of free GFP by the band intensities of full length Atg15-GFP plus free GFP. In the case of Ape1 transport, the calculation method is the same. Therefore, we think that differences in the loadings used in the experiments are theoretically removed by this calculation. The same method is used to estimate the activity of Ape1 maturation in Figures 5D, 6C, 7C, 8C, 9B, 10B and 11C. In the case of ALP assay, the amount of proteins used for the assays were normalized with protein concentrations measured by the BCA assay.

(Specific comment 11)
Many of the blots and fluorescence panels (other than those which include statistical analysis) lack reproducibility statements.

(Answer to the specific comment 11)
We appreciate the reviewer for the comment on reproducibility. We understand your comment to do as much statistical analysis as possible on the data that we present. The following figures lack statistical analysis: Figures 2, 6D, 7D, 8D, 9C and 11D, but the remaining figures include statistical analysis. In Figure 2, we just show the localization of Atg15-GFP in several strains. The transport of Atg15-GFP to the vacuole and maturation of prApe1 using the strains are presented in Figure 3, which includes statistical analysis. In the other panels (6D, 7D, 8D, 9C and 11D), we show only numbers below fluorescent images. We have modified to add standard deviations to the numbers and performed statistical tests for the results of 6D, 7D, 8D, 9C and 11D.
Response to the reviewer #2:

We are grateful to the reviewer #2 for the useful suggestions, which helped us to improve our manuscript considerably. As indicated below, we took these suggestions into account in the revised version of our manuscript.

(Major comment 1)
The authors make fusions of Atg15 with Pho8, yet find that the protein is almost exclusively anchored to the membrane, whereas it is luminal when sorted via the MVB pathway. However, there is almost no change in activity. One difficulty in this analysis is that the authors always follow the GFP-tagged protein, or better the GFP-fluorescence when they do imaging. Is it possible to quantify the amount of Atg15 in the lumen by using an Atg15-specific antibody? This would be more informative as the amounts of Atg15 may differ.

(Answer to the major comment 1)
We appreciate the reviewer for the helpful comment. In previous studies, Atg15 and C-terminally 3×haemagglutinin-tagged Atg15 has been detected as a ~70-kDa or ~75-kDa band, respectively (Teter et al. 2001; Epple et al. 2001). In this study, we constructed C-terminally 2×GFP-tagged Atg15 strains. Atg15-2×GFP is detected as ~120-kDa band by immunoblot analysis with anti-GFP antibodies (Figure 3A). The size of Atg15-2×GFP is reasonable in comparison to previous studies (70-kDa plus 54-kDa). We performed immunoblot analysis with anti-Atg15 antibodies (kindly provided from Dr. Yoshinori Ohsumi) using the same cell lysates. However, we could not detect any band specifically detected in wild-type cells (Attached Figure 1A). We next tried to detect the lipase domain using GFP-Pho8TMD-Atg15C expressing cells. GFP-Pho8TMD-Atg15C overexpressed from the pRS426 plasmid was detected by immunoblot analysis with anti-GFP antibodies but the one expressed from the pRS316 plasmid, which is detectable in this study with anti-GFP antibodies (see Figure 6), was not detected because exposure time was quite short (Attached Figure 1B). GFP-Pho8TMD-Atg15C was also detected with anti-Atg15 antibodies but no other Atg15C-specific band was detected (Attached Figure 1B). These results suggest two possibilities, a very small amount of Atg15C is sufficient for degradation of Cvt bodies and autophagic bodies, or the current antibodies against Atg15 do not recognize Atg15C inside vacuolar lumen. Unfortunately, we cannot detect the active form of Atg15C at present.

(Major comment 2a)
The authors need to introduce their different parts with a hypothesis. I felt that this was lacking in some parts. For instance, why do they do C-terminal truncations? What do we learn from this?

(Answer to the major comment 2a)
We thank the reviewer for the kind comment. We have added the purpose of the C-terminal truncation
experiments in page 10 that “As shown above, the GFP moiety is cleaved off from Atg15-GFP in a manner dependent on Pep4 (Figure 3A). Pep4 is a vacuolar endopeptidase that activates Prb1 and Pho8 by cleaving them (Ammerer et al., 1986). If Atg15 is activated by Pep4, a proper Atg15 truncation mutant might be active in pep4Δ cells.” We have also add the result that “We examined whether these truncation mutants could bypass the activity of Pep4 with atg15Δpep4Δ cells expressing them by the Ape1 maturation assay, showing that no Ape1 maturation was detected in all truncation mutants (Supplemental Figure S5).” at the end of the section.

(Major comment 2b)
Why do the address the role of W466?
(Answer to the major comment 2b)
According to the reviewer’s advice, we have modified the text in the W466 section to “The observations described above suggest that Atg15 lacking the 55th residue from the extreme C-terminus (W466) is severely defective in degradation of Cvt bodies / autophagic bodies (Figure 8).”

(Major comment 2c)
Why do they make multiple truncations? I understand that they want to figure out how Atg15 is functioning and why it still requires activation by Pep4, but this only becomes clear when reading the discussion.
(Answer to the major comment 2c)
We appreciate the reviewer for the kind suggestion. According to the reviewer’s suggestion, we added the text that “Finally, we examined whether Atg15ΔN49ΔC54 requires activation by Pep4. mApe1 did not appear in atg15Δpep4Δ cells expressing Atg15ΔN49ΔC54 (Supplemental Figure S7), showing that this truncation mutant cannot bypass the activation by Pep4.” at the end of the result section.

(Minor comment 3a)
"Constructs for...." - I find this title misleading. "Sorting of Atg15 via the AP-3 pathway maintains its hydrolytic activity" would be a more suitable title.
(Answer to the minor comment 3a)
Thank you for the thoughtful suggestion by the reviewer #2. We have changed the title accordingly.

(Minor comment 3b)
Page 10....we think - I would say..."we conclude".
(Answer to the minor comment 3b)
We appreciate the kind advice by the reviewer. We have modified the manuscript according to the reviewer’s advice.
(Minor comment 3c)
W466 residue - not clear why this is mutated.
(Answer to the comment 3c)
We appreciate the question raised by the reviewer. We have added the description explaining why the W466 residue is mutated as “Moreover, W466 is a highly conserved residue at the extreme C-terminal region among Atg15 orthologs (Supplemental Figure S4). Hence, to examine the importance of the residue...”

(Minor comment 3d)
Atg15 is a hydrolase - there should be a homology model that could be used to explain the function of each residue. Such a model would be useful as well as a model describing the biogenesis of Atg15.
(Answer to the comment 3d)
Thank you for the reviewer for the comment. We show alignment of Atg15 orthologs from various fungal species in Supplemental Figure S4. We have added the reference to the text as “Moreover, Teter et al. suggests that several highly conserved residues constitute the catalytic triad: D387 or D421 and H435 (Supplemental Figure S4).”
RE: Manuscript #E20-07-0500R
TITLE: “Atg15 in Saccharomyces cerevisiae consists of two functionally distinct domains”

Dear Dr. Suzuki:

First, let me apologize for the long wait in the evaluation of your manuscript. This was due largely to the strong difference of opinion between the two experts who reviewed your manuscript and to the winter holiday season.

Referee 1 was clearly not satisfied with your responses to the first evaluation, whereas referee 2 was. I have gone through the manuscript myself and even though I am not familiar with all of the details of the autophagy work, I can see that your manuscript does have some new findings that would be useful to scientists in the field. Therefore, I would like to give you the opportunity to revise your manuscript a final time. You should take into account the comments of referee 1 to make sure that your findings are properly put into the context of previous work in the field and be very clear about where questions have been resolved and where they have not, even if they were clear previous to your studies. It is important to state where things have already been shown, even if you provide confirmatory evidence. It is useful when other groups come to the same conclusions as previous studies. This raises the confidence in the original findings. There is also some issues about the assays you use and the rationale. Please make it clear if what you measure is a direct or an indirect readout concerning the questions you pose. I don't agree with everything that referee 1 says, for instance, I think that it is fine for you to call the "putative catalytic triad" as you do. I will not go through every comment of Referee 1, but I think that you can see how to respond with textual changes in your manuscript by my guidelines above.

I would highly appreciate seeing a revised document where I can directly analyse the changes you have made in the text because I will take a final decision without returning the manuscript to the referees.

Best regards,

Howard Riezman
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Suzuki,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): Link Not Available

Authors of Articles and Brief Communications whose manuscripts have returned for minor revision ("revise only") are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.
Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

The following are my comments on the authors' rebuttal and the revised manuscript, including the original referee comments:

(Specific comment 1)
The impetus for the work described in Figure 1 and the associated text is unclear. Atg15 has been implicated in autophagic body breakdown and is known not to be defective in autophagosome formation. Why are the authors testing for effects of ATG15 deletion on IM expansion? Is there some underlying scientific rationale for this?

(Author response to the specific comment 1):
We thank the reviewer for the fundamental question. It is known that the completion of autophagy is required for protein synthesis to adapt to severe environmental conditions by supplying free amino acids (Onodera and Ohsumi, 2004; Suzuki SW et al., 2010). In Figure 1, we examine the activity of expansion of autophagic membranes, which correlates with the activity of autophagosome formation (Suzuki et al., 2013), in atg15Δ cells defective in supplying free amino acids, resulting in negative results. We have added the reason to the second and third paragraphs in page 5.

Referee response: The authors are confusing and conflating two distinct concepts, 'autophagosome completion' and the capacity if the cell to carry out normal autophagic flux. In this manuscript they are making claims regarding the potential function of Atg15 in autophagosome expansion. However such a function was already ruled out by previous publications, rendering the exercise unnecessary. Therefore, I do not think that they are properly addressing my criticism.

(Specific comment 2)
The authors need to prove that the structures which they label as 'IM' are indeed intermediates in the formation of autophagosomes. They could do this by making a movie showing that the intermediate ultimately closes and fuses with the vacuole, but as it stands there is no evidence that these are autophagic intermediates.

(Author response to specific comment 2): 
"We appreciate the comment to our analysis. The reviewer suggests an experiment to take a movie showing that the 'IM's ultimately close to become mature autophagosomes. Previously, we have shown that the cup-shaped 'IM's visualized by Ape1 overexpression are long-lasting structures at the maximum size but cannot become mature autophagosomes (Suzuki et al., 2013). Moreover, we have take a movie the cup-shaped 'IM's physiologically disintegrate when the giant Ape1 complex is too large to be enclosed (Suzuki et al., 2013). As the reviewer points out, the structures which we label as 'IM' might not be true intermediates of autophagosomes. However, it is true that the lengths of the 'IM's reflect the magnitude of the activity of autophagosome formation (Suzuki et al., 2013). According to the reviewer's advice, we have modified to use 'autophagic membranes (AM)' for the cup-shaped Atg8-labeled membranes instead of 'IM's in the manuscript.""

Referee response: I believe that the authors, together with additional workers in the field (including the Suzuki et al paper from 2013), are falling into a logical trap. Their reasoning, as I see it, is that since overexpression of Ape1 generates a complex that is too large for autophagosomes to engulf, then by its nature, its assembly is a valid reflection on the mechanism of autophagosome formation. The assumption here is that the only reasonable explanation for the block in Cvt trafficking observed in cells that are over-expressing Ape1 is that the resultant Cvt complex is simply too large. This seemingly innocuous and self-evident assumption ignores several crucial pieces of information. Ape1 has been shown to directly interact, pair-wise, with at least three or four different factors that are essential for Cvt trafficking. Thus, overexpression of Ape1 is equally likely to lead to squelching of these interactions, leading to dead end molecular complexes that are not forming functional autophagosomes. No attention has been paid to this possibility in any of the recent publications utilizing Ape1 overexpression to study autophagosome formation, including Suzuki et al (2013). In agreement with this second hypothesis, it has been shown in other systems, that very large structures, on the order of the super-Ape1 complex and even larger, can indeed be engulfed by autophagosomes without a hiccup.

Author response to specific comment 2:
"However, it is true that the lengths of the 'IM's reflect the magnitude of the activity of autophagosome formation (Suzuki et al., 2013)."

referee response: I disagree with this statement, and also with the conclusion in the 2013 paper. The authors must demonstrate that the structures they are viewing are bona-fide intermediates before they can make statements on autophagosome formation based on these experiments.

Author response to specific comment 2:
"According to the reviewer's advice, we have modified to use 'autophagic membranes (AM)' for the cup-shaped Atg8-labeled membranes instead of 'IM's in themanuscript."

referee response: This was not my advice, and the issue is not semantic ...changing from IM to AM does not improve the situation

referee response: Beyond the general problem with the authors' reasoning, I also have a problem with the rationale for carrying out these studies in the first place. Assays for autophagosome expansion have been around for at least 20 years and beyond the problem pointed out above, there is no novelty in asking whether expansion is defective in atg15 mutants. It was shown by the Teter et al paper, as well as in the Epple et al publications, that Atg15 is not required for autophagosome formation but in autophagic body degradation. There is no reason to doubt those earlier results, and the data presented here on this issue, in this manuscript, do not further our knowledge on this topic.

Author response to specific comment 3:
"We thank the reviewer for the question about the significance of our analysis. As far as we know, there is no study estimating the activity of autophagosome membrane biogenesis in atg15Δ cells quantitatively (Epple et al., 2001; Teter et al., 2001). Here we show autophagosome forming activity is maintained in atg15Δ cells, which are defective in degradation of autophagic bodies(Figures 1 and S1).We think that this fact is worth mentioning."

referee response: The previous studies demonstrated that atg15 delta cells accumulate autophagic bodies to the same extent and with the same sizes as do pep4 delta cells. This demonstrated that normal sized autophagosomes are formed in pep15 delta cells. In addition, protease protection studies in the Teter et al publication demonstrated that atg15Δ cells are able to form completed autophagosomes. Finally, the use of a flawed system (as I perceive it-see above) to analyze autophagosome expansion greatly detracts from any minor value which we could have gained from a more stringent investigation of autophagosome biogenesis in atg15Δ cells, and paves the way for more confusion in the future.

(Specific comment 4)
The section dealing with trafficking of Atg15 through the MVB pathway is essentially a recap of the studies published by Ulrike Epple in Michael Thumm's lab and are not sufficiently novel.

(Authors' response to specific comment 4)
"We appreciate the reviewer for the concern about the novelty of our trafficking analysis. We understand the reviewer on the point of novelty. In previous studies, immunofluorescence microscopy using haemagglutinin-tagged Atg15 has been used for localization analysis of Atg15 (Epple et al., 2001; Epple et al. 2003). Here we have shown that GFP-tagged Atg15 strains can be useful both for localization analysis by fluorescence microscopy (Figure 2) and for quantification of Atg15 trafficking to the vacuolar lumen by immunoblot analysis (Figures 3). In these figures, we present these strains as new tools for future studies on Atg15.We think that this result would be worth showing."

referee response: This is nice but the manuscript is presented as a research article, and not a technical note. You need to present data which furthers our understanding of the system, and adding "tools" is not a sufficiently valid premise for publication (unless you wish to publish a technical note).

Specific comment 8:
The finding that the double apl5 vps4 mutant can fully suppress the autophagy defect of atg15 mutants requires more attention and a significant amount of work.

(Authors' response to specific comment 8)
"We thank the reviewer for the interest in our results and for your advice. We are also very interested in this phenotype: Does the maturation of Ape1 depend on Pep4? and so on. However, we feel this research theme goes beyond this paper. As the reviewer suggests, this phenomenon is potentially very interesting and important. We will analyze this phenomenon in greater
referee response: This result is troubling. If you claim (as others did before you) that the role of Atg15 is to degrade autophagic bodies in the vacuole via its lipase activity, then how do you explain this result??! It is not compatible with what we know of Atg15 function nor of Apl5 and Vps4.
At a minimum, the authors should have tested whether double deletion of APL5 and VPS4 equally suppresses other autophagy mutants, in order to see whether this suppression is specific to atg15Δ.

(Specific comment 9)
The heading "Residues S332, D387, and H435 are the putative catalytic triad" is nonsensical. Either they ARE or they ARE NOT. It is of little to no interest to the reader that they are putative. Have the authors scanned all the aspartate, serine, and histidine residues in the sequence? Have they carried out in vitro affinity labeling experiments with transition state analogs? It is mildly interesting that these residues are important for biological activity, but more stringent standards are required before they can link this with the catalytic mechanism.

(Authors' response to specific comment 9)
We thank the reviewer for the advice about the catalytic mechanism. As the reviewer points out, we might just analyze the phenotypes of D387A and D421 mutants (Figure 7). However, our experiments clearly show that D387 is an important residue of the two aspartic acid residues described in Teter et al. as candidates for the residues consisting of the catalytic triad (Figure 3B in Teter et al., 2001). We think this result is worth reporting. We understand that structural analysis is necessary to provide a stringent proof of the catalytic mechanism, and we are now in the process of the experiments using the minimum regions (residues 50-466) identified in this study. To show that we need more stringent results to prove that these residues correspond to the catalytic triad, we have added the statement that "Structural analysis will be necessary to prove whether these residues truly consist of the catalytic triad" at the end of this section.

referee response: Yes, but despite these disclaimers here in the rebuttal, the authors still maintain their original claims in the abstract and elsewhere.
If the authors wish to publish such claims they need at a minimum 1) To cite a similar lipase with a known catalytic triad, in which the required analysis was carried out. 2) To show homology of Atg15 to the other lipase, showing the corresponding residues in Atg15 and testing their respective mutations. No amount of narrowing down the 416 AA "essential" region will be enough. You can do mutations until you are blue in the face, but unless someone does some actual biochemistry somewhere down the line, you will not know the catalytic mechanism.

Additional specific comment:
New figure 3A is still not making sense. If Atg15 is reaching the vacuole but is not being degraded in pep4Δ cells, then we expect that the Atg15-GFP band should increase in intensity at the expense of the free GFP. Then why is the intensity of the chimera band still as weak in the pep4Δ mutant, as that observed in cells which are able to traffic Atg15 to the vacuolar lumen and release free GFP?

Reviewer #2 (Remarks to the Author):
The authors answered my requests. I have no further questions.
February 13, 2021

Manuscript #E20-07-0500R
Revised manuscript for *Molecular Biology of the Cell*

“Atg15 in *Saccharomyces cerevisiae* consists of two functionally distinct domains”

by

Eri Hirata, Kyo Shirai, Tatsuya Kawaoka, Kosuke Sato, Fumito Kodama and Kuninori Suzuki

Dear Dr. Howard Riezman:

We are most grateful to you and the reviewers for the helpful comments on the revised version of our manuscript. We have taken all of these comments into account, and we hereby submit a revised version of our manuscript as a *Research Article* paper. We carefully read the comments of you and the reviewer #1, and revised the manuscript accordingly. All answers to the comments are attached to this letter.

We hope that the revised manuscript is now suitable for publication in *Molecular Biology of the Cell*. We would be glad to respond to any further questions and comments that you may have. We thank you very much for your interest and assistance.

Sincerely yours,

[Signature]

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Response to the monitoring editor:

We are grateful to the monitoring editor for the kind suggestions, which helped us to improve our manuscript considerably. As indicated below, we took these suggestions into account in the revised version of our manuscript. We have found that the reviewer #1 raised questions without regard to our discussion in the discussion section. What do you think about including a reference to discussion in the manuscript?

[Comment #1]
You should take into account the comments of referee 1 to make sure that your findings are properly put into the context of previous work in the field and be very clear about where questions have been resolved and where they have not, even if they were clear previous to your studies.
[Answer #1]
Thank you for your suggestion. We write point-by-point answers to the reviewer #1’s comments according to your suggestions. We try to clarify the resolved and unresolved questions.

[Comment #2]
It is important to state where things have already been shown, even if you provide confirmatory evidence. It is useful when other groups come to the same conclusions as previous studies. This raises the confidence in the original findings.
[Answer #2]
We appreciate your thoughtful suggestions. We try to state where things have already been shown.

[Comment #3]
There is also some issues about the assays you use and the rationale. Please make it clear if what you measure is a direct or an indirect readout concerning the questions you pose.
[Answer #3]
Thank you for your suggestion. We try to clarify what we measure is a direct or an indirect ones.

[Comment #4]
I don't agree with everything that referee 1 says, for instance, I think that it is fine for you to call the "putative catalytic triad" as you do.
[Answer #4]
Thank you for your comment. I will use the term “putative catalytic triad” in the revised manuscript.
Response to the reviewer #1:

We are grateful to the reviewer #1 for the useful suggestions, which helped us to improve our manuscript considerably. As indicated below, we took these suggestions into account in the revised version of our manuscript.

[Question #1]
(Specific comment 1)
The impetus for the work described in Figure 1 and the associated text is unclear. Atg15 has been implicated in autophagic body breakdown and is known not to be defective in autophagosome formation. Why are the authors testing for effects of ATG15 deletion on IM expansion? Is there some underlying scientific rationale for this?

(Authors response to the specific comment 1):
We thank the reviewer for the fundamental question. It is known that the completion of autophagy is required for protein synthesis to adapt to severe environmental conditions by supplying free amino acids (Onodera and Ohsumi, 2004; Suzuki SW et al., 2010). In Figure 1, we examine the activity of expansion of autophagic membranes, which correlates with the activity of autophagosome formation (Suzuki et al., 2013), in atg15Δ cells defective in supplying free amino acids, resulting in negative results. We have added the reason to the second and third paragraphs in page 5.

Referee response: The authors are confusing and conflating two distinct concepts, 'autophagosome completion' and the capacity if the cell to carry out normal autophagic flux. In this manuscript they are making claims regarding the potential function of Atg15 in autophagosome expansion. However such a function was already ruled out by previous publications, rendering the exercise unnecessary. Therefore, I do not think that they are properly addressing my criticism.

[Answer #1]
We do not deny the results obtained from previous studies, but rather clarify new aspects that have not been analyzed before. As the reviewer #1 points out, there is no doubt that Atg15 is a lipase essential for autophagic body breakdown. In this study, we try to clarify whether the defect in autophagic body breakdown in atg15Δ cells directly or indirectly affects the size of autophagosomes with our quantitative method (Suzuki et al., 2013; Kawaoka et al., 2017). We have moved the introductory sentences to the first paragraph of the result section and modified the text in page 5 to clarify the primary role of Atg15 is to degrade autophagic bodies. Moreover, we have explained unresolved questions there.
The authors need to prove that the structures which they label as 'IM' are indeed intermediates in the formation of autophagosomes. They could do this by making a movie showing that the intermediate ultimately closes and fuses with the vacuole, but as it stands there is no evidence that these are autophagic intermediates.

"We appreciate the comment to our analysis. The reviewer suggests an experiment to take a movie showing that the 'IM's ultimately close to become mature autophagosomes. Previously, we have shown that the cup-shaped 'IM's visualized by Ape1 overexpression are long-lasting structures at the maximum size but cannot become mature autophagosomes (Suzuki et al., 2013). Moreover, we have took a movie the cup-shaped 'IM's physiologically disintegrate when the giant Ape1 complex is too large to be enclosed (Suzuki et al., 2013). As the reviewer points out, the structures which we label as 'IM' might not be true intermediates of autophagosomes. However, it is true that the lengths of the 'IM's reflect the magnitude of the activity of autophagosome formation (Suzuki et al., 2013). According to the reviewer's advice, we have modified to use 'autophagic membranes (AM)' for the cup-shaped Atg8-labeled membranes instead of 'IM's in the manuscript."

Referee response: I believe that the authors, together with additional workers in the field (including the Suzuki et al paper from 2013), are falling into a logical trap. Their reasoning, as I see it, is that since overexpression of Ape1 generates a complex that is too large for autophagosomes to engulf, then by its nature, its assembly is a valid reflection on the mechanism of autophagosome formation. The assumption here is that the only reasonable explanation for the block in Cvt trafficking observed in cells that are over-expressing Ape1 is that the resultant Cvt complex is simply too large. This seemingly innocuous and self evident assumption ignores several crucial pieces of information. Ape1 has been shown to directly interact, pair-wise, with at least three or four different factors that are essential for Cvt trafficking. Thus, overexpression of Ape1 is equally likely to lead to squelching of these interactions, leading to dead end molecular complexes that are not forming functional autophagosomes. No attention has been paid to this possibility in any of the recent publications utilizing Ape1 overexpression to study autophagosome formation, including Suzuki et al (2013). In agreement with this second hypothesis, it has been shown in other systems, that very large structures, on the order of the super-Ape1 complex and even larger, can indeed be engulfed by autophagosomes without a hiccup.
“I believe that the authors, together with additional workers in the field (including the Suzuki et al paper from 2013), are falling into a logical trap. Their reasoning, as I see it, is that since overexpression of Ape1 generates a complex that is too large for autophagosomes to engulf, then by its nature, its assembly is a valid reflection on the mechanism of autophagosome formation.”

As the reviewer points out, we certainly think that autophagic membranes accumulate according to the valid mechanisms in Ape1 overexpressed cells.

“The assumption here is that the only reasonable explanation for the block in Cvt trafficking observed in cells that are over-expressing Ape1 is that the resultant Cvt complex is simply too large.”

Yes, we agree with the reviewer.

“This seemingly innocuous and self evident assumption ignores several crucial pieces of information. Ape1 has been shown to directly interact, pair-wise, with at least three or four different factors that are essential for Cvt trafficking. Thus, overexpression of Ape1 is equally likely to lead to squelching of these interactions, leading to dead end molecular complexes that are not forming functional autophagosomes.”

We agree to the possibility that dead end molecular complexes that are not functional autophagosomes are formed. However, we showed that the cup-shaped structures were membrane-bound by electron microscopy and their shapes changed dynamically by timelapse fluorescence microscopy in Suzuki et al (2013). Moreover, the AM length well reflected the magnitude of autophagy. The reviewers of that paper agreed to the point that this approach somehow reflected the activity of autophagosome formation. Based upon this knowledge, we analyze the magnitude of autophagosome formation activity in \textit{atg15Δ} cells in this study.

“No attention has been paid to this possibility in any of the recent publications utilizing Ape1 overexpression to study autophagosome formation, including Suzuki et al (2013).”

I understand the reviewer’s concern. However, Ape1 overexpression is just used to block the completion of autophagosome formation to estimate autophagic membrane expansion activity but not used to estimate the activity of the Cvt pathway.
“In agreement with this second hypothesis, it has been shown in other systems, that very large structures, on the order of the super-Apel complex and even larger, can indeed be engulfed by autophagosomes without a hiccup.”

I understand the reviewer’s point. As the reviewer points out, autophagosomes can engulf larger natural cargoes than the Ape1 complex. By contrast, the giant Ape1 complex is not a natural cargo. We think this is a reason why autophagosomes cannot engulf the giant Ape1 complex. The disturbance of pair-wise interactions might be one of the reasons for this as the reviewer points out. We just use this assay to estimate the activity of autophagic membrane expansion. No other mechanistic conclusions have drawn from this assay.
[Question #3]
Author response to specific comment 2:
"However, it is true that the lengths of the 'IM's reflect the magnitude of the activity of autophagosome formation (Suzuki et al., 2013)."

referee response: I disagree with this statement, and also with the conclusion in the 2013 paper. The authors must demonstrate that the structures they are viewing are bona-fide intermediates before they can make statements on autophagosome formation based on these experiments.

[Answer #3]
We are perplexed by this reviewer's comment. Our 2013 paper was duly reviewed by reviewers and published. We believe that a substantial proof should be provided to deny the conclusion of the paper. There are certainly many points that I agree to the criticisms of the reviewer #1 on our 2013 paper. However, we do not think it is the role of the reviewer of this paper to deny the conclusion of our 2013 paper. In this paper, we have drawn only minimal conclusions using this assay. This is because we are well aware of the inherent problems that this assay may have.
[Question #4]
Author response to specific comment 2:
"According to the reviewer's advice, we have modified to use 'autophagic membranes (AM)' for the cup-shaped Atg8-labeled membranes instead of 'IM's in the manuscript."

referee response: This was not my advice, and the issue is not semantic ...changing from IM to AM does not improve the situation

referee response: Beyond the general problem with the authors' reasoning, I also have a problem with the rationale for carrying out these studies in the first place. Assays for autophagosome expansion have been around for at least 20 years and beyond the problem pointed out above, there is no novelty in asking whether expansion is defective in atg15 mutants. It was shown by the Teter et al paper, as well as in the Epple et al publications, that Atg15 is not required for autophagosome formation but in autophagic body degradation. There is no reason to doubt those earlier results, and the data presented here on this issue, in this manuscript, do not further our knowledge on this topic.

[Answer #4]
“Assays for autophagosome expansion have been around for at least 20 years and beyond the problem pointed out above, there is no novelty in asking whether expansion is defective in atg15 mutants.”

We thank the reviewer for the helpful suggestion. To avoid misunderstandings, we have added the explanation that the primary role of Atg15 is to degrade autophagic bodies in the first paragraph of the result section. Moreover, we have added the explanation why we performed this experiment.

“It was shown by the Teter et al paper, as well as in the Epple et al publications, that Atg15 is not required for autophagosome formation but in autophagic body degradation. There is no reason to doubt those earlier results, and the data presented here on this issue, in this manuscript, do not further our knowledge on this topic.”

We do not doubt the earlier results. We have added the explanation that “The primary role of Atg15 is to degrade autophagic bodies by its lipase activity (Epple et al., 2001; Teter et al., 2001)” in the first paragraph of the result section. However, it is not known whether degradation of autophagic bodies affects the size of autophagosomes. We try to analyze this point by quantitative analysis using Ape1-overexpressed cells although the result is negative.
[Question #5]
Author response to specific comment 3:
"We thank the reviewer for the question about the significance of our analysis. As far as we know, there is no study estimating the activity of autophagosome membrane biogenesis in atg15Δ cells quantitatively (Epple et al., 2001; Teter et al., 2001). Here we show autophagosome forming activity is maintained in atg15Δ cells, which are defective in degradation of autophagic bodies (Figures 1 and S1). We think that this fact is worth mentioning."

referee response: The previous studies demonstrated that atg15 delta cells accumulate autophagic bodies to the same extent and with the same sizes as do pep4 delta cells. This demonstrated that normal sized autophagosomes are formed in pep15 delta cells. In addition, protease protection studies in the Teter et al. publication demonstrated that atg15Δ cells are able to form completed autophagosomes. Finally, the use of a flawed system (as I perceive it—see above) to analyze autophagosome expansion greatly detracts from any minor value which we could have gained from a more stringent investigation of autophagosome biogenesis in atg15Δ cells, and paves the way for more confusion in the future.

[Answer #5]
“The previous studies demonstrated that atg15 delta cells accumulate autophagic bodies to the same extent and with the same sizes as do pep4 delta cells. This demonstrated that normal sized autophagosomes are formed in pep15 delta cells.”

As the reviewer points out, accumulation of autophagic bodies in atg15Δ cells has been already reported using the electron microscope (Epple et al., 2001). However, the size of autophagic bodies (autophagosomes) has never quantified. In the absence of quantified data, we can not conclude that “normal sized autophagosomes are formed”. We believe that the quantitative analysis done in this study is the first data suggesting that normal sized autophagosomes are formed in atg15Δ cells.

“In addition, protease protection studies in the Teter et al. publication demonstrated that atg15Δ cells are able to form completed autophagosomes.”

We agree with the reviewer on this point. We do not deny the result that completed autophagosomes are formed in atg15Δ cells.

“Finally, the use of a flawed system (as I perceive it—see above) to analyze autophagosome expansion greatly detracts from any minor value which we could have gained from a more stringent investigation of autophagosome biogenesis in atg15Δ cells, and paves the way for more confusion in the future.”
As the reviewer points out, our Ape1 overexpression system is not flawless. However, we obtained a result that the AM length in wild-type cells was longer than that in *atg2Δ* cells (Figure 1). Moreover, we showed that the AM length in *atg15Δ* cells was comparable to that in wild-type cells (Figure 1). With these control data, we believe that this analysis provides meaningful data.
(Specific comment 4)
The section dealing with trafficking of Atg15 through the MVB pathway is essentially a recap of the studies published by Ulrike Epple in Michael Thumm's lab and are not sufficiently novel.

(Authors' response to specific comment 4)
"We appreciate the reviewer for the concern about the novelty of our trafficking analysis. We understand the reviewer on the point of novelty. In previous studies, immunofluorescence microscopy using haemagglutinin-tagged Atg15 has been used for localization analysis of Atg15 (Epple et al., 2001; Epple et al. 2003). Here we have shown that GFP-tagged Atg15 strains can be useful both for localization analysis by fluorescence microscopy (Figure 2) and for quantification of Atg15 trafficking to the vacuolar lumen by immunoblot analysis (Figures 3). In these figures, we present these strains as new tools for future studies on Atg15. We think that this result would be worth showing."

referee response: This is nice but the manuscript is presented as a research article, and not a technical note. You need to present data which furthers our understanding of the system, and adding "tools" is not a sufficiently valid premise for publication (unless you wish to publish a technical note).

[Answer #6]
We appreciate the reviewer for the suggestion. As the reviewer suggests, Epple et al., 2003 presents the data showing that the transport of Atg15 is defective in class E vps mutants by indirect immunofluorescence microscopy. However, Ape1 maturation in these cells appears normal (Epple et al., 2003). To explain this apparent contradiction, they discuss the possibility that “the small amounts that might still reach the vacuole lumen are sufficient for lysis of almost all autophagic bodies.” In Figure 3, our quantitative analysis clearly shows that the transport of Atg15-GFP is partially inhibited and that Atg15-GFP is still transported to the vacuolar lumen in vps4Δ cells. Without functional Atg15-GFP, we cannot carry out this experiment. We have already discussed this point in the “Activity of Atg15 in mutants of the MVB pathway” in the discussion section. In addition, we have modified the text (page 7) to clarify this point.
Specific comment 8:
The finding that the double apl5 vps4 mutant can fully suppress the autophagy defect of atg15 mutants requires more attention and a significant amount of work.

(Authors' response to specific comment 8)
"We thank the reviewer for the interest in our results and for your advice. We are also very interested in this phenotype: Does the maturation of Ape1 depend on Pep4? and so on. However, we feel this research theme goes beyond this paper. As the reviewer suggests, this phenomenon is potentially very interesting and important. We will analyze this phenomenon in greater depth in future."

Referee response: This result is troubling. If you claim (as others did before you) that the role of Atg15 is to degrade autophagic bodies in the vacuole via its lipase activity, then how do you explain this result?! It is not compatible with what we know of Atg15 function nor of Apl5 and Vps4. At a minimum, the authors should have tested whether double deletion of APL5 and VPS4 equally suppresses other autophagy mutants, in order to see whether this suppression is specific to atg15Δ.

[Answer #7]
We appreciate the reviewer for the suggestive comment. According to the reviewer’s advice, we generated apl5Δvps4Δatg1Δ and apl5Δvps4Δatg2Δ cells. Immunoblot analysis shows that double deletion of APL5 and VPS4 does not suppress at least atg1Δ and atg2Δ deletions. This result suggests that completion of autophagosome formation is required for the suppression in apl5Δvps4Δ background cells. We have modified the text in page 9 and Supplemental Figure S3 to show this finding.
The heading "Residues S332, D387, and H435 are the putative catalytic triad" is nonsensical. Either they ARE or they ARE NOT. It is of little to no interest to the reader that they are putative. Have the authors scanned all the aspartate, serine, and histidine residues in the sequence? Have they carried out in vitro affinity labeling experiments with transition state analogs? It is mildly interesting that these residues are important for biological activity, but more stringent standards are required before they can link this with the catalytic mechanism.

We thank the reviewer for the advice about the catalytic mechanism. As the reviewer points out, we might just analyze the phenotypes of D387A and D421 mutants (Figure 7). However, our experiments clearly show that D387 is an important residue of the two aspartic acid residues described in Teter et al. as candidates for the residues consisting of the catalytic triad (Figure 3B in Teter et al., 2001). We think this result is worth reporting. We understand that structural analysis is necessary to provide a stringent proof of the catalytic mechanism, and we are now in the process of the experiments using the minimum regions (residues 50-466) identified in this study. To show that we need more stringent results to prove that these residues correspond to the catalytic triad, we have added the statement that "Structural analysis will be necessary to prove whether these residues truly consist of the catalytic triad" at the end of this section.

referee response: Yes, but despite these disclaimers here in the rebuttal, the authors still maintain their original claims in the abstract and elsewhere.

If the authors wish to publish such claims they need at a minimum 1) To cite a similar lipase with a known catalytic triad, in which the required analysis was carried out. 2) To show homology of Atg15 to the other lipase, showing the corresponding residues in Atg15 and testing their respective mutations. No amount of narrowing down the 416 AA "essential" region will be enough. You can do mutations until you are blue in the face, but unless someone does some actual biochemistry somewhere down the line, you will not know the catalytic mechanism.

[Answer #8]
“Yes, but despite these disclaimers here in the rebuttal, the authors still maintain their original claims in the abstract and elsewhere.”

We thank the reviewer for the suggestion. We have searched the words “catalytic triad” in the text but
mostly they are described as “putative catalytic triad”. We have inserted one “putative” to the text (page 10). In the abstract, we just describe “Finally, we identified H435 as one of the residues composing the putative catalytic triad”. We do not claim that we have identified the catalytic triad. Could you tell us the points that our “original claims in the abstract and elsewhere”?  

“If the authors wish to publish such claims they need at a minimum 1) To cite a similar lipase with a known catalytic triad, in which the required analysis was carried out. 2) To show homology of Atg15 to the other lipase, showing the corresponding residues in Atg15 and testing their respective mutations. No amount of narrowing down the 416 AA "essential" region will be enough. You can do mutations until you are blue in the face, but unless someone does some actual biochemistry somewhere down the line, you will not know the catalytic mechanism.”

We agree with the reviewer on this point. We have searched the similar lipase using online databases but Atg15 orthologs are found only in fungal species (Supplemental Figure S4). As the reviewer points out, extensive mutational analysis is meaningless in the absence of biochemical analysis. Currently, we do not have a method to measure the lipase activity of Atg15. As discussed in the “The substrate specificity of Atg15” of the discussion section, we think this important to understand the molecular mechanisms of degradation of autophagic bodies by Atg15. The next project we should do is to purify and crystalize Atg15.
[Question #9]
Additional specific comment:

New figure 3A is still not making sense. If Atg15 is reaching the vacuole but is not being degraded in pep4Δ cells, then we expect that the Atg15-GFP band should increase in intensity at the expense of the free GFP. Then why is the intensity of the chimera band still as weak in the pep4Δ mutant, as that observed in cells which are able to traffic Atg15 to the vacuolar lumen and release free GFP?

[Answer #9]
We agree to this comment by the reviewer #1. We did several experiments prior to submission but obtained similar results. Thus, we do not have the satisfactory answer to this question. Instead, we have already discussed this point in the “The C-terminal region of Atg15 is responsible for its enzymatic activity” section in the discussion section.
Response to the reviewer #2:

We are grateful to the reviewer #2 for reviewing our manuscript. Because the reviewer did not suggest any revisions, we have not attached any answers.
Dear Dr. Suzuki,

I have now read through your responses to the reviewer comments and your manuscript and I am happy to recommend its acceptance for publication in Molecular Biology of the Cell.

Best regards,

Howard Riezman

Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Suzuki:

Congratulations on the acceptance of your manuscript.

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