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Binding of the Atg1/ULK1 kinase to the ubiquitin-like protein Atg8 regulates autophagy

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(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.)

1st Editorial Decision 31 January 2012

Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees have now evaluated it, and their comments are shown below. As you will see, while all three referees consider the study as interesting in principle, it becomes clear that further work will be required to develop the study sufficiently to justify publication here. I will not repeat all points here, but the Atg1/Atg13 interaction and its role needs to be analysed in more depth as put forward by all three referees. Furthermore the functional significance of the Atg1/Atg8 interaction in autophagy needs to be analysed by additional approaches. On balance, and given the interest expressed by the referees in principle, we would be able to consider a revised manuscript in which you need to address the referees' criticisms - in particular those mentioned above - in an adequate manner and to their satisfaction. Please do not hesitate to contact me at any time in case you would like to discuss any aspect of the revision further.

I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance or rejection of your manuscript will therefore depend on the completeness of your responses in this revised version as well as on the final assessment by the referees.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

In this manuscript, Kraft et al investigate the interaction between key autophagy-regulating (ATG) proteins Atg1/ULK1, Atg13 and Atg8/LC3/GABARAP and their effect on the execution of the autophagy pathway. Understanding mechanisms of autophagy is important given the increasingly recognized role of autophagy in health and disease.

In the first part of the manuscript, Kraft et al demonstrate that, unlike previously thought, the interaction between the apical Ser/Thr kinase Atg1/ULK1 and its regulatory subunit Atg13 is constitutive and is not regulated by the nutrition status of the yeast. The authors pinpoint two amino acids (Phe and Val) that may be responsible for the interaction between Atg1 and Atg13 and, by mutating them, show that the tight interaction between Atg1 and Atg13 is essential for the kinase activity of Atg1. Surprisingly, this defect does not severely disturb the autophagic pathways in yeast. Given this discrepancy and the lack of thorough characterization of the Atg1/Atg13 interaction surface, the true significance of the interaction remains unclear.

The second part of the manuscript focuses on the discovery of the novel interaction between Atg1 and ATG8, the ubiquitin-like modifier covalently conjugated to the lipid specific to autophagic structures (autophagosomes). A conserved ATG8/LC3-interacting region (LIR) is identified within Atg1 and its mammalian homologue ULK1. Using LIR wild type and mutant Atg1 constructs, the authors demonstrate that yeast Atg1 is recruited to the autophagic membranes and become degraded in the vacuole. The data on mammalian ULK1 are less convincing and more work is required to claim the general role for the ATG8/Atg1 or ULK1 interactions across the phylogenetic tree.

Specific points:

1) Fig. 1A: it is not clear from the text or the figure legend how Atg1-TAP and Atg13-GFP are identified on the silver stained gel. Were the bands analyzed by MassSpec? If it was done by Western blotting shown in Fig. 1B, why rapamycin and not starvation sample is shown? Please clarify.

2) Identification of FV as the amino acids responsible for the Atg1:Atg13 interaction is based on the loss-of-function evidence (no binding in Y2H and IP). It is still formally possible that the interaction between Atg1 and Atg13 (in yeast or mammalian cells) via FV is indirect. Use of an isolated Atg13 peptide containing the FV sequence and Atg1 expressed in and purified from bacteria in binding assays can be used to demonstrate the direct link between Atg1:ATG13 via the FV sequence of Atg13.

3) Vac8 is a binding partner of Atg13. Its interaction by IP with Atg13 cannot be used to claim that Atg13-FV mutants do not affect Atg1 complex formation. Please correct the statement in the text.

4) Fig. 1D clearly shows abrogation of ATG1 kinase activity when Atg13 FV mutant is used to

reconstitute Atg13-deficient yeast cells. Since the Atg1 kinase-dead mutant T226A inhibits both Cvt and autophagy pathway (Fig. 5A), it is unclear why Cvt pathway and autophagy are only minimally inhibited by the Atg13 FV mutant in Fig. 3A and 3B. Any explanations for this disconnect?

5) On several figures (e.g. Fig. 3A/3B and 4A), Atg1 LIR mutants (EYE and VE) run on the gel slightly faster than their WT counterpart. Is that due to reduced autophosphorylation of Atg1? How does this observation relate to the data from the Atg1 kinase assay shown in Fig. 3D? Please comment.

6) Fig. 4A: Atg1 is enriched in the membrane (pellet) fraction of yeast cell lysates but the data for the VE mutant do really not look convincing. Is the second LIR mutant (EYE) to a lesser extent recruited to the membranes than the wild-type Atg1? In the floatation experiments, Atg1 was not lipid-associated. Is there a conflict between these two pieces of experimental evidence or there are technical differences in yeast extract preparations, which justify the differences in the statements?

7) Fig 4B: HA-ULK1 is claimed to be autophagosome-associated based on the colocalization with GFP-LC3 and WIPI2. Why not to use in this assay GABARAP or GATE-16, which should bind ULK1 with much higher affinity than LC3B (Fig 2E). Is there any biochemical evidence that endogenous ULK1 is membrane associated and this association is LIR-dependent? A FRET assay using fluorescently labeled ULK1 and LC3/GABARAP could be used to demonstrate the direct interaction between ULK1 and LC3/GABARAP in vivo. Finally, interaction between endogenous ULK1 and LC3/GABARAP would be more informative than that between fusion proteins.

8) Fig 5: these data very nicely demonstrate the validity of the idea that Atg1 and Atg3 (via Atg1) are indeed transported into the yeast vacuole for the degradation. Is there any evidence that mammalian ULK1/ULK2 complex also becomes depleted upon starvation or selective autophagy induction? Only such experiment would justify the generalization of the role of LIR-mediated Atg1/ULK1:Atg8/LC3/GABARAP interaction in targeting Atg1/ULK1 kinase for degradation, which might in turn regulate the strength of the autophagy response.

Referee #2

This manuscript entitled "Binding of the Atg1/ULK1 kinase to the ubiquitin-like protein Atg8 regulates autophagy" by Kraft C. et. al. describes the characterization of the regulation mechanism of Atg1. The authors first found that binding between Atg1 and Atg13 is not regulated, but constitutive, as opposed to previous reports. They identified crucial amino acids in Atg13 for binding to Atg1, and a mutant failed to activate Atg1 activity. However, the effect on autophagy is not as strong as the one of the atg13 null mutant. Then, they found a LIR motif in Atg1. The mutant shows reduced Ape1 maturation, and most remarkably, failure to be transported to the vacuole via autophagy. A similar mutation in mammalian ULK1 also affects autophagy to some degree.

In general, the data are clean and convincing. The story is potentially interesting, but this referee expects it to be more sophisticated to be published in this journal. In addition, as the data regarding Atg13 binding are contradictory to other studies, more data need to be presented.

Specific comments;

1. Additional evidence is needed regarding the change of Atg13-Atg1 binding by nutrient conditions. First, the Atg13-TAP purification data need to be presented that the authors mentioned as data not shown. Second, the authors did not provide the method for Figure 1A. Is TEV protease used to release Atg1? If the IgG-sepharose is just treated with SDS-PAGE sample buffer, is there any difference in the result? Third, in figure 1C, the authors presented the IP of HA-tagged Atg1. Is the binding to Atg13 changed in this system by nutrient availability?

2. The most prominent effect of the Atg1-VE mutant is the failure in its delivery to the vacuole. It would be an interesting possibility to test if Atg1-VE expressing cells fail to terminate autophagy after long starvation because of the presence of Atg1 in the cytoplasm. The Pho8D60 method will be better than GFP-Atg8 processing in this assay. The other effects seem so subtle that I am not certain

if there is significance.

Minors;
1. Figure 5B, Atg13 FV mutant. The Atg1-GFP signal is absent at 6 hr. Is this reproducible? Then, Atg13 binding may affect the stability of Atg1.
2. Page 4, 2nd line from the bottom. The reference, Mizushima et al, is inappropriate.

Referee #3

EMBO Kraft et al.

This is a potentially interesting paper, but it is premature. I will not highlight the positive points, but will only mention my concerns.

1. Throughout the paper the authors use Atg13-GFP or Atg13-TAP. They need to demonstrate that both of these constructs are functional. The same applies to Atg1 fusions.

2. The authors use flotation to suggest that the Atg1-Atg13 interaction does not depend on lipid. It would be helpful if they also looked at the interaction in the presence of a low percentage of Triton X-100.

3. The authors show that the mutation of Atg13-FV did not affect Atg1 complex formation because the former could still bind Vac8. This is not adequate to examine Atg1 complex formation. It is not at all clear if Vac8 is truly a part of the Atg1 complex, and its function is certainly not known in this regard. In contrast, Atg17 is a component of the complex and it is required for Atg1 kinase activity, and the same is even true for Atg11. It makes much more sense to examine the binding of these proteins. At the end of this section, on page 9, the authors suggest that Atg1 requires additional activators. Again, why not examine Atg17 at the very least?

4. The Atg1-VE mutant had no effect on autophagy based on GFP-Atg8 processing. This assay is not adequate. The authors also need to carry out a Pho8Δ60 assay. At the end of this section on page 11, the authors state that the Atg1-Atg8 interaction is "functionally important in vivo." However, they have only shown a partial block in the Cvt pathway and apparently no effect on autophagy, Atg1 kinase activity or Atg1 localization. This is why they need to extend the autophagy analysis; otherwise the interaction does not appear to be very important.

5. The authors show that Atg1 is enriched in membrane fractions along with Ape1 and conclude that it is associated with autophagosomes and Cvt vesicles. This is too much of a leap of faith. All that they can say is that it is associated with membranes, and even this conclusion would be strengthened by including a control with detergent.

6. The time course in Fig. S2C is over a period of days. It is not even clear if autophagy still occurs over this length of time. In Fig. 5A, the authors see free GFP being generated as early as 4 hours. Thus, the time course of degradation needs to be much shorter.

In conclusion, I think that the authors need to further examine the nature of the Atg1 complex, which includes Atg11, Atg17, Atg29 and Atg31. In addition, they need to expand their assays for monitoring autophagy. If the interaction mutants only have a partial defect in the Cvt pathway and no effect on autophagy, I do not think this paper will be of adequate significance to justify publication in EMBO J.

1st Revision - authors' response 13 June 2012

Point-by-point reply to the reviewer's comments:

General revisions:

1) Two reviewers asked for additional evidence and controls to substantiate the claim that the Atg1-Atg13 interaction is functionally important but not regulated by nutrient starvation. We have thus significantly worked on this aspect and now include several new experiments (revised Figure 1, and Suppl. Figures S1B,C,D). First, we demonstrate using *E. coli* expressed, purified fragments that the interaction is direct, and requires the FV-residues. Second, we have expanded our co-immunoprecipitation analysis, which revealed that the amount of the Atg1-Atg13 complex is comparable irrespective of the cell lysis, epitope tag, or if the cells were grown in nutrient-rich, rapamycin-treated or starvation conditions. We controlled that complex formation did not artificially occur in the extract after cell lysis, and excluded the possibility that the used epitope-tags did alter the function of the fusion proteins. Finally, we have used a novel methylation-based *in vivo* interaction assay, which confirmed that the Atg1-Atg13 complex is stable in different growth conditions. Together, these data strongly suggest that in response to nutrient starvation, autophagy is not triggered by regulating the interaction of Atg1 and its activator Atg13.

2) As suggested by the reviewers, we have now included additional evidence demonstrating that the Atg8-Atg1 interaction is conserved and functionally important on autophagosomes *in vivo*. First, we have extended the analysis of mammalian ULK1, and show that HA-tagged ULK1 not only binds GABARAP *in vitro*, but also co-immunoprecipitates with GFP-tagged GABARAP *in vivo* in a manner that is highly dependent on the LIR motif on ULK1. Furthermore, we show by immunofluorescence that HA-ULK1 colocalizes with GFP-GABARAP on WIPI2-positive autophagosomes (revised Figure 2F, S3B and 4C). Moreover, the number of ULK1-positive structures was significantly reduced in the LIR-mutant of ULK1, suggesting that efficient association of ULK1 to autophagosomes requires its interaction with mammalian Atg8 (Figure 4E). Interestingly, the total number of WIPI2 spots was increased after expression of the ULK1-LIR mutant (Figure 4D,E), implying that WIPI2-positive autophagosomes are stalled at an early stage during autophagy. Second, we used the well-established pho8D60 assay to further quantify the autophagy defect of the Atg1-LIR mutant in yeast. Interestingly, this assay revealed a strong autophagy defect of the single Atg1-LIR mutant (revised Figure 3), which is consistent with a defect in the maturation of autophagosomes.

Specific points raised by the referees:

Referee #1

1) *Fig. 1A: it is not clear from the text or the figure legend how Atg1-TAP and Atg13-GFP are identified on the silver stained gel. Were the bands analyzed by MassSpec? If it was done by Western blotting shown in Fig. 1B, why rapamycin and not starvation sample is shown? Please clarify.*

As described in the general points above, we have changed and expanded the analysis of the Atg1-Atg13 complex. In particular, we have replaced the original Figure 1 and included a new set of co-immunoprecipitation experiments with different tags on Atg1 and Atg13 (HA and TAP), using immunoblotting "on-bead" or after TEV elution for detection of the proteins (Figure S1D). Furthermore, we compared the Atg1-Atg13 complex in cells grown in rich, starvation and rapamycin-containing media in a single experiment. The original silver gel was moved to Supplementary Figure S1C, and we state in the revised text that the bands have been analyzed and clearly identified by mass spectrometry. Importantly, we have used a novel methylation-based *in vivo* interaction assay, which confirmed that the Atg1-Atg13 complex is stable *in vivo* under different growth conditions (new panel F).

2) *Identification of FV as the amino acids responsible for the Atg1:Atg13 interaction is based on the loss-of-function evidence (no binding in Y2H and IP). It is still formally possible that the interaction between Atg1 and Atg13 (in yeast or mammalian cells) via FV is*

indirect. Use of an isolated Atg13 peptide containing the FV sequence and Atg1 expressed in and purified from bacteria in binding assays can be used to demonstrate the direct link between Atg1:ATG13 via the FV sequence of Atg13.

As suggested by the reviewer, we purified the minimal interaction domains of Atg1 and Atg13 and performed *in vitro* binding assays, which clearly show the inability of Atg13-FV to bind Atg1 (revised Figure 1, new panel B). This new experiments demonstrate that the interaction is direct, and does not require post-translational modifications, at least within the binding sites.

3) *Vac8 is a binding partner of Atg13. Its interaction by IP with Atg13 cannot be used to claim that Atg13-FV mutants do not affect Atg1 complex formation. Please correct the statement in the text.*

We have expanded this analysis, and now analyzed several interaction partners of Atg1, including Atg11, Atg17, Atg29 and Vac8. Interestingly, whereas Atg11, 17, 29 no longer associated with Atg1 in an Atg13-FV mutant strain, Vac8 remains bound although the interaction may be reduced. We are not aware of any publication clearly showing that loss of Atg13 results in loss of Vac8 binding to Atg1. We included these new co-immunoprecipitation experiments of Atg1 with Atg17, Atg29 and Vac8 in the revised Figure 1, new panel C, and state the decrease in binding of Vac8 in the revised text: "…while the interaction with Vac8, a putative complex member, was only slightly reduced…"

4) *Fig. 1D clearly shows abrogation of ATG1 kinase activity when Atg13 FV mutant is used to reconstitute Atg13-deficient yeast cells. Since the Atg1 kinase-dead mutant T226A inhibits both Cvt and autophagy pathway (Fig. 5A), it is unclear why Cvt pathway and autophagy are only minimally inhibited by the Atg13 FV mutant in Fig. 3A and 3B. Any explanations for this disconnect?*

In a pho8D60 assay, the effect of the Atg13-FV mutant was similar to *atg13D* cells (revised Figure 1, new panel H), whereas the Atg13-FV mutant showed only a partial defect when analyzed with the GFP-Atg8 and Ape1 processing assays (revised Figure 1, panel I). This 'higher sensitivity' of the pho8D60 assay has previously been recognized and discussed in several publications, as for instance (Cheong et al, 2005). We speculate in the revised text that Atg13 may have additional roles in autophagy independent of activating Atg1, which could explain the differential effect of Atg13-FV vs. *atg13D* in the kinase assays. Specifically, we now write on page 10: "Taken together, we conclude that Atg13 binding to Atg1 is important for efficient Cvt pathway and autophagy function, however, Atg13 might have an additional role in autophagy progression that is independent of Atg1 binding." The Atg1-T226A mutant has no detectable kinase activity, whereas Atg1 isolated from *atg13-FV* and *atg13D* cells maintains some low, residual activity. We believe that this difference explains the less pronounced *in vivo* defects in the Cvt pathway.

5) *On several figures (e.g. Fig. 3A/3B and 4A), Atg1 LIR mutants (EYE and VE) run on the gel slightly faster than their WT counterpart. Is that due to reduced autophosphorylation of Atg1? How does this observation relate to the data from the Atg1 kinase assay shown in Fig. 3D? Please comment.*

As observed by the reviewer, the Atg1-EYE and VE mutant proteins migrate slightly faster on SDS-PAGE. This mobility change may indeed be due to differential phosphorylation, but unlikely involves the autophosphorylation sites, as the Atg1-T226A mutant doesn't show faster migration on SDS-PAGE. Alternatively, this mobility change might be due to the net charge caused by the E to A change, which often results in a different migration behavior on SDS-PAGE. We are currently following up on these possibilities in the course of another study.

6) *Fig. 4A: Atg1 is enriched in the membrane (pellet) fraction of yeast cell lysates but the data for the VE mutant do really not look convincing. Is the second LIR mutant (EYE) to a lesser extent recruited to the membranes than the wild-type Atg1? In the floatation experiments, Atg1 was not lipid-associated. Is there a conflict between these two pieces of experimental evidence or there are technical differences in yeast extract preparations, which justify the differences in the statements?*

As suggested by reviewers 1 and 2, we have extended the yeast fractionation experiments, demonstrating that a fraction of Atg1 was associated with membranes. As shown in the revised Figure 4 (new panel A), Triton-X100 treatment of the pellet fraction enriched for membrane vesicles and autophagosomes, released Atg1 into the supernatant. Most of Atg1 in the pellet fraction is protected from proteinase K digestion, implying that this Atg1 pool is inside intact autophagosomes (Figure S3E). However, the amount of the Atg1-LIR mutant present in this pellet fraction was reduced, suggesting that binding of Atg8 recruits Atg1 to autophagosomes.

The floatation experiments were performed after freezer-mill lysis of the yeast cells, which also disrupts organelles. These experiments exclude the possibility that during this type of extract preparation, co-immunoprecipitation of Atg1 and Atg13 is not mediated by lipids. For the membrane pelleting experiments, however, yeast cells were gently spheroplasted followed by low speed pelleting of membranes, which preserves cellular compartments such as autophagosomes and therefore also 'weaker' membrane interactions and organelle enclosed proteins. We have clarified this difference in the revised text on page 24: "Note that in contrast to spheroplasting, freezermilling disrupts organelle-enclosed particles."

7) *Fig 4B: HA-ULK1 is claimed to be autophagosome-associated based on the colocalization with GFP-LC3 and WIPI2. Why not to use in this assay GABARAP or GATE-16, which should bind ULK1 with much higher affinity than LC3B (Fig 2E). Is there any biochemical evidence that endogenous ULK1 is membrane associated and this association is LIR-dependent? A FRET assay using fluorescently labeled ULK1 and LC3/GABARAP could be used to demonstrate the direct interaction between ULK1 and LC3/GABARAP in vivo. Finally, interaction between endogenous ULK1 and LC3/GABARAP would be more informative than that between fusion proteins.*

As suggested by the reviewer, we now show co-immunoprecipitation of GABARAP with ULK1 (revised Figure 2, new panel F) as well as co-localization of GABARAP with ULK1 and WIPI2 (revised Figure 4, new panel C). Unfortunately, we failed to immunoprecipitate endogenous LC3 and GABARAP using the commercially available antibodies. However, we now demonstrate binding of GABARAP to endogenous ULK1 (new Supplementary Figure S3B). FRET-assays to probe interactions *in vivo* are certainly powerful, but not simple to develop and quantify, and thus beyond the scope of the present manuscript. Furthermore FRET-assays often require overexpression of proteins, which would not greatly advance our current understanding of these interactions *in vivo.* Interestingly, it has previously been reported that ULK1 associates with membranes (Chan et al, 2009). We now refer to this publication in the text: "It has previously been observed that ULK1 associates with membranes (Chan et al, 2009),…".

8) *Fig 5: these data very nicely demonstrate the validity of the idea that Atg1 and Atg13 (via Atg1) are indeed transported into the yeast vacuole for the degradation. Is there any evidence that mammalian ULK1/ULK2 complex also becomes depleted upon starvation or selective autophagy induction? Only such experiment would justify the generalization of the role of LIR-mediated Atg1/ULK1:Atg8/LC3/GABARAP interaction in targeting Atg1/ULK1 kinase for degradation, which might in turn regulate the strength of the autophagy response.*

It is clear from our unpublished results that ULK1 levels rapidly decrease upon starvation in mammalian cells, but we have not been able to block this decrease using either MG132 or bafilomycin A alone. This observation suggests that ULK1 degradation upon autophagy may be mediated by more than one mechanism, and thus requires additional work. We are thus at present not able to clearly address the nature and role of autophagy in ULK1 degradation in mammals.

Referee #2

1. *Additional evidence is needed regarding the change of Atg13-Atg1 binding by nutrient conditions. First, the Atg13-TAP purification data need to be presented that the authors mentioned as data not shown. Second, the authors did not provide the method*

for Figure 1A. Is TEV protease used to release Atg1? If the IgG-sepharose is just treated with SDS-PAGE sample buffer, is there any difference in the result? Third, in figure 1C, the authors presented the IP of HA-tagged Atg1. Is the binding to Atg13 changed in this system by nutrient availability?

As outlined in the general part 1, we have greatly expanded our analysis of the Atg1-Atg13 interaction and regulation. Briefly, we now analyze Atg1-TAP co-immunoprecipitation experiments using release of Atg1-TAP from the beads either by SDS-PAGE sample buffer or TEV cleavage (revised Figures 1E and S1C,D). Furthermore, we performed co-immunoprecipitation experiments with two different tags on Atg1 (TAP and HA, Figures 1D,E), as well as the TAP-tag on Atg13 (Figure 1E, right panel). Most importantly however, we now include two new experiments to strengthen that the Atg1-Atg13 interaction is nutrient-independent *in vivo*. Specifically, we applied an *in vivo* methylation assay to demonstrate that binding of Atg13 to Atg1 is not altered by growth conditions *in vivo* (M-track, (Zuzuarregui et al, 2012), revised Figure 1, new panel F). Finally, we show that the Atg1-Atg13 interaction is direct using *E. coli* purified proteins (revised Figure 1, new panel B). Together, these experiments strongly support and expand the previous finding that the interaction of Atg1 and Atg13 is not regulated by the availability of nutrients.

2. *The most prominent effect of the Atg1-VE mutant is the failure in its delivery to the vacuole. It would be an interesting possibility to test if Atg1-VE expressing cells fail to terminate autophagy after long starvation because of the presence of Atg1 in the cytoplasm. The Pho8D60 method will be better than GFP-Atg8 processing in this assay. The other effects seem so subtle that I am not certain if there is significance.*

As suggested by the reviewer and outlined in the general part 2, we performed pho8D60 autophagy assays and further analyzed autophagosomes in cells expressing the Atg1-VE mutant. Importantly, the effect of the Atg1-LIR single mutants is strong in this assay. We included these new results in the revised Figures 3B and S3C, and changed the text accordingly.

Minors;

1. *Figure 5B, Atg13 FV mutant. The Atg1-GFP signal is absent at 6 hr. Is this reproducible? Then, Atg13 binding may affect the stability of Atg1.*

We have replaced this panel by a more representative experiment (revised Figure 5B).

2. *Page 4, 2nd line from the bottom. The reference, Mizushima et al, is inappropriate.*

We have removed this reference.

Referee #3

1. *Throughout the paper the authors use Atg13-GFP or Atg13-TAP. They need to demonstrate that both of these constructs are functional. The same applies to Atg1 fusions.*

All fusion proteins used in this study are functional. As suggested by the reviewer we now include these experiments in Supplementary Figure S1E.

2. *The authors use flotation to suggest that the Atg1-Atg13 interaction does not depend on lipid. It would be helpful if they also looked at the interaction in the presence of a low percentage of Triton X-100.*

The aim of these experiments was to demonstrate that the conditions used for co-immunoprecipitation didn't cause artificial binding of Atg1 and Atg13 due to lipids. Therefore the floatation analysis was performed in the same buffer as the co-immunoprecipitation experiments. However, we addressed association of Atg1 with autophagosomes and controlled its dependence using Triton X-100 (revised Figure 4A). In these experiments, spheroplasting instead of freezer milling was used to gently lyse the cells and preserve organelles, vesicles and autophagosomes. Most importantly however, we now included two new experiments to strengthen the data that the

Atg1-Atg13 interaction is nutrient-independent. Specifically, we use an *in vivo* methylation assay to demonstrate that this interaction was not altered by the availability of nutrients *in vivo* (M-track, (Zuzuarregui et al, 2012), Figure 1F). Furthermore we show that this interaction is direct using *E. coli* purified proteins (Figure 1B).

3. *The authors show that the mutation of Atg13-FV did not affect Atg1 complex formation because the former could still bind Vac8. This is not adequate to examine Atg1 complex formation. It is not at all clear if Vac8 is truly a part of the Atg1 complex, and its function is certainly not known in this regard. In contrast, Atg17 is a component of the complex and it is required for Atg1 kinase activity, and the same is even true for Atg11. It makes much more sense to examine the binding of these proteins. At the end of this section, on page 9, the authors suggest that Atg1 requires additional activators. Again, why not examine Atg17 at the very least?*

As suggested by the reviewer, we now performed co-immunoprecipitation experiments of Atg11, Atg17, Atg29 and Vac8 with Atg1. In contrast to Vac8, all four other components specifically co-immunoprecipitate with Atg1 in the presence of wild-type but not the FV-mutant of Atg13. These data provide new insight into the structure of the Atg1 complex, which we now discuss in the revised text: "Co-immunoprecipitation experiments confirmed that Atg13-FV was unable to bind Atg1 (Figure 1C). Interestingly, its association with Atg11, Atg17 and Atg29 was also abolished, while the interaction with Vac8, a putative complex member, was only slightly reduced (Figure 1C and Figure S1B). We concluded that F468 and V469 are required for the ability of Atg13 to directly interact with Atg1 and stabilize the Atg1 kinase complex."

4. *The Atg1-VE mutant had no effect on autophagy based on GFP-Atg8 processing. This assay is not adequate. The authors also need to carry out a Pho8*D*60 assay. At the end of this section on page 11, the authors state that the Atg1-Atg8 interaction is "functionally important in vivo." However, they have only shown a partial block in the Cvt pathway and apparently no effect on autophagy, Atg1 kinase activity or Atg1 localization. This is why they need to extend the autophagy analysis; otherwise the interaction does not appear to be very important.*

As suggested by the reviewer and discussed in the general part 2, we performed the pho8D60 assay with the Atg1-LIR mutants. Using this read-out, the autophagy defect of the LIR mutants is strong, almost comparable to the kinase-inactive Atg1 mutant (Figure 3B). Together with the analysis of autophagosomes, these results suggest that Atg1 plays an important function during autophagosome maturation. We now changed the text accordingly, and discuss the difference in the autophagy assays in the revised text.

5. *The authors show that Atg1 is enriched in membrane fractions along with Ape1 and conclude that it is associated with autophagosomes and Cvt vesicles. This is too much of a leap of faith. All that they can say is that it is associated with membranes, and even this conclusion would be strengthened by including a control with detergent.*

As suggested by the reviewer, we now included a detergent control as well as a protease protection experiment in the revised Figure 4A and S3E.

6. *The time course in Fig. S2C is over a period of days. It is not even clear if autophagy still occurs over this length of time. In Fig. 5A, the authors see free GFP being generated as early as 4 hours. Thus, the time course of degradation needs to be much shorter.*

We demonstrate the Atg1 degradation by several means: First, we used immunoblotting after induction of autophagy (Figure 5A,B) and second, Atg1-GFP levels were quantified *in vivo* by fluorescent microscopy (Figure 5C and S3H). Atg1 degradation starts rapidly after autophagy induction. However, we removed the panel mentioned above to avoid confusion.

In conclusion, I think that the authors need to further examine the nature of the Atg1 complex, which includes Atg11, Atg17, Atg29 and Atg31. In addition, they need to expand their assays for monitoring autophagy. If the interaction mutants only have a partial defect in the Cvt pathway and no effect on autophagy, I do not think this paper will be of adequate

significance to justify publication in EMBO J.

As suggested by the reviewer and described above (point 3), we expanded the analysis of the Atg1 complex to include Atg11, Atg17, Atg29 and Vac8. Furthermore, we performed pho8D60 assays as requested, which shows a strong phenotype for the Atg1-LIR mutants. These data are now presented in the revised Figures 1C and S1B, and 3B. Together with the additional experiments requested by the other reviewers, these new findings significantly expand and substantiate the notion that Atg8 recruits Atg1 to autophagosomes, where it may be required to regulate autophagosome maturation.

References:

Chan EY, Longatti A, McKnight NC, Tooze SA (2009) Kinase-inactivated ULK proteins inhibit autophagy via their conserved C-terminal domains using an Atg13-independent mechanism. *Molecular and cellular biology* **29:** 157-171

Cheong H, Yorimitsu T, Reggiori F, Legakis JE, Wang CW, Klionsky DJ (2005) Atg17 regulates the magnitude of the autophagic response. *Mol Biol Cell* **16:** 3438-3453

Zuzuarregui A, Kupka T, Bhatt B, Dohnal I, Mudrak I, Friedmann C, Schuchner S, Frohner IE, Ammerer G, Ogris E (2012) M-Track: detecting short-lived protein-protein interactions in vivo. *Nat Methods* Epub

2nd Editorial Decision 06 July 2012

Thank you for sending us your revised manuscript. In the meantime, the referees have seen it again (please see below). While referees 1 and 2 are satisfied with the revision, referee 3 still raises a number of major concerns. At this point, I would thus like to ask you to address or respond to these points in a further amended version of the manuscript.

In addition, there are a number of editorial issues that need further attention:

* Please add scale bars together with an explanation to figures 3D, 5C, S2B.

* Please add the number of independent repeats into the legend of figure 4D.

* Please clarify for figures S1D (B) and S2C whether all lanes come from the same gel and include this information into the legend.

* We now generally encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide files comprising the original, uncropped and unprocessed scans of all gels used in the figures? We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

Thank you for your kind cooperation. We are looking forward to receiving your amended manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

The authors have responded to all raised issues and the manuscript is now acceptable for publication.

Referee #2

I am satisfied with the revised version.

Referee #3

1. In Fig. 1B, the authors do not indicate how much (%) of the input is shown on the gel. The amount of Atg13 pulled down is quite small, and if this is only a small percent of the total input, it would be very unsatisfactory, especially considering that this is an in vitro analysis.

2. The above is particularly an issue in light of the data shown in Fig. 1C, where the Atg13 FV mutant pulls down a considerable amount of Vac8. The authors pass this off with a minor mention, but why does the Atg13 FV mutant, which should not interact with Atg1, pull down Vac8, which should be interacting via Atg13? It appears that 30-50% of Vac8 is brought down. It is important to note that the FV mutation does not affect the interaction of Atg13 with its binding partners (in which case Vac8 might be less affected), but rather its interaction with Atg1. Thus, the (indirect) binding of Atg1 with any of these other proteins should be affected to the same extent.

3. Another major concern is seen with the migration of Atg13 in Fig. 1C and 1D. In rich conditions, Atg13 should be hyperphosphorylated, but that does not appear to be the case here. This is quite problematic in Fig. 1D where the authors examine the interaction of Atg1 with Atg13. First, they need to show the total Atg13. Second, it is possible that they are only looking at the interaction of Atg1 with dephosphorylated Atg13, and therefore missing the fact that the hyperphosphorylated form does not bind Atg1. Third, is Atg13 phosphorylation being maintained under their experimental conditions? If not, that is obviously a problem for the reasons indicated. This same concern applies to Fig. 1E.

4. For clarification, in Fig. S1E are all of these constructs transformed into the corresponding deletion strains?

5. Fig. S1G has no positive control, so it is not very useful.

6. The concern about Atg13 phosphorylation also pertains to Fig. 1F. This is a critical issue because the authors want to make the point that Atg13 binding to Atg1 is constitutive. They may be correct, but they need to clearly demonstrate that Atg13 is normally phosphorylated in "rich" conditions and that it maintains this phosphorylation in their various assays that examine interaction with Atg1.

7. On page 12, "Figure 1B" should be "Figure 3B".

8. Considering the discrepancy among the autophagy assays (i.e., the lack of a clear defect with the Atg1 mutants based on Ape1 processing or GFP-Atg8 processing), the authors should present the Pho8Δ60 data as units of activity rather than relative ALP activity. This would allow the reader to determine whether the assay was robust.

9. On page 13, the authors examine Atg1 (Atg8 binding) and Atg13 double mutants, and based on the more severe phenotype conclude that this is an indication that Atg8 and Atg13 act on Atg1 at different steps of autophagy. This is not an appropriate conclusion at this stage of the analysis. If the two mutations simply result in a stronger defect at the same part of the pathway the authors would observe the same result.

10. On page 14, the authors refer to Pep12 as a vacuolar membrane protein. I am not aware of any data that suggest Pep12 is on the vacuole membrane. It is generally considered to be an endosome marker.

11. The protease protection experiment that is hidden away in Figure S3E is not very adequate. First, the authors do not include a control for a protease-sensitive protein. Second, Atg1 migrates at two different positions. This makes me wonder if the lower position corresponds to protease-sensitive protein. Third, the loss of Atg1 (and presumably Pep12) upon the addition of detergent alone is problematic. The authors claim that this is due to the release of intracellular proteases. However, if that is the explanation then the propeptide of Ape1 should also be removed, but that does not appear to be the case.

12. As in the previous version of the paper, the authors make the claim that the appearance of Atg1 in a membrane-associated fraction means that it is recruited to autophagosomes by Atg8. I still do not understand how they can make this claim. What defines these membranes as autophagosomes?

13. Similarly, the authors examine the localization of ULK1 in mammalian cells and conclude that it is located at autophagosomes based on colocalization with GABARAP and WIPI2. However, both GABARAP and WIPI2 may be localized at earlier structures including the omegasome. Furthermore, the authors find an increase in WIPI2-positive dots in the ULK1-DFP mutant and conclude that these are accumulated autophagosomes. It seems as likely, perhaps more so, that these are not autophagosomes, but rather are autophagosome precursors. This is an important point because the authors are trying to assert that Atg1/ULK1 act at a late step of the process.

14. The authors state as data not shown that Atg1 is degraded and is stabilized in a pep4 mutant. Rather than showing just the data for Atg1-GFP they should also show the data for the endogenous protein.

15. The analysis of Atg1-Atg13 degradation in the vacuole is not sufficient. At present, this is simply a phenomenon. Is this degradation important? Is there a physiological significance?

2nd Revision - authors' response 15 July 2012

Referee #3

1. In Fig. 1B, the authors do not indicate how much (%) of the input is shown on the gel. The amount of Atg13 pulled down is quite small, and if this is only a small percent of the total input, it would be very unsatisfactory, especially considering that this is an in vitro analysis.

The input is 5.5% of the total Atg13 used in the binding assay. This *in vitro* binding assay is performed under saturating binding conditions, i.e. an excess of Atg13 is used. Quantification of the coomassie gel results in a 10:1 ratio of Atg1 to Atg13. Taking into account that coomassie staining depends on the size of the protein, the data imply an almost 1:1 complex of the purified proteins (the GST-Atg1 fragment is approx. 70kDa, and the Atg13 fragment 9.5 kDa). We now state that the binding assays were performed with an excess of the Atg13 fragment, indicate the percent of input and explain the binding ratios in the Figure legend and the corresponding Material and Method section:
"The ratio of Atg13 bound to GST-Atg1 was quantified using ImageJ software and normalized by the size of the respective proteins. The ratio quantified from the coomassie stained gel of Atg13 to GST-Atg1 was 0.11, whereas the size ratio is 0.13. Therefore the binding ratio is roughly 1:1."

2. The above is particularly an issue in light of the data shown in Fig. 1C, where the Atg13 FV mutant pulls down a considerable amount of Vac8. The authors pass this off with a minor mention, but why does the Atg13 FV mutant, which should not interact with Atg1, pull down Vac8, which should be interacting via Atg13? It appears that 30-50% of Vac8 is brought down. It is important to note that the FV mutation does not affect the interaction of Atg13 with its binding partners (in which case Vac8 might be less affected), but rather its interaction with Atg1. Thus, the (indirect) binding of Atg1 with any of these other proteins should be affected to the same extent.

We are not aware of any publication clearly showing that loss of Atg13 also results in loss of Vac8 binding to Atg1. However, Scott *et al.,* 2000 showed that the interaction of Vac8 with Atg13 by two-hybrid analysis is dependent on Atg1. In our experiments, Vac8 binding to the Atg1-Atg13 complex is specific, and no significant background binding is detected under the used conditions. However, while binding of Atg17 and Atg29 to Atg1 was abolished in *atg13-FV* cells, the interaction with Vac8 was reduced by approximately 50% (Figure 1C). Based on these results, it is likely that Vac8 possesses binding sites for both Atg1 and Atg13, and therefore a significant fraction is able to bind Atg1 even in the absence of Atg13.

3. *Another major concern is seen with the migration of Atg13 in Fig. 1C and 1D. In rich conditions, Atg13 should be hyperphosphorylated, but that does not appear to be the case here. This is quite problematic in Fig. 1D where the authors examine the interaction of Atg1 with Atg13. First, they need to show the total Atg13. Second, it is possible that they are only looking at the interaction of Atg1 with dephosphorylated Atg13, and therefore missing the fact that the hyperphosphorylated form does not bind Atg1. Third, is Atg13 phosphorylation being maintained under their experimental conditions? If not, that is obviously a problem for the reasons indicated. This same concern applies to Fig. 1E.*

We disagree with the reviewer and would like to point out that Atg13 in Figure 1D, lane 1, ("rich"), shows a characteristic mobility shift, indicating that phosphorylated Atg13 can bind Atg1. We now explicitly mention this phosphorylation-shift of Atg13 bound to HA-Atg1 under rich conditions in the final Figure legend. The extent of the retarded migration is similar when analyzing endogenous, untagged Atg13 in lysates prior to immunoprecipitation, using the described method for freezer-milling lysate preparation and gel electrophoresis and buffer system. Importantly, our IP buffer contains a cocktail of several potent phosphatase inhibitors, and we have indeed verified several of the published (and unpublished) phosphorylation sites by mass spectrometry after immunoprecipitating TAP-tagged Atg13. Some of these sites have been claimed to be exclusively phosphorylated under rich conditions (Kamada *et al*., 2010).
Moreover, the kinase activity of immunopurified Atg1 towards *in vitro* substrates remains high using the same cell lysis/extract procedure, but is rapidly reverted by prior lambda-phosphatase treatment (Atg1 requires an activating phosphorylation on T226, see Kijanska *et al*. 2010 and Yeh *et al*. 2010). Together, these results make it unlikely that phosphatases released by cell lysis dephosphorylate Atg13 or Atg1 during the immunoprecipitation incubations.

In contrast to untagged Atg13, we were never able to detect a significant mobility shift of TAP-, GFP- or Suv-tagged Atg1 and Atg13 in cell extracts or TCA-precipitated samples, and now explicitly mention this fact in the corresponding Figure legend. We can't exclude that a fraction of hyperphosphorylated Atg13 remains resistant to Atg1 binding. Most importantly however, our data demonstrate that the amount of Atg13 bound to Atg1 doesn't change when comparing cells grown in rich and starvation conditions. This is most clearly exemplified in the methylation assays (Figure 1D), which monitors the interaction of Atg1 and Atg13 *in vivo*, and excludes the possibility that dephosphorylation of Atg13 in the extracts during immunoprecipitation obscures the regulation. Please note, that we do not claim that starvation-induced changes in Atg13 phosphorylation is not important to regulate autophagy, but we consider it very unlikely based on the presented results that Atg13 phosphorylation alters binding of Atg1 and Atg13.

4. *For clarification, in Fig. S1E are all of these constructs transformed into the corresponding deletion strains?*

All these strains are endogenously tagged as shown in the strain list. Endogenous tagging and expression of the relevant proteins from CEN-plasmids in the corresponding deletion strains showed the same result. We now explain this fact in the final Figure legends.

5. *Fig. S1G has no positive control, so it is not very useful.*

As a positive control we used the well-established flotation of caveolin1 from HeLa cells. While we agree with the reviewer that a protein from yeast which was already characterized by flotation gradients would be better, we were not aware of any protein matching the experimental parameters at the time of experimentation. Thus, we consider caveolin as a 'technical control' for the sucrose density gradients, and have now adjusted the legend of Suppl. Figure S1G.

6. *The concern about Atg13 phosphorylation also pertains to Fig. 1F. This is a critical issue because the authors want to make the point that Atg13 binding to Atg1 is constitutive. They may be correct, but they need to clearly demonstrate that Atg13 is normally phosphorylated in "rich" conditions and that it maintains this phosphorylation in their various assays that examine interaction with Atg1.*

Please also see the response to point 3. Both Suv- and H3-tagged Atg1 and Atg13 are fully functional, as judged by their ability to rescue the Ape1 processing defect observed in the corresponding deletions. In addition, in the methylation-interaction assay, the cells are lysed in TCA, and therefore all phosphorylations should be maintained. So far, we could never detect phosphorylation-induced shifts of tagged Atg1 or Atg13 in our gel and buffer system. We now explicitly mention this fact in the legend of Figure 1.

7. *On page 12, "Figure 1B" should be "Figure 3B".*

We thank the reviewer for spotting this mistake and have changed this cross-reference accordingly.

8. *Considering the discrepancy among the autophagy assays (i.e., the lack of a clear defect with the Atg1 mutants based on Ape1 processing or GFP-Atg8 processing), the authors should present the Pho8Δ60 data as units of activity rather than relative ALP activity. This would allow the reader to determine whether the assay was robust.*

A similar “discrepancy” between the GFP-Atg8 cleavage assay and the pho8D60 assay has been reported in several publications, e.g. *atg17D* (Cheong et al, 2005) and *tlg2D* (Nair et al., Cell 2011). We have carefully quantified and controlled our pho8D60 assays, and as deduced from the error bars, the experimental variations are minimal and the assays thus very robust and reproducible. Absolute values differ between publications, and our ALP activity values are comparable to the ones published in the publications by the Klionsky laboratory (the ALP activity amounts to approx. 90 units in wild-type, compared to 10 units in *atg1D* cells (-> 9x induction)). To be sure that the ALP measurements are performed in the linear range of the enzyme, we have used different sample dilutions. To inform readers about this control, we added the following sentence in the final Materials and Methods section: “To ensure the linear range of the enzymatic reaction, the assay was performed with two different sample concentrations”. In most publications, the pho8D60 data are shown as relative values, which allows for a better comparison between different assays. However, as requested by the reviewer, we now also show the absolute values in the new Supplementary Figure S2C and S3C.

9. *On page 13, the authors examine Atg1 (Atg8 binding) and Atg13 double mutants, and based on the more severe phenotype conclude that this is an indication that Atg8 and Atg13 act on Atg1 at different steps of autophagy. This is not an appropriate conclusion at this stage of the analysis. If the two mutations simply result in a stronger defect at the same part of the pathway the authors would observe the same result.*

We now adjusted the text, and changed 'most likely' to 'possibly': "Together, these data demonstrate that Atg8 and Atg13 mutually act on Atg1 to regulate its function *in vivo*, **possibly** at different steps of the autophagy pathway."

10. *On page 14, the authors refer to Pep12 as a vacuolar membrane protein. I am not aware of any*

data that suggest Pep12 is on the vacuole membrane. It is generally considered to be an endosome marker.

We thank the reviewer for pointing out this discrepancy and changed the text as suggested to 'endosomal membrane'.

11. *The protease protection experiment that is hidden away in Figure S3E is not very adequate. First, the authors do not include a control for a protease-sensitive protein. Second, Atg1 migrates at two different positions. This makes me wonder if the lower position corresponds to protease-sensitive protein.*

Both Atg1 and Pep12 migrate slightly faster in all pellet fractions, including the pellet fractions that were neither treated with proteinase K nor TX100. Therefore it is highly unlikely that the mobility shift comes from protease cleavage; rather the mobility shift is due to different composition of the samples. It is commonly observed that a protein shows a different mobility in a complex mixture such as a total extract compared to an immunoprecipitation sample.

Third, the loss of Atg1 (and presumably Pep12) upon the addition of detergent alone is problematic. The authors claim that this is due to the release of intracellular proteases. However, if that is the explanation then the propeptide of Ape1 should also be removed, but that does not appear to be the case.

We have performed similar experiments in a *pep4D* background, which lack the activity of the major vacuolar proteases. Under these conditions, Atg1 is stable in the TX100 sample, strongly suggesting that its degradation in the *ypt7D* extracts is indeed due to the release of vacuolar proteases. It is possible that Ape1 oligomers are less susceptible to the released proteases compared to Atg1.

12. *As in the previous version of the paper, the authors make the claim that the appearance of Atg1 in a membrane-associated fraction means that it is recruited to autophagosomes by Atg8. I still do not understand how they can make this claim. What defines these membranes as autophagosomes?*

We now rephrased this in the final manuscript and state the observation more carefully: "Membrane-associated Atg1 was resistant to proteolytic cleavage by proteinase K, indicating that part of Atg1 **may be** protected in completed autophagosomes (Figure S3E). Importantly, membrane-association of Atg1 depended on a functional LIR motif, as the Atg1-VE mutant showed a decreased fractionation with membranes (Figure 4B). Together, this biochemical analysis suggests that binding of Atg1 to Atg8 recruits Atg1 to **membranes, which most likely represent** autophagosomes."

13. *Similarly, the authors examine the localization of ULK1 in mammalian cells and conclude that it is located at autophagosomes based on colocalization with GABARAP and WIPI2. However, both GABARAP and WIPI2 may be localized at earlier structures including the omegasome. Furthermore, the authors find an increase in WIPI2-positive dots in the ULK1-DFP mutant and conclude that these are accumulated autophagosomes. It seems as likely, perhaps more so, that these are not autophagosomes, but rather are autophagosome precursors. This is an important point because the authors are trying to assert that Atg1/ULK1 act at a late step of the process.*

We have altered the text to account for the possibility that the structures we observe and quantify could be a mixture of early structures (including phagophores and omegasomes) and autophagosomes.

14. *The authors state as data not shown that Atg1 is degraded and is stabilized in a pep4 mutant. Rather than showing just the data for Atg1-GFP they should also show the data for the endogenous protein.*

The Atg1-GFP as well as the YFP-Atg1 strains are fully functional, as judged by their ability to rescue the Ape1 processing defect observed in the corresponding deletions. We have analyzed the degradation with the tagged proteins to compare vacuolar degradation of Atg1 by immunoblotting and microscopy.

15. *The analysis of Atg1-Atg13 degradation in the vacuole is not sufficient. At present, this is simply a phenomenon. Is this degradation important? Is there a physiological significance?*

The data clearly demonstrate that a fraction of Atg1 and Atg13 is degraded in the vacuole in an Atg8-dependent manner. This is consistent with recent findings in *A. thaliana*, suggesting that the Atg1-Atg13 complex localizes to autophagosomes and is targeted to the plant vacuole (Suttangkakul *et al*., 2011). Moreover, we show that Atg8-mediated membrane recruitment of Atg1 is important for its function in Cvt and autophagy. We agree with the reviewer that the physiological significance of vacuolar Atg1 degradation is not clear at present. This is not easy however to address experimentally, as Atg8-binding to Atg1 on the one hand promotes but on the other hand also restricts autophagy. Unlike autophagy mutants, *atg1-LIR* strains survive when exposed to starvation conditions, but this result is tricky to interpret as the two opposing functions may balance each other. Thus, specific Atg1-alleles that can separate the early functions and its vacuolar degradation will be necessary to examine whether Atg1 degradation indeed restricts autophagic flux *in vivo*. Nevertheless, in our opinion, it remains an attractive and plausible hypothesis that is certainly worth discussing as such in the manuscript.

