EMBO *reports*

# The deubiquitinating enzyme USP20 stabilizes ULK1 and promotes autophagy initiation

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**Review timeline:**

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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.)

1st Editorial Decision 24 May 2017

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, while all referees in principle agree on the potential interest of your findings, they also raise a number of - often overlapping - issues that would need to be addressed before publication. In particular, referee 1 and 2 indicate that better immunofluorescence and co-localization data are required and that a quantitative assessment of the data should be provided. Moreover, referee 1 and 2 agree that the autophagy assays should be extended and the lysosomal degradation pathway better characterized. Also the rescue experiments should be extended along the lines suggested by referee 1 and 3. Also point 9 of referee 2 should be addressed in the revision, i.e., is polyubiquitinated ULK1 indeed degraded? Also further data on the impact of ubiquitination on degradation can be provided.

However, upon further discussion with the referees we think that it is beyond the scope of the current study to address the effect of USP20 on mTOR or AMPK signaling (ref #2, point 1) and to investigate the importance of polyubiquitin-binding proteins on ULK1 degradation (ref #2, point 8).

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete

 

point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Regarding data quantification, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information is currently incomplete and must be provided in the figure legends. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

**********
REFEREE REPORTS

Referee #1:

In the manuscript by Kim et al., the authors uncover a direct interaction between the DUB USP20 and ULK1. They show that USP20 regulates ULK1 stability and hence autophagy. Intriguingly, the authors propose that loss of USP20 drives lysosomal, rather than proteasomal, degradation of ULK1. Finally, the authors show that loss of USP20 increases apoptosis under prolonged starvation conditions.
I feel this is a good body of work and presents convincing evidence for a role of USP20 in ULK1

regulation. I think the manuscript will be of interest to wide readership, including those interested in targeting autophagy therapeutically. I am therefore in favour of publishing this article, providing the points below can be addressed.

Main points:
1) The actual autophagy assays presented in Figure 3 are not yet convincing. In panel 3A, the authors should include a flux-style image with bafilomycin to show that autophagosome induction is blocked rather than their turnover being increased. These data should also be quantified. The authors do try address this in panel 3B, but the changes are very small, likely due to the fact that autophagic turnover of p62 can be very variable. For this panel, the authors should also blot their lysates for LC3, as I'm sure this will show a greater flux than p62, and hence be more convincing.
2) Related to this, the IF images of ULK1 and USP20 are inconclusive (Fig2D). Both just show a general diffuse staining pattern. Upon autophagy induction, ULK1 localises to phagophores and these puncta are easily visible by IF. Do the authors see co-localisation of USP20 to these induced ULK1 puncta (and can they quantify this). These data perhaps should be in Figure 3?
3) The authors show that loss of ULK1 upon siUSP20 can be rescued by exogenous WT USP20, but not catalytically dead. These are very nice experiments, but I think it is key to show that WT, but not dead, USP20 also rescues the autophagy defect.
4) The lysosomal turnover of ULK1 upon loss of USP20 is unexpected, given that multiple publications have suggested that ULK1 is not degraded in the lysosome. If the authors' arguments are true (small molecules will have off-target effects), then ULK1 should be detected in lysosomes. Therefore, upon loss of USP20 do the authors see co-localisation of ULK1 with lysosomal markers (such as LAMP1) when lysosome function is inhibited?
5) In Figure 5, the authors should quantify the decrease in ULK1 binding to USP20 with respect to total ULK1. The TCL ULK1 is overexposed in the blots, but it is decreasing nonetheless.
6) As above, the IF image is not clear in Fig 5D. The authors should quantify ULK1 puncta co-localisation if possible.
7) In Figure 6D, what is the level of ULK1 overexpression with respect to endogenous ULK1? It is well known that overexpression of ULK1 can block autophagy (see Chan et al., 2007 JBC), do the authors know that autophagy induction is fine here? With respect to this, the authors may wish to discuss recent work that shows ULK1 can induce apoptosis independently of autophagy (Joshi et al., 2016 CDD).

Minor point:
On page 7, relating to Fig 2E, the authors state "immunoprecipitation assay using antibody against endogenous ULK1 showed the endogenous interactions between USP20 and ULK1 in vivo". I do not think demonstrating an interaction in cell lystates constitutes in vivo evidence. the "in vivo" should be removed.

Referee #2:

In this manuscript, the authors performed screening for ULK1-specific deubiquitinase (DUB) and identified USP20 as the DUB of ULK1. They found that the knockdown of USP20 decreased ULK1 expression and that USP20 directly interacts with ULK1, suggesting that USP20 is required for ULK1 stability. They further showed that the knockdown of USP20 inhibits autophagy induction and decreases cellular survival under starvation. Although ULK1 is polyubiquitinated, the degradation of ULK1 is via the lysosomal-dependent process and not via proteasomes. From these findings, the authors concluded that USP20 stabilizes ULK1 by forming the ULK1-USP20 complex and has a role in the initiation of autophagy.
Although the manuscript was well written and most experiments were well performed, the following points are missing: the role of ubiquitination of ULK1, degradation of ULK1 through the lysosomal pathway, and regulation of the ULK1-USP20 interaction.
The authors should consider the following:

1. It is known that ULK1 interacts with mTOR and AMPK. Does USP20 affect the stability of mTOR or AMPK?

2. Figure 2B: When autophagy is inhibited in USP20 siRNA cells, the amount of p62 in these cells should increase compared with that in control siRNA cells before chloroquine treatment. The

 

expression of p62 may be affected by USP20.

3. Figure 3: To observe autophagy, further experiments such as the degradation of p62 and the amount and ratio of LC3-I and LC3-II during starvation should be performed.

4. It is important to consider that the deubiquitination of ULK1 by USP20 has a role in ULK1 stability and autophagy.

5. The authors mentioned that ULK1 is degraded through the lysosomal-dependent pathway. However, a description of this pathway is missing. Is this autophagy or something novel process?

6. Page 9: The authors stated that USP20 prevents lysosome-mediated ULK1 degradation through the deubiquitination of ULK1. However, they should clarify whether there is any evidence to support that the ubiquitination of ULK1 is required for the degradation of ULK1.

7. The authors should clarify whether the ubiquitination of ULK1 increases when USP20 dissociates from ULK1 during starvation.

8. The authors should consider that if the degradation of ULK1 depends on the polyubiquitination of ULK1, proteins containing the ubiquitin-binding domain, including p62, OPT, NDP52, TAX1BP1, and NBR1, may be related to degradation.

9. When USP20 was knocked down or dissociated from ULK1 during starvation, ULK1 was polyubiquitinated and the molecular weight of ULK1 increases. Thus, the reduction of ULK1 band near 140kDa may be due to the polyubiquitination of ULK1 but not by degradation.

Referee #3:

This is a very interesting manuscript suggesting a role for the deubiquitylase USP20 in the stabilization of ULK1, a serine-threonine kinase with a key function in the initiation of autophagy. The authors show that depletion of USP20 reduces the levels of ULK1 and interferes with autophagosome formation. USP20 is shown to bind directly to ULK1 and this association is disrupted upon prolonged autophagy induction and may be linked to autophagic cell death.

Overall the data are clear and well presented, with the exception of some immunofluorescence experiments for which the figures (2D and 5D) are very fuzzy and of very low quality. Since these really do not add to the story I would suggest removing them from the manuscript.

A second general point concerns the lack of quantitative assessments of the significance of some of the data - eg Figure 1C, Figure 4C, Figure 4D, Figure 5C. In those instances where a quantitation is shown, it should be clarified whether n=3 refers to technical replicates or independent experiments. Finally, for statistical analysis of an experiment with more than two conditions a t-test is inappropriate.

I have a series of specific concerns regarding the execution and interpretation of some experiments which I am listing below.

1. Figure 1D and 1F: Given that the basal levels of ULK1 are much reduced in the USP20 knockdown cells, and western blotting is a very non-linear detection approach, in the knockdown cells ULK1 levels drop rapidly below detection.
It is therefore very important to rerun these gels and load more protein for the lysates from the USP20 knockdown cells in order to compare similar starting signals that allow analysis of the relative turnover rate.

2. Figure 2C and D - the text states that these figures demonstrate that USP20 binds directly to ULK1. This is incorrect unless Myc-ULK1 used in 2C is recombinant and purified from bacteria or elsewhere. Figure 2D shows a very fuzzy immunofluorescence image that does not inform on the colocalization of the two proteins - they both are excluded from the nucleus but subcellular structures cannot be made out. Even if the image was clearer this would not inform on a physical

 

interaction. With regards to the imaging, it should be noted that USP20 has been localized to the endoplasmic reticulum which is of course interesting in the context of autophagy induction. I would also have expected some discussion of previously identified substrates of USP20.

3. Figure 4C is missing a control, namely the mock (IgG) IP from cells in which USP20 is depleted. The Ub-HRP TCL blot is very overexposed so it is impossible to see whether the levels of Ub in the three lanes differ. Have the cells in Figure 4C also been treated with proteasome inhibitor to stabilize the ubiquitylated proteins?

4. Figure 4E - I did not quite understand the point of this figure - as the effect of overexpression has already been shown in Figure 1G. More interesting would be to assess the ability of the CI mutant to rescue the knockdown phenotype (ie ULK1 levels) to complement Figure 1C. This should ideally be quantified over several experiments.

5. Figure 5A - the loss of ULK1 in starved cells is not surprising - presumably it simply gets degraded as a component of the autophagosome. The authors should test whether the starvation induced loss of ULK1 is dependent on autophagy by knocking down an autophagy gene required for a later step ie ATG5 or others.

6. Figure 5B and C - the dissociation of USP20 from ULK1 is again interesting but may need to be interpreted in view of the membrane compartments (ER and phagophore respectively) that the proteins are associated with as autophagy progresses. Rigourous analysis of this would require higher resolution confocal microscopy.

7. Figure 5D - these images are very poor - and I am really unsure as to what I am meant to see here.

8. Figures 5C-E: Given that USP20 depletion results in a decrease in ULK1 levels and ULK1 is important for cell survival, it is not surprising that depleting USP20 may accelerate the onset of apoptosis upon prolonged autophagy. Restoring cell viability by re-expressing ULK1 is a good way to demonstrate that the effect on cell survival is due to the stabilizing effect of USP20 on ULK1. However the results of the FACS experiments in Figure E are marginal and compare two different cell lines. These data need to be quantified over several experiments to determine whether the differences seen are significant. In addition it would be more convincing to see that re-expression of catalytically active ULK1 (but not a catalytically inactive mutant of ULK1) in USP20 siRNA treated cells can rescue the cells.

9. There are quite a lot of typographic errors in the manuscript, the first one is in the title, followed by the abstract (pucta). These as well as stylistic errors (see Intro: "a number of evidence..."; page 13: treatment with - not of - lysosomal inhibitors; page 12 - these results were similar to - not with) should be corrected.

1st Revision - authors' response 25 October 2017

***Point-by-point responses to reviewers' comments***

**Referee #1:**

**Main points:**

Q1) The actual autophagy assays presented in Figure 3 are not yet convincing. In panel 3A, the authors should include a flux-style image with bafilomycin to show that autophagosome induction is blocked rather than their turnover being increased. These data should also be quantified. The authors do try address this in panel 3B, but the changes are very small, likely due to the fact that autophagic turnover of p62 can be very variable. For this panel, the authors should also blot their lysates for LC3, as I'm sure this will show a greater flux than p62, and hence be more convincing.

**Answer**: We agree with the reviewer's concern. According to the reviewer's comment, we performed a flux-style analysis and immunoblot assays again in control and USP20-depleted HeLa cells. The flux-style analysis was performed with the GFP-RFP-LC3 expression system. As shown

 

in Fig. 3A, USP20 depletion impaired induction of LC3 puncta emitting green and red fluorescence. This result indicated that USP20 is required for induction of autophagy at the initiation step, not by blocking LC3 turnover at later steps. These results were described and quantitated as Fig. 3A of the revised manuscript and the previous Fig. 3A was deleted.
To further support these results, we examined the LC3II to LC3I ratio in USP20-depleted cells. Immunoblot analysis supported our immunofluorescence results. The LC3II to LC3I ratio was significantly decreased in USP20-depleted HeLa cells under starvation with HBSS, compared to the control cells. These results were included and quantitated in Fig. 3B-F of the revised manuscript. In contrast, p62 protein which has been known as an adaptor of autophagy was significantly increased in control HeLa cells in the presence of chloroquine (CQ) and p62 expression was decreased in control cells under nutrient starvation (Fig. EV2 in the revised manuscript). However, p62 expression at the basal state was slightly decreased in USP20-depleted cells, which were not treated with CQ. In addition, the decreased expression of p62, which is a typical process shown in starvation-induced autophagy, was not augmented in USP20-depleted cells under starvation (Fig. EV2 in the revised manuscript). Although we do not know the exact mechanism about these observations yet, it is possible that p62 is another target of USP20 protein regarding autophagy. Therefore, we do not exclude the possibility that p62 expression might be directly or indirectly regulated by USP20-mediated autophagy process. These findings were described in Fig. EV2 and the Results and Discussion of the revised manuscript.

2) Related to this, the IF images of ULK1 and USP20 are inconclusive (Fig2D). Both just show a general diffuse staining pattern. Upon autophagy induction, ULK1 localises to phagophores and these puncta are easily visible by IF. Do the authors see co-localisation of USP20 to these induced ULK1 puncta (and can they quantify this). These data perhaps should be in Figure 3?

**Answer**: We agree with the reviewer's comments. We attempted to examine the colocalization of HA-tagged USP20 and endogenous ULK1 by immunofluorescence in HeLa cells which were transfected with HA-USP20 plasmid again, but we did not obtain relevant images because they were observed as diffuse forms at a basal state. Therefore, we deleted the previous Fig. 2D. Nevertheless, our immunoprecipitation analysis clearly showed the interaction between USP20 and ULK1 (Fig. 2A-D in the revised manuscript). In addition, we investigated the localization of ULK1 into lysosomes. Because our results in this study indicated that the dissociation of USP2-ULK1 complex initiates at 4 h post-starvation and ULK1 is subject to degradation through the lysosome pathway (Fig. 4F, Fig. 5A and Fig. 5C-D in the revised manuscript), we examined the localization of ULK1 into lysosomes in control and USP20-depleted HeLa cells. mCherry-ULK1 was significantly localized to lysosomes in USP20-depleted cells (Fig. 6C in the revised manuscript). Also, live cell images regarding the localization of wild-type mCherry-ULK1-WT and mCherry-ULK1-9KR mutant indicated that ULK1 localization into lysosomes during nutrient starvation requires ubiquitination of the carboxy terminal domain of ULK1 (Fig. 6D in the revised manuscript). These results were included in Fig. 6C and D and described in the Results in the revised manuscript.

3) The authors show that loss of ULK1 upon siUSP20 can be rescued by exogenous WT USP20, but not catalytically dead. These are very nice experiments, but I think it is key to show that WT, but not dead, USP20 also rescues the autophagy defect.

**Answer**: To examine whether exogenous wild-type USP20 can rescue the autophagy defect, we initially generated USP20-depleted HeLa cells by USP20-specific siRNA and subsequently transfected siUSP20-resistant wild-type USP20 plasmid or catalytically-inactive (CI) USP20 mutant plasmid into USP20-depleted HeLa cells. After starvation with HBSS, we examined the LC3II to LC3I ratio by immunoblot analysis. The ratio of LCII to LC3I was significantly increased upon starvation in the control HeLa cells, which were transfected with the control siRNA (siCON), whereas this ratio was decreased in USP20-depleted cells expressing empty vector (Mock). However, ectopic expression of wild-type USP20 in USP20-depleted cells showed an increased ratio of LC3II to LC3I under starvation again, whereas expression of catalytically-inactive USP20 did not. These results suggest that wild-type USP20 rescues the autophagy defect of USP20-depleted HeLa cells under starvation. These new results, including quantitation, were included in Fig. 4E and described in the Results of the revised manuscript.

4) The lysosomal turnover of ULK1 upon loss of USP20 is unexpected, given that multiple publications have suggested that ULK1 is not degraded in the lysosome. If the authors' arguments

 

are true (small molecules will have off-target effects), then ULK1 should be detected in lysosomes. Therefore, upon loss of USP20 do the authors see co-localisation of ULK1 with lysosomal markers (such as LAMP1) when lysosome function is inhibited?

**Answer**: As pointed out by the reviewer, ULK1 was detected in lysosomes. To visualize ULK1 easily in lysosomes, we utilized mCherry-ULK1 rather than GFP-ULK1 in combination with lysotracker, a functionally relevant lysosomal marker. In control HeLa cells, mCherry-ULK1 was localized to the cytosol with a diffuse staining pattern. Upon USP20 depletion, however, cytoplasmic localization of mCherry-ULK1 disappeared and mCherry-ULK1 significantly colocalized with lysotracker. These new results, including quantitation, were included in Fig 6C and described in the Results in the revised manuscript.

5) In Figure 5, the authors should quantify the decrease in ULK1 binding to USP20 with respect to total ULK1. The TCL ULK1 is overexposed in the blots, but it is decreasing nonetheless.

**Answer**: To clearly address the decreased binding of ULK1 to USP20 in the presence of rapamycin, we repeated the immunoprecipitation and immunoblot analysis. After HeLa cells were co-transfected with Flag-USP20 and HA-ULK1, cells were subsequently treated with rapamycin during the indicated time. USP20 bound to ULK1 in a steady-state in the absence of rapamycin and this binding of ULK1 with USP20 gradually decreased upon rapamycin treatment and nearly disappeared at 8 h post-treatment. In addition, USP20 binding with ULK1 disappeared at 8 h post-nutrient starvation. The binding kinetics was also quantitated by Image J program. These new results, including quantitation, were included in Fig. 5C and D and described in the Results of the revised manuscript.

6) As above, the IF image is not clear in Fig 5D. The authors should quantify ULK1 puncta co-localisation if possible.

**Answer**: We agree with the reviewer's comment. We deleted Fig. 5D of the original manuscript. We tried to obtain a higher resolution image regarding the dissociation of USP20 from ULK1 at 8 h post-nutrient starvation. Unfortunately, because considerable amounts of cells were being progressed into cell death at 8 h post-starvation, we could not obtain relevant confocal images regarding the dissociation of USP20 from ULK1. In spite of the failure of immunofluorescence imaging, the immunoprecipitation/immunoblot analysis, showing interaction of two proteins (Fig. 5D) and the polyubiquitination of ULK1 (Fig 5E) at 8 h post-starvation robustly suggests that USP20 is dissociated from ULK1 at 8 h post-starvation. Furthermore, the localization of wild-type mCherry-ULK1 and ubiquitination-defective mCherry-ULK1-9KR mutant under starvation supported the lysosome-dependent degradation of ULK1 together with ULK1 dissociation from USP20 (Fig. 6C and D in the revised manuscript). These new results were described in the Results of the revised manuscript.

7) In Figure 6D, what is the level of ULK1 overexpression with respect to endogenous ULK1? It is well known that overexpression of ULK1 can block autophagy (see Chan et al., 2007 JBC), do the authors know that autophagy induction is fine here? With respect to this, the authors may wish to discuss recent work that shows ULK1 can induce apoptosis independently of autophagy (Joshi et al., 2016 CDD).

**Answer**: We performed immunoblot analysis using anti-Flag and anti-ULK1 antibodies to examine the overexpression levels of ULK1 with respect to endogenous ULK1. Expression levels of exogenous wild-type Flag-ULK1 and related ULK1 mutants in stable cell lines, including quantitation, were described in Fig. 7D and E of the revised manuscript.
To answer the second question of the reviewer, we examined LC3II to LC3I ratio in ULK1-overexpressing HeLa cells under starvation. Unlike the previous result reported by Chan et al, wild-type ULK1 or catalytically active (CA) ULK1 overexpression did not inhibit the increased ratio of LC3II to LC3I, suggesting that ULK1 overexpression dose not block autophagy in our system (Fig. EV3 in the revised manuscript). The reason seems to be due to the different amounts of overexpressed ULK1 protein used in the two different experiments. In addition, a role of ULK1, which was suggested in this study, seems to be different with the results reported by Joshi et al. Joshi and colleagues suggested a role of ULK1 in cell death induced by oxidative stress whereas our

 

results demonstrate a role of ULK1-USP20 axis in the early step of autophagy induction under nutrient starvation. This explanation was added in the Discussion of the revised manuscript.

**Minor point:**
On page 7, relating to Fig 2E, the authors state "immunoprecipitation assay using antibody against endogenous ULK1 showed the endogenous interactions between USP20 and ULK1 in vivo". I do not think demonstrating an interaction in cell lystates constitutes in vivo evidence. the "in vivo" should be removed.

**Answer**: We agree with the reviewer's comment. We deleted the "in vivo" in the revised manuscript.

**Referee #2:**

1. It is known that ULK1 interacts with mTOR and AMPK. Does USP20 affect the stability of mTOR or AMPK?

**Answer**: Although it is an important question, it is beyond the scope of our current study, as the editor has mentioned.

2. Figure 2B: When autophagy is inhibited in USP20 siRNA cells, the amount of p62 in these cells should increase compared with that in control siRNA cells before chloroquine treatment. The expression of p62 may be affected by USP20.

**Answer**: We think that this comment is related to Fig. 3B of the original manuscript, and not Fig. 2B. To clearly show the expression of p62 regarding the autophagy, we re-examined p62 expression in the absence or presence of chloroquine (CQ). After HeLa cells were reverse-transfected with two independent USP20-specific or control (siCON) siRNAs and subsequently treated with chloroquine (CQ), we analyzed p62 expression by immunoblot analysis. p62 expression was significantly increased upon CQ treatment in control HeLa cells (Fig. EV2 in the revised manuscript). However, p62 expression at the basal state was slightly decreased in USP20-depleted cells, which were not treated with CQ. In addition, the decreased expression of p62, which is a typical process shown in starvation-induced autophagy, was not augmented in USP20-depleted cells under starvation (Fig. EV2 in the revised manuscript). Although we do not know the exact mechanism about these observations yet, it is possible that p62 is another target of USP20 proteins regarding autophagy. Therefore, we do not exclude the possibility that p62 expression might be directly or indirectly regulated by USP20-mediated autophagy process. These findings were described in Fig. EV2 and the Results and Discussion of the revised manuscript.

3. Figure 3: To observe autophagy, further experiments such as the degradation of p62 and the amount and ratio of LC3-I and LC3-II during starvation should be performed.

**Answer**: This comment is similar to the question #1 of the reviewer #1. We examined the LC3II to LC3I ratio in USP20-depleted cells during starvation with HBSS. The LC3II to LC3I ratio was significantly decreased in USP20-depleted HeLa cells upon the starvation with HBSS compared to control cells (Fig. 3C and E in the revised manuscript).
In contrast, p62 level at a steady-state was slightly decreased in USP20-depleted cells, which were not treated with CQ, as we mentioned in the above question #2. Furthermore, the decreased expression of p62, which is a typical process shown in starvation-induced autophagy, was not augmented in USP20-depleted cells under starvation (Fig. EV2 in the revised manuscript). Although we do not know the exact mechanism about these observations yet, it is possible that p62 is another target of USP20 proteins regarding autophagy. Therefore, we do not exclude the possibility that p62 expression might be directly or indirectly regulated by USP20-mediated autophagy process. These findings were described in Fig. EV2 and the Results and Discussion of the revised manuscript.

4. It is important to consider that the deubiquitination of ULK1 by USP20 has a role in ULK1 stability and autophagy.

 

**Answer**: Although we already presented results regarding the role of USP20 regarding ULK1 stability and autophagy in the original manuscript, our new results also support a role of USP20 in ULK1 stability and autophagy. ULK stability was significantly decreased in USP20-depleted HeLa cells upon cycloheximide treatment and expression of the catalytically inactive mutant of USP20 (USP20-CI), which is impaired for deubiquitinase activity, did not increase ULK1 stability, compared to the expression of wild-type USP20. In addition, a flux-style analysis indicated that USP20 depletion impaired the induction of LC3 puncta (Fig. 3A in the revised manuscript). Furthermore, the decreased ratio of LC3II to LC3I in USP20-depleted HeLa cells under starvation with HBSS was restored by ectopic expression of wild-type USP20, but not by the catalytically inactive mutant of USP20 (USP20-CI) (Fig. 4E in the revised manuscript). Considering these results, it is evident that USP20 is required for ULK1 stability and autophagy induction.

5. The authors mentioned that ULK1 is degraded through the lysosomal-dependent pathway. However, a description of this pathway is missing. Is this autophagy or something novel process?

**Answer**: In this manuscript, we showed that USP20 plays an important role in autophagy initiation through stabilizing the ULK1 protein at the basal level. This ULK1 stabilization requires USP20 deubiquitinase activity, resulting in the prevention of lysosome-dependent ULK1 degradation at the basal level. Furthermore, our data demonstrate that the ULK1-USP20 interaction at the basal level is decreased during autophagy induction, eventually promoting the next steps in autophagy. This is the first report about a novel molecular mechanism regarding the deubiquitination of ULK1 during autophagy induction. These findings are described and summarized in the Results and Discussion of the revised manuscript.

6. Page 9: The authors stated that USP20 prevents lysosome-mediated ULK1 degradation through the deubiquitination of ULK1. However, they should clarify whether there is any evidence to support that the ubiquitination of ULK1 is required for the degradation of ULK1.

**Answer**: To answer the reviewer's comment, we generated the ubiquitination-defective ULK1 9KR mutant, and provided data that the localization of mCherry-ULK1-9KR mutant to lysosomes is significantly decreased under starvation, compared to wild-type mCherry-ULK1 (Fig. 6D in the revised manuscript). Considering the other result showing the increased ubiquitination of ULK1 in USP20-depleted HeLa cells at 8 h post-starvation when USP20 was dissociated from ULK1 (Fig. 5E in the revised manuscript), these results indicated that ULK1 ubiquitination is required for ULK1 degradation. These new results are included in Fig. 5E and 6D and described in the Results in the revised manuscript.

7. The authors should clarify whether the ubiquitination of ULK1 increases when USP20 dissociates from ULK1 during starvation.

**Answer**: To answer the reviewer's comment, we examined whether ULK1 ubiquitination is increased at 8 h post-starvation, which is when USP20 dissociates from ULK1. Endogenous ubiquitination experiments indicated that ULK1 polyubiquitination is significantly increased at 8 h post-starvation. These results are consistent with the time point showing the dissociation of USP20 from ULK1. These new results are included in Fig. 5E and described in the Results in the revised manuscript.

8. The authors should consider that if the degradation of ULK1 depends on the polyubiquitination of ULK1, proteins containing the ubiquitin-binding domain, including p62, OPT, NDP52, TAX1BP1, and NBR1, may be related to degradation.

**Answer**: Although it is an interesting question, it is beyond the scope of our current study, as the editor has mentioned.

9. When USP20 was knocked down or dissociated from ULK1 during starvation, ULK1 was polyubiquitinated and the molecular weight of ULK1 increases. Thus, the reduction of ULK1 band near 140kDa may be due to the polyubiquitination of ULK1 but not by degradation.

**Answer**: Our present findings demonstrate that USP20 stabilizes ULK1 through ULK1 deubiquitination at the basal level. Therefore, USP20 depletion significantly caused ULK1

 

degradation. In particular, our new results regarding endogenous ubiquitination of ULK1 at 8 h post-starvation showed that the dissociation of USP20 from ULK1 significantly induces ULK1 polyubiquitination. That is, when UPS20 is dissociated from ULK1 during starvation, polyubiquitination of ULK1 is increased upon autophagy induction, eventually causing the degradation of ULK1, as shown in Fig. 5E of the revised manuscript.

**Referee #3:**

1. Figure 1D and 1F: Given that the basal levels of ULK1 are much reduced in the USP20 knockdown cells, and western blotting is a very non-linear detection approach, in the knockdown cells ULK1 levels drop rapidly below detection.
It is therefore very important to rerun these gels and load more protein for the lysates from the USP20 knockdown cells in order to compare similar starting signals that allow analysis of the relative turnover rate.

**Answer**: To clearly address the decreased stability of ULK1 in USP20-depleted cells, we adjusted the amount of starting materials and re-examined the turnover rate of ULK1 upon cycloheximide treatment. ULK1 stability was significantly decreased in USP20-depleted cells and increased upon ectopic expression of USP20. We replaced the previous Fig. 1D and F with the new results in the revised manuscript.

2. Figure 2C and D - the text states that these figures demonstrate that USP20 binds directly to ULK1. This is incorrect unless Myc-ULK1 used in 2C is recombinant and purified from bacteria or elsewhere. Figure 2D shows a very fuzzy immunofluorescence image that does not inform on the colocalization of the two proteins - they both are excluded from the nucleus but subcellular structures cannot be made out. Even if the image was clearer this would not inform on a physical interaction. With regards to the imaging, it should be noted that USP20 has been localized to the endoplasmic reticulum which is of course interesting in the context of autophagy induction. I would also have expected some discussion of previously identified substrates of USP20.

**Answer**: We think that the reviewer's comment regarding Fig. 2C seems to be due to a misunderstanding. The pull-down experiment shown in Fig. 2C was performed with the purified GST-USP20 protein and *in vitro* translated Myc-ULK1 protein. GST-USP20 was purified from *E.coli* and Myc-ULK1 was prepared by using an *in vitro* translation kit (TNT-coupled SP6 reticulocyte lysate system). This procedure was mentioned in the Experimental Procedures. Therefore, this result obtained from i*n vitro* pull-down assay revealed that USP20 directly interacts with ULK1.
Although we tried to re-examine the colocalization of HA-tagged USP20 and endogenous ULK1 by immunofluorescence in HeLa cells which were transfected with HA-USP20 plasmid, we did not obtain relevant images because they were still observed as diffuse forms at a basal state. Therefore, we removed the previous Fig. 2D regarding the colocalization of two proteins. Nevertheless, our immunoprecipitation and immunoblot analysis clearly showed the interaction between USP20 and ULK1 (Fig. 2A-D and Fig. 5C-D in the revised manuscript).
Furthermore, we agree with the reviewer's comment about the localization of USP20 to the endoplasmic reticulum. Because USP20 has been reported to be localized to the ER in certain report, it should be interesting to examine how USP20 localization to the ER may be related to autophagy induction and thus we are planning to do relevant experiments in a future study. The localization of USP20 into the ER and its substrates were discussed in the revised manuscript.

3. Figure 4C is missing a control, namely the mock (IgG) IP from cells in which USP20 is depleted. The Ub-HRP TCL blot is very overexposed so it is impossible to see whether the levels of Ub in the three lanes differ. Have the cells in Figure 4C also been treated with proteasome inhibitor to stabilize the ubiquitylated proteins?

**Answer**: To answer the reviewer's comment, we re-examined the ubiquitination of endogenous ULK1 and we added the IgG control for immunoprecipitation in USP20-depleted cells. When endogenous ULK1 was immunoprecipitated by anti-ULK1 antibody and subsequently immunoblotted by anti-ubiquitin antibody, ULK1polyubiquitination in USP20-depleted cells was significantly increased compared to control cells. In addition, when anti-IgG antibody was used for immunoprecipitation in USP20-depleted cells as a negative control, the polyubiquitinated bands of

 

ULK1 were not observed. Immunoblot analysis using anti-ubiquitin antibody in total cell lysates showed that the ubiquitinated proteins are equally loaded. These new results were included in Fig. 4C of the revised manuscript.
We have not examined the ubiquitinated protein in the presence of proteasome inhibitor because our results indicated that ULK1 is degraded by the lysosomal pathway. In addition, our new result about the increased ubiquitination of ULK1 at 8 h post-starvation in USP20-depleted HeLa cells strongly supported our conclusion that ubiquitination of ULK1 is required for ULK1 degradation when ULK1 was dissociated from USP20 (Fig. 5E in the revised manuscript).

4. Figure 4E - I did not quite understand the point of this figure - as the effect of overexpression has already been shown in Figure 1G. More interesting would be to assess the ability of the CI mutant to rescue the knockdown phenotype (ie ULK1 levels) to complement Figure 1C. This should ideally be quantified over several experiments.

**Answer**: We showed that ectopic expression of wild-type USP20 increases expression of ULK1 in Fig. 1G whereas we emphasized that the deubiquitinase activity of USP20 plays an important role in ULK1stability of, as shown in Fig. 4E. According to the reviewer's comment, we examined the ULK1 levels in USP20-depeleted HeLa cells in which siRNA resistant wild-type Flag-USP20 or catalytically inactive (CI) mutant were respectively transfected. Like the previous Fig. 1C, wild-type USP20 rescued the ULK1 levels, but the catalytic inactive mutant did not. Therefore, the previous Fig. 1C was replaced with a new Fig. 1C in the revised manuscript. To further demonstrate that the deubiquitinase activity of USP20 plays an important role in the autophagy defect of USP20-depleted HeLa cells, we examined whether the catalytically inactive (CI) mutant or wild-type USP20 protein rescues the autophagy flux in USP20-depleted cells. As shown in Fig. 4E of the revised manuscript, wild-type USP20 rescued the autophagy flux of USP20-depleted cells, but the CI mutant USP20 protein did not. These results suggest that the deubiquitinase activity of USP20 is required for autophagy induction. These results, including quantitation, are included in Fig. 4E and described in the Results of the revised manuscript.

5. Figure 5A - the loss of ULK1 in starved cells is not surprising - presumably it simply gets degraded as a component of the autophagosome. The authors should test whether the starvation induced loss of ULK1 is dependent on autophagy by knocking down an autophagy gene required for a later step ie ATG5 or others.

**Answer**: To address the reviewer's comment, we examined the expression of ULK1 in ATG KO and wild-type MEFs under starvation with HBSS. ULK1 expression gradually decreased in both wild-type and ATG5 KO MEFs until 4 h post-starvation and disappeared after 8 h in both cells. These results indicated that the loss of ULK1 under nutrient starvation is independent of later steps of autophagy such as ATG5. These new results were included in Fig. 5B and described in the Results of the revised manuscript.

6. Figure 5B and C - the dissociation of USP20 from ULK1 is again interesting but may need to be interpreted in view of the membrane compartments (ER and phagophore respectively) that the proteins are associated with as autophagy progresses. Rigourous analysis of this would require higher resolution confocal microscopy.

**Answer**: To obtain higher resolution images regarding the dissociation of USP20 from ULK1, we initially transfected HA-USP20 into HeLa cells, which were subsequently incubated for 1 h and 8 h under starvation with HBSS, and examined colocalization of HA-USP20 and endogenous ULK1 using anti-HA and anti-ULK1 antibodies. Unfortunately, because considerable amounts of cells were progressing into cell death at 8 h post-starvation, we could not obtain relevant confocal images regarding the dissociation of USP20 from ULK1. We agreed the reviewer's points that higher resolution images are required to interpret USP20-ULK1 dissociation in terms of alteration in membrane associated USP20 or ULK1 localization. To this end, elaborate detection system such as super-resolution microscopy using membrane markers (ER and/or autophagosome) and high quality endogenous antibodies for USP20 and ULK1. We hope to continue this study in the future.
In spite of the failure of immunofluorescence imaging, the immunoprecipitation/immunoblot analysis, showing interaction dynamics of two proteins (Fig. 5C and D) and the polyubiquitination of ULK1 (Fig. 5E) at 8 h post-starvation in USP20-depleted cells robustly suggests that USP20 is

 

dissociated from ULK1 at 8 h post-starvation. These findings were described in the Results of the revised manuscript.

7. Figure 5D - these images are very poor - and I am really unsure as to what I am meant to see here.

**Answer**: This question is same with question #6. We deleted the previous Fig. 5D from the revised manuscript.

8. Figures 5C-E: Given that USP20 depletion results in a decrease in ULK1 levels and ULK1 is important for cell survival, it is not surprising that depleting USP20 may accelerate the onset of apoptosis upon prolonged autophagy. Restoring cell viability by re-expressing ULK1 is a good way to demonstrate that the effect on cell survival is due to the stabilizing effect of USP20 on ULK1. However the results of the FACS experiments in Figure E are marginal and compare two different cell lines. These data need to be quantified over several experiments to determine whether the differences seen are significant. In addition it would be more convincing to see that re-expression of catalytically active ULK1 (but not a catalytically inactive mutant of ULK1) in USP20 siRNA treated cells can rescue the cells.

**Answer**: We think that the reviewer is commenting on Fig. 6C-E, not Fig. 5C-E in the previous manuscript. To answer the reviewer's question, we generated a HeLa cell line that stably expressed a catalytically active (CA) mutant as well as a catalytically inactive mutant (CI) of ULK1. After these stable cells were depleted by USP20-specific siRNA and incubated for 12 h under starvation with HBSS, we performed FACS analysis to measure cell death in these cells. Expression of CA mutant or wild-type USP20 protein partially rescued cell death upon prolonged autophagy whereas expression of the CI mutant of ULK1 did not. These new results, including quantitation, were included in Fig. 7E and described in the Results of the revised manuscript.

9. There are quite a lot of typographic errors in the manuscript, the first one is in the title, followed by the abstract (pucta). These as well as stylistic errors (see Intro: "a number of evidence..."; page 13: treatment with - not of - lysosomal inhibitors; page 12 - these results were similar to - not with) should be corrected.

**Answer**: We corrected typos and grammatical errors in the revised manuscript.

2nd Editorial Decision 29 November 2017

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see the referees are positive about the study and request mostly clarifications to figures, text and the methods. Moreover, referee 3 has some remaining concerns regarding the statistical analysis and quantification (see also my comments below).
In addition referee 3 and 1 have some remaining concerns that should be addressed experimentally to strengthen the conclusiveness and impact of the paper. Both referees note that ULK1 is still degraded in ATG5-null MEFs and referee 3 suggest to consider and test the possibility that ULK1 is degraded via the proteasome in this context. Referee 3 is also concerned that the evidence for a direct interaction between USP20 and ULK1 based on the current data is rather weak. Also this point should be clarified. Depending on the data provided the revised version might be sent back to referee 3 for a final evaluation.

From the editorial side, there are also a few things that we need:

- Please clearly indicate if the number of experiments refers to three independent experiments or technical replicates in the figure legends. You have noted this in the Materials and Methods section, but the information should also be easily accessible and understandable from looking at the figures and their legends. Also the term "Replicate" in the source data files could in theory also refer to a technical replicate. To resolve this ambiguity you could for example include a short legend in the first line of the excel file that states that the measurements are derived from three biological/independent replicates or three independent experiments.

 

- Source data: please split the file into one excel file per figure. Please also change the name of the source data files relating to EV figures since they currently refer to "Supplementary Figure 2/3" instead of " Figure EV2/3".
The source data excel files should then be uploaded using the Source Data file type. They will be linked to the respective figures in the html version of your manuscript.
Source Data for EV figures can unfortunately not be linked to the respective figure due to technical reasons. Please provide these as a single zip file called Source Data for Expanded View. Please submit this file type via the Expanded View File in the manuscript submission system.

- Please remove the legends from the EV figures and provide them as separate paragraph after the main figure legends (Expanded View Figure legends).

- Please provide the Appendix tables (primer, siRNA, antibodies) as a single pdf file called Appendix. The Appendix contains a table of content on the first page including page numbers, and then the tables and their legends.
Alternatively, you could integrate the siRNA sequences into the methods section since it reports only three sequences and provide the Appendix tables 1 and 2 as Expanded View tables. In this case they can remain in the Excel format, the legend is inserted into the first line of the Excel file. If you choose this option, please rename the tables to Table EV1 and also change the callouts in the text accordingly.

- Please provide up to five keywords (on the first manuscript page)

- Fig. 7G and 7H are never mentioned in the main text. Please provide the respective callouts where appropriate.

I look forward to seeing a final version of your manuscript as soon as possible.

**********
REFEREE REPORTS

Referee #1:

This is a re-review of the manuscript by Kim et al., which described the role of USP20 in deubiquitinating ULK1 and regulating autophagy. The manuscript is much improved after revision and I can recommend publication after the following small points are addressed.
1) In revised Figure 7, the authors do not refer to 7G and H correctly in the text.
2) Related to this, the authors state that ULK1 S556D is constitutively active. However, this residue is not essential for ULK1 activity and in the paper referenced, I think they state it is inactive. Perhaps the authors could change the phrase to "reportedly constitutively active" as this has not been demonstrated (and indeed shows the same effect as WT ULK in this manuscript).
3) The fact that ULK1 is still lysosomally degraded in ATG5-null MEFs is surprising as conventional autophagy is blocked. How then is ULK getting into the lysosome? Some speculation on this in the discussion is needed, especially as the authors still state on p16: "dissociation of USP20 from ULK1 during autophagy initiation may cause the transition of ULK1 localization into lysosome, resulting in ULK1 degradation. Our finding that dissociation of USP20 from its substrate causes the localization of the substrate into autophagosomes, which fuse with lysosomes to form autolysosomes". This cannot be true as ULK1 still gets degraded when there are no autophagosomes (conventional ones at least).

Referee #2:

The authors have made a substantial effort and included new data to address the comments made by this reviewer. I believe that the current manuscript becomes suitable for publication.

Referee #3:

 

I appreciate all the work that the authors have put into their revised manuscript, which is now much improved. There are some remaining issues that I think should be addressed before publication. The new experiments are very helpful and informative but some of the information is incomplete and representation or interpretation need to be clarified. My comments to these points are listed below.

1. Figure 1D - the position of the 140 MW marker in relation to ULK1 appears to be incorrect when compared to other panels in this Figure (1B, 1C, 1F and 1G).

2. Figure 1C: This rescue experiment is a very important - has this only been done once? This needs to be quantified over several biological replicates.

3. Figure 1D: I am confused as to why the actin levels are not higher in the USP20 knockdown samples if the protein loading was adjusted to bring the ULK1 starting level to the same level as for the control? The legend also does not indicate that the loading was adjusted? Likewise in Figure 1F one would expect to see higher amount of ULK1 in the starting material of the Flag-USP20 transfected cells - based on the results from Figure 1G, unless the loading was adjusted. If the latter is correct one would expect to see reduced levels of actin in the samples co-expressing Flag-USP20. Can this be clarified?

4. Figure 2C - indeed I had misunderstood the figure. However the Coomassie blue staining indicates that the majority of the USP20 protein is broken down to a GST-size degradation fragment which is in vast excess over the amount of GST in the control sample. How was the GST-USP20 generated - there is no mention of this in the method section, and the IVT methods contain no detail as to how this experiment was performed or analysed? Can the authors exclude the possibility that they are merely picking up the µg amounts of GST-USP20 with their Myc antibody, seeing as band they detect in the Myc western blot runs at the same molecular weight as the faint GST-USP20 band? Seeing as the authors go on to show that the UCH domain alone is sufficient for the interaction, such a shorter fragment may be more appropriate to strengthen the point that the interaction is direct. As it stands, I think this is weak evidence and this claim for a direct interaction should be toned down at least in the abstract of the manuscript.

5. Text Page 7 referring to Figure 2E - It should read "Three truncated mutants of USP20", - not "ULK1".

6. Figure 3A - The IF data are very hard to see on these images - I suggest presenting the individual channels in greyscale to increase the dynamic range. The scal bar is missing. What does "magnification 200x mean? The objective should be mentioned in the method section. What image analysis program was used for B?.
Figure 3C-E - The analysis of LC3 conversion is very helpful but the text describing the results needs some work.
Page 8, second paragraph, 4th line: "this ratio was decreased in USP20-depleted cells" - this as well as the corresponding statistical analysis in Figure 3D is confusing. The comparison here should be between untreated and HBSS-starved samples - is USP20 depleted cells do not respond with a corresponding increase in LC3II-LC3I ratio. Overall the ratio is very similar in untreated control and untreated siUSP20 cells.
Page 9: line 2: "ATG13 phosphorylation is not augmented in USP20 depleted cells during starvation." The P-ATG13 data are confusing since the P-ATG13 signal is also not increased in starved control cells - but actually decreases at 1 hour and is barely changed at 0.5 h. The big difference lies in the starting levels - ie basal levels of P-ATG13 according to this western blot. It is unclear what that means, but the interpretation of the data as given is confusing and incorrect.
Page 9 - second paragraph - this is unintelligible. I would suggest removing the p62 data from the manuscript.

7. Page 10: line 2 - "An in vitro ubiquitination assay" - this should be "de-ubiquitination"

8. Figure 4B: Why does the DUB assay buffer include ATP?
Figure 4E: n=3 - are these independent experiments that were analysed? Given that the experiment contained more than two samples, an Anova with multiple comparisons test would be more appropriate than a t-test.

 

9. Figure 5 A and B - in starved cells, ULK1 degradation is Baf sensitive and may well occur through autophagy - ie ULK1 associated with autophagosomes is degradaed together with the autophagosomes - however without ATG5, turnover is clearly slower and may proceed via the proteasome. It would be interesting to see ULK1 turnover in ATG5 KO cells {plus minus} Baf and Proteasome inhibitors as well as {plus minus} USP20 to see whether USP20 is still relevant for ULK1 stability in the absence of the autophagy machinery.

10. Page 11 - last line: at steady state - not in a steady state

11. Figure 5C - n=3 - are these independent experiments that were analysed? Given that the experiment contained more than two samples, an Anova with multiple comparisons test would be more appropriate than a t-test.

12. Figure 6A - the fact that Ni-NTA pulls down ULK1 in the absence of HisUb suggests that ULK1 binds non-specifally to Ni-NTA beads - is that the case or is there another interpretation?

13. Figure 7 G and H - I do not find this fully convincing given that this is not a true rescue as the experiment is conducted in different stable cell lines, and only one clone is analysed, leaving the possibility that we are looking at slight clonal variation to starvation.
In addition, the error bars are very small indicating that the n=3 relates to triplicates - this is misleading and does not inform on the statistical significance between the cell lines. As for other quantitations that concern experiments with more than two samples - an ANOVA would be more appropriate - however there is little interest in showing variation between technical replicates.

14. Discussion page 16:
As discussed above (point 9) , the authors' observation that USP20 depletion promotes the lysosome dependent degradation of ULK1 does not prove that degradation of ULK1 in starved cells is a) via the lysosome and b) triggered by USP20 dissociation. Given that USP20 depletion prevents inititttion of autophagy, this is hard to test. It is however possible that during autophagy ULK1 is degraded in autophagosomes (which will be sensitive to Bafiilomycin, and the turnover during autophagy and the turnover in USP20 depleted cells are two independent processes. This caveat could be acknowledged in the discussion.

Overall I still find this a very interesting manuscript, however there are some instances where the data are presented in a potentially confusing or misleading way - unnecessarily so, as the core data are convincing. I apologise for the delay, but this review took a lot of time - I think it is an important piece of work and important to get it right.

2nd Revision - authors' response 26 December 2017

***Point-by-point responses to reviewers' and editorial comments***

**Referee #1:**

This is a re-review of the manuscript by Kim et al., which described the role of USP20 in deubiquitinating ULK1 and regulating autophagy. The manuscript is much improved after revision and I can recommend publication after the following small points are addressed.

Q1) In revised Figure 7, the authors do not refer to 7G and H correctly in the text.

**Answer**: We have referred to Fig. 7G and H in the text of the revised manuscript.

Q2) Related to this, the authors state that ULK1 S556D is constitutively active. However, this residue is not essential for ULK1 activity and in the paper referenced, I think they state it is inactive. Perhaps the authors could change the phrase to "reportedly constitutively active" as this has not been demonstrated (and indeed shows the same effect as WT ULK in this manuscript).

 

**Answer**: We agree with the reviewer's comment because it is not clear whether ULK1 S556D is constitutively active in autophagy. According to the reviewer's suggestion, we changed the phrase "catalytically active" to "reportedly constitutive active" in the revised manuscript (line 9, page 15).

Q3) The fact that ULK1 is still lysosomally degraded in ATG5-null MEFs is surprising as conventional autophagy is blocked. How then is ULK getting into the lysosome? Some speculation on this in the discussion is needed, especially as the authors still state on p16: "dissociation of USP20 from ULK1 during autophagy initiation may cause the transition of ULK1 localization into lysosome, resulting in ULK1 degradation. Our finding that dissociation of USP20 from its substrate causes the localization of the substrate into autophagosomes, which fuse with lysosomes to form autolysosomes". This cannot be true as ULK1 still gets degraded when there are no autophagosomes (conventional ones at least).

**Answer**: To clarify our observation that ULK1 is degraded in ATG5-null MEFs, we examined the expression of ULK1 at 8 h post-starvation in the absence or presence of BafA1 and MG132 in ATG5 wild-type and ATG5-null MEFs. In ATG5 wild-type MEFs, ULK1 degradation at 8 h was inhibited by the lysosomal inhibitor BafA1, but not by MG132. In contrast, the decreased ULK1 levels observed at 8 h in ATG5-null MEFs was restored by the proteasome inhibitor MG132, but not by BafA1. These results indicate that ULK1 degradation is largely mediated by the lysosome-dependent pathway in normal MEFs whereas ULK1 degradation in autophagy-deficient contexts such as ATG5-null MEFs is mainly mediated by the proteasome-dependent pathway. That is, under autophagy-deficient conditions, ULK1 is likely to be degraded through the proteasome pathway to decrease the ULK1 levels. Therefore, our results indicate that ULK1 degradation is regulated by both lysosome-dependent and proteasome-dependent pathways.
Based on these results together with our previous findings, we speculate that ULK1, under normal cellular contexts, is localized to lysosomes during nutrient starvation conditions and degraded through the lysosome-dependent pathway after dissociation from USP20. In contrast, because ULK1 should be degraded in autophagy-deficient cellular contexts during starvation, degradation in this context is mediated by the proteasome pathway. These results were included in the new Fig. 5C and our speculations were described in the Results and Discussion of the revised manuscript.

**Referee #3**:

Q1) Figure 1D - the position of the 140 MW marker in relation to ULK1 appears to be incorrect when compared to other panels in this Figure (1B, 1C, 1F and 1G).

**Answer**: We corrected the position of ULK1 molecular weight marker of Fig. 1D in the revised manuscript.

Q2) Figure 1C: This rescue experiment is a very important - has this only been done once? This needs to be quantified over several biological replicates.

**Answer**: This rescue image was representative of three independent experiments. This statement was included at the end of the legend of Fig. 1, in which we described the following sentence "The data in (B)-(G) are representative of three independent experiments with similar results." In addition, quantitation of the rescue experiments was included in the new Fig. 1C of the revised manuscript.

Q3) Figure 1D: I am confused as to why the actin levels are not higher in the USP20 knockdown samples if the protein loading was adjusted to bring the ULK1 starting level to the same level as for the control? The legend also does not indicate that the loading was adjusted? Likewise in Figure 1F one would expect to see higher amount of ULK1 in the starting material of the Flag-USP20 transfected cells - based on the results from Figure 1G, unless the loading was adjusted. If the latter is correct one would expect to see reduced levels of actin in the samples co-expressing Flag-USP20. Can this be clarified?

**Answer**: In this experiment, we adjusted the amounts of total proteins loaded and this was mentioned in the legend in the revised manuscript. When we performed this experiment, the exposure time to detect other proteins such as USP20 and b-actin was variable because we had to

 

adjust the band intensity of the initial amount of ULK1 in control and USP20 knockdown samples. Also, we would like to note that it is more important to focus on the relative ratio of ULK1 to b-actin expression in the same immunoblot. That is, we normalized the ULK1 level for b-actin expression in the same blot and compared how much the normalized ULK1 is decreased between siCON and siUSP20 blots. Therefore, although b-actin appeared to be lower in USP20-knockdown samples due to the short exposure time, it is evident that ULK1 was rapidly degraded compared to the siCON-expressing sample, when the relative levels of ULK1 and b-actin expression are compared.
In the revised manuscript, the blot showing b-actin expression in siCON-expressing control samples was replaced with one exposed for a short time and the new results were moved to Fig EV2. We think that the results regarding the reduction of basal levels of ULK1 in USP20 knockdown cells shown in the initial manuscript are more reasonable to demonstrate a role of USP20 stabilizing ULK1. Therefore, we added the results in which the initial amounts of ULK1 were not adjusted to the new Fig. 1D and moved the results in which the amounts of ULK1 were adjusted to Fig EV2.

Q4) Figure 2C - indeed I had misunderstood the figure. However the Coomassie blue staining indicates that the majority of the USP20 protein is broken down to a GST-size degradation fragment which is in vast excess over the amount of GST in the control sample. How was the GST-USP20 generated - there is no mention of this in the method section, and the IVT methods contain no detail as to how this experiment was performed or analysed? Can the authors exclude the possibility that they are merely picking up the µg amounts of GST-USP20 with their Myc antibody, seeing as band they detect in the Myc western blot runs at the same molecular weight as the faint GST-USP20 band? Seeing as the authors go on to show that the UCH domain alone is sufficient for the interaction, such a shorter fragment may be more appropriate to strengthen the point that the interaction is direct. As it stands, I think this is weak evidence and this claim for a direct interaction should be toned down at least in the abstract of the manuscript.

**Answer**: The generation of the GST-USP20 fusion protein and the detailed IVT procedure were mentioned in the "*in vitro* pull-down assay" of the Methods section of the revised manuscript. GST-USP20 fusion proteins were expressed in *E.coli* and purified using a glutathione column. Because GST fusion proteins are generally abnormal in *E.coli*, most fusion proteins, including GST-USP20, were rapidly degraded during the purification steps. Therefore, the observation that GST-USP fusion proteins were weakly stained by Coomassie blue staining, compared to GST alone, is generally acceptable in the field of molecular biology. Also, the GST pull-down assay is a general method to detect the direct interaction between two proteins. Although considerable amounts of GST-USP20 fusion proteins were degraded, our results indicated that GST proteins, which were stably expressed and used as a negative control, are not bound to Myc-ULK1 whereas GST-USP20 proteins, which were expressed as a low level, are still bound to Myc-ULK1. Considering other results such as co-immunoprecipitation and immunoprecipitation assays for endogenous proteins together, we can propose the direct interaction between ULK1 and UPS20. To confirm the previous results, we repeated the *in vitro* pull-down assay with GST-USP20 fusion protein which were degraded to a lesser extent. We observed the same results, and the old Fig. 2C data was replaced with new data. Although it is a good suggestion that we examine the interaction between UCH domain of USP20 and ULK1, this experiment is not absolutely necessary to support our current conclusion. Based on our findings and the reviewer's suggestion, we deleted the word "directly" from the abstract of the revised manuscript.

Q5) Text Page 7 referring to Figure 2E - It should read "Three truncated mutants of USP20", - not "ULK1".

**Answer**: We corrected the typo to USP20.

Q6) Figure 3A - The IF data are very hard to see on these images - I suggest presenting the individual channels in greyscale to increase the dynamic range. The scal bar is missing. What does "magnification 200x mean? The objective should be mentioned in the method section. What image analysis program was used for B?

**Answer**: In Fig. 3A, we displayed the scale bar, although the scale bars were already indicated in each inset as a red bar. Although we converted the individual channels to greyscale, the quality of the images was not significantly improved. However, to support our present IF data, the images

 

presented in greyscale were included in Fig.EV3 of the revised manuscript. The resolution of Fig. 3A images was increased in the revised manuscript. In Fig. 3B, “magnification x200” was deleted from the legend of Fig. 3B and the image analysis program was already described in the legend of Fig. 3B.

Figure 3C-E - The analysis of LC3 conversion is very helpful but the text describing the results needs some work.
Page 8, second paragraph, 4th line: "this ratio was decreased in USP20-depleted cells" - this as well as the corresponding statistical analysis in Figure 3D is confusing. The comparison here should be between untreated and HBSS-starved samples - is USP20 depleted cells do not respond with a corresponding increase in LC3II-LC3I ratio. Overall the ratio is very similar in untreated control and untreated siUSP20 cells.

**Answer**: We agree with the reviewer’s concern. To avoid confusion, we performed statistical analysis of the LC3II to LC3I ratio between untreated and HBSS-starved USP20-depleted cells and described the significance in Fig. 3D. We also changed the previous sentence to the following in the revised manuscript; “HBSS-induced augmentation of the LC3II to LC3I ratio was not observed in USP20-deplted cells, suggesting that USP20 is required for autophagy induction.”

Page 9: line 2: "ATG13 phosphorylation is not augmented in USP20 depleted cells during starvation." The P-ATG13 data are confusing since the P-ATG13 signal is also not increased in starved control cells - but actually decreases at 1 hour and is barely changed at 0.5 h. The big difference lies in the starting levels - ie basal levels of P-ATG13 according to this western blot. It is unclear what that means, but the interpretation of the data as given is confusing and incorrect.

**Answer**: This is our mistake. An incorrect immunoblot was inserted in Fig. 4E by error. We inserted the correct immunoblots of phospho-ATG13 and total ATG13 and the quantitation results in Fig. 4D and 4F of the revised manuscript.

Page 9 - second paragraph - this is unintelligible. I would suggest removing the p62 data from the manuscript.

**Answer**: We deleted the previous sentence-“we do not exclude the possibility that p62 expression might be directly or indirectly regulated by the USP20-mediated autophagy process”- in the revised manuscript.

Q7) Page 10: line 2 - "An in vitro ubiquitination assay" - this should be "de-ubiquitination"

**Answer**: We corrected “ubiquitination” into “deubiquitination” in the revised manuscript.

Q8) Figure 4B: Why does the DUB assay buffer include ATP?
Figure 4E: n=3 - are these independent experiments that were analysed? Given that the experiment contained more than two samples, an Anova with multiple comparisons test would be more appropriate than a t-test.

**Answer**: We performed the DUB assay according to the method previously reported (Ref 49: Lee et al. Cell Death Differ. 2015. 22:1463-76). *In vitro* DUB assays using ATP were described in other papers such as the following example: Dupont S et al., FAM/USP9x, a deubiquitinating enzyme essential for TGFbeta signaling, controls Smad4 monoubiquitination. Cell 2009. 136(1):123-35.
In Fig. 4E, n=3 means three independent experiments. According to the reviewer’s suggestion, we re-analyzed and described the results by two-way ANOVA followed by Sidak’s multiple comparison test. According to the reviewer’s suggestion, all data in the revised manuscript were re-analyzed and described by one-way or two-way ANOVA using GraphPad Prism 5 software.

Q9) Figure 5 A and B - in starved cells, ULK1 degradation is Baf sensitive and may well occur through autophagy - ie ULK1 associated with autophagosomes is degradaed together with the autophagosomes - however without ATG5, turnover is clearly slower and may proceed via the proteasome. It would be interesting to see ULK1 turnover in ATG5 KO cells {plus minus} Baf and Proteasome inhibitors as well as {plus minus} USP20 to see whether USP20 is still relevant for ULK1 stability in the absence of the autophagy machinery.

 

**Answer**: This question is similar to question #3 of reviewer 1. According to the reviewer's suggestion, we examined the expression of ULK1 at 8 h post-starvation in the absence or presence of BafA1 and MG132 in ATG5 wild-type and ATG5-null MEFs. In ATG5 wild-type MEFs, ULK1 degradation at 8 h was inhibited by the lysosomal inhibitor BafA1, but not by MG132. In contrast, the decreased levels of ULK1 observed at 8 h in ATG5-null MEFs was restored by the proteasome inhibitor MG132, but not by BafA1. These results indicate that ULK1 degradation is largely mediated by the lysosome-dependent pathway in normal MEFs whereas ULK1 degradation is mainly mediated by the proteasome-dependent pathway in autophagy-deficient contexts such as ATG5-null MEFs. That is, under autophagy-deficient conditions, ULK1 is likely to be degraded through the proteasome pathway to decrease ULK1 levels. Therefore, it appears clear that ULK1 degradation is regulated by both the lysosome-dependent and proteasome-dependent pathway. Based on these results together with our previous findings, we speculate that ULK1, under normal cellular contexts, is localized to lysosomes during nutrient starvation conditions and degraded through the lysosome-dependent pathway after dissociation from USP20. In contrast, because ULK1 should be degraded in autophagy-deficient cellular contexts during starvation, degradation in this context is mediated by the proteasome pathway. These results were included in the new Fig. 5C and our speculations were described in the Results and Discussion of the revised manuscript.

Q10) Page 11 - last line: at steady state - not in a steady state

**Answer**: We corrected the words according to the reviewer's suggestion.

Q11) Figure 5C - n=3 - are these independent experiments that were analysed? Given that the experiment contained more than two samples, an Anova with multiple comparisons test would be more appropriate than a t-test.

**Answer**: In Fig. 5C, n=3 means three independent experiments. According to the reviewer's suggestion, we re-analyzed results by one-way ANOVA followed by Dunnett's multiple comparisons test and described in Fig. 5D of the revised manuscript.

Q12) Figure 6A - the fact that Ni-NTA pulls down ULK1 in the absence of HisUb suggests that ULK1 binds non-specifally to Ni-NTA beads - is that the case or is there another interpretation?

**Answer**: 6XHis-tagged proteins can be purified with Ni-NTA columns. However, it is common that additional bands are purified using Ni-NTA beads as these proteins naturally contain metal-binding pockets or several His amino acids on their surface. As shown in Fig. 6A, unmodified ULK1 bound to Ni-NTA beads even without expression of His-Ub, suggesting that ULK1 contains charged amino acids on their surface, which have affinity for the Ni-NTA beads. Despite non-specific binding of ULK1 to the beads, there are no ubiquitinated proteins with high molecular weight above unmodified ULK1 proteins. Therefore, there is no problem in interpreting these results regarding ULK1 ubiquitination.

Q13) Figure 7 G and H - I do not find this fully convincing given that this is not a true rescue as the experiment is conducted in different stable cell lines, and only one clone is analysed, leaving the possibility that we are looking at slight clonal variation to starvation.
In addition, the error bars are very small indicating that the n=3 relates to triplicates - this is misleading and does not inform on the statistical significance between the cell lines. As for other quantitations that concern experiments with more than two samples - an ANOVA would be more appropriate - however there is little interest in showing variation between technical replicates.

**Answer**: In Fig. 7G and H, we performed the experiments by using three independent colonies isolated during the generation of each cell line. This statement was mentioned in the legend of Fig. 7G and H of the revised manuscript. In Fig. 7H, we re-analyzed and described the results by two-way ANOVA followed by Sidak's multiple comparison test.

Q14) Discussion page 16:
As discussed above (point 9), the authors' observation that USP20 depletion promotes the lysosome dependent degradation of ULK1 does not prove that degradation of ULK1 in starved cells is a) via the lysosome and b) triggered by USP20 dissociation. Given that USP20 depletion prevents

 

initiation of autophagy, this is hard to test. It is however possible that during autophagy ULK1 is degraded in autophagosomes (which will be sensitive to Bafiilomycin, and the turnover during autophagy and the turnover in USP20 depleted cells are two independent processes. This caveat could be acknowledged in the discussion.

**Answer**: According to the reviewer's suggestion, we mentioned in the Discussion of the revised manuscript that ULK1 may be degraded in autophagosomes during autophagy and turnover of ULK1 during autophagy and turnover of ULK1 in USP20-depleted cells could be two independent processes.

**Editorial concerns:**
From the editorial side, there are also a few things that we need:

Q1) Please clearly indicate if the number of experiments refers to three independent experiments or technical replicates in the figure legends. You have noted this in the Materials and Methods section, but the information should also be easily accessible and understandable from looking at the figures and their legends. Also the term "Replicate" in the source data files could in theory also refer to a technical replicate. To resolve this ambiguity you could for example include a short legend in the first line of the excel file that states that the measurements are derived from three biological/independent replicates or three independent experiments.

**Answer**: We indicated that the number of experiments refers to three independent experiments at the end of all figure legends. We also mentioned in the first line of the excel file of the statistics source data that measurements are derived from three independent experiments.

Q2) Source data: please split the file into one excel file per figure. Please also change the name of the source data files relating to EV figures since they currently refer to "Supplementary Figure 2/3" instead of " Figure EV2/3".
The source data excel files should then be uploaded using the Source Data file type. They will be linked to the respective figures in the html version of your manuscript.
Source Data for EV figures can unfortunately not be linked to the respective figure due to technical reasons. Please provide these as a single zip file called Source Data for Expanded View. Please submit this file type via the Expanded View File in the manuscript submission system.

**Answer**: We split the file into one excel file per figure in the revised manuscript. Also, we changed the names of the source data files such as Source data for Figure EV2 and uploaded as a single zip file called Source Data for Expanded View.

Q3) Please remove the legends from the EV figures and provide them as separate paragraph after the main figure legends (Expanded View Figure legends).

**Answer**: We removed the legends from the EV figures and provided the Expanded View Figure legends as a text file, according to the editor's suggestion.

Q4) Please provide the Appendix tables (primer, siRNA, antibodies) as a single pdf file called Appendix. The Appendix contains a table of content on the first page including page numbers, and then the tables and their legends.
Alternatively, you could integrate the siRNA sequences into the methods section since it reports only three sequences and provide the Appendix tables 1 and 2 as Expanded View tables. In this case they can remain in the Excel format, the legend is inserted into the first line of the Excel file. If you choose this option, please rename the tables to Table EV1 and also change the callouts in the text accordingly.

**Answer**: We converted all Appendix tables into a single pdf file called Appendix.

Q5) Please provide up to five keywords (on the first manuscript page)

**Answer**: We provided five key words on the first manuscript page.

 

Q6) Fig. 7G and 7H are never mentioned in the main text. Please provide the respective callouts where appropriate.

**Answer**: We mentioned Fig.7G and 7H in the revised manuscript.

2nd Editorial Decision 26 January 2018

Thank you for your patience while we have reviewed your revised manuscript.

As you will see from the reports below, also referee 3 is now positive about its publication in EMBO reports. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor editorial issues/corrections have been addressed.

Thank you for your contribution to EMBO reports.

**********
REFEREE REPORT

Referee #3:

The authors have addressed my key concerns and in my opinion this paper should be published without further delay

 

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

| |
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| Corresponding Author Name: Seok Hee Park |
| Journal Submitted to: EMBO Reports |
| Manuscript Number: EMBOR-2017-44378V3 |

**Reporting Checklist For Life Sciences Articles (Rev. June 2017)**

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

## A- Figures

### 1. Data

**The data shown in figures should satisfy the following conditions:**

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

### 2. Captions

**Each figure caption should contain the following information, for each panel where they are relevant:**

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

**In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.**

## B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

| | |
|---|---|
| 1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size? | Based on the literature, we chose the sample size routinely used in the field ofmolecular and cellular biology regarding quantitative real-time RT-PCR, quantitation of immunoblotted bands, quantitation of LC3 puncta, the localization of mCherry-ULK1, and quantitation of apoptic cell death. All experiments are independently repeated at three times. This statement was described at the end of each figure legend (p33-42) and each expanded view figure legend (p43-45) and experimental procedures (p25). |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used. | NA |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | NA |
| 3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe. | In all experiments, cells were randomly allocated into each experimental group and treated with the indicated reagents. |
| For animal studies, include a statement about randomization even if no randomization was used. | NA |
| 4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe. | NA |
| 4.b. For animal studies, include a statement about blinding even if no blinding was done | NA |
| 5. For every figure, are statistical tests justified as appropriate? | Yes, statistical signficance was calculated by one-way or two-way ANOVA using GraphPad Prism 5 software and P values were described in each figure legend and experimental procedures (p25). |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. | Statistical signficance was calculated by one-way or two wasy ANOVA using GraphPad Prism 5 software. Staticstics source data are provided as a separate excel file per figure. This statement was described in experimental procedures (p25). |
| Is there an estimate of variation within each group of data? | The variation of data analyzed by one-way or two-way ANOVA were described as the mean ± S.D. or ± SEM of three independent experiments. This statement was described in each figure legend and experimental procedures (p25). |
| Is the variance similar between the groups that are being statistically compared? | Yes, If n=3, we statistically analyzed similar results obtained from three independent experiments. If n>3, we usually analyzed the variance similar between the groups by another statistical method such as Levene's test. |

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## C- Reagents

| | |
|---|---|
| 6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right). | The companies, catalog numbers, species and dilution ratio for primary antibodies used in this study were described in Appendix table S2. |
| 7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. | HEK293T, HeLa, HCT116, HT29 cells were purchased from the American Type Culture Collection (ATCC). ATG5 KO MEFs were kindly provided by Dr. Heesun Cheong (National Cancer Center, Korea). The cell lines have not been authenticated in present study. Our lab always test for mycroplasma contamination before performing the experiments. This statement was described in the Experimental Procedures (p19). |

* for all hyperlinks, please see the table at the top right of the document

## D- Animal Models

| | |
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| 8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals. | NA |
| 9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments. | NA |
| 10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance. | NA |

## E- Human Subjects

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| 11. Identify the committee(s) approving the study protocol. | NA |
| 12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. | NA |
| 13. For publication of patient photos, include a statement confirming that consent to publish was obtained. | NA |
| 14. Report any restrictions on the availability (and/or on the use) of human data or samples. | NA |
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## F- Data Accessibility

| | |
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| 18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>e. Proteomics and molecular interactions | NA |
| 19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right). | NA |
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| 21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information. | NA |

## G- Dual use research of concern

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| 22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could. | NA |