THE EMBO JOURNAL

TRIM27 cooperates with STK38L to inhibit ULK1-mediated autophagy and promote tumorigenesis

Yi Yang, Yifu Zhu, Shuai Zhou, Peipei Tang, Ran Xu, Yuwei Zhang, Dongping Wei, Jian Wen, Rick F. Thorne, Xu Dong Zhang, Jun-lin Guan, Lianxin Liu, Mian Wu, Song Chen
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Transaction Report:

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

1st Editorial Decision **8th Nov 2021**

Dear Dr Mian Wu,

Thank you again for the submission of your manuscript (EMBOJ-2021-109777) to The EMBO Journal, as well as for your patience with our response. Your study has been sent to three experts, and we have received reports from all of them, which I enclose below.

As you will see, the referees acknowledge the potential interest and value of your results, although they also express major concerns, which need to be addressed before they can be supportive of publication at the EMBO Journal. In more detail, expert #3 states that the mechanistic details of how TRIM27 exerts its dual independent activities are not sufficiently resolved and need further evaluation (ref#3, pt.3). Further, this referee points to major issues on the in vivo relevance of an autophagy-dependent function of TRIM27 in breast cancer (ref#3, pt.4). Related, reviewer #1 states that the causalities and details of involvement of ULK1 stability downstream of TRIM27-STK38L are not conclusively addressed (ref#1, pts1,2). Finally, the referees list a number of additional biochemical experiments, missing controls and issues with the data presentation and discussion of the results, that would need to be addressed to achieve the level of robustness needed for The EMBO Journal.

Given the referees' overall interest and detailed comments, I would like to invite you to submit a revised version of the manuscript, addressing the issues raised. As you know it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

In light of the extensive experimentation requested by the reviewers, I would appreciate if you could contact me during the next weeks via e.g. a video call to discuss your perspective on the comments and potential plan for revisions.

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

When submitting your revised manuscript, please carefully review the instructions below.

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

Best regards,

Daniel Klimmeck

Daniel Klimmeck, PhD
Senior Editor
The EMBO Journal

Instruction for the preparation of your revised manuscript:

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

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

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

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

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

manuscript.

6) It is mandatory to include a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition).
In case you have no data that requires deposition in a public database, please state so in this section. Note that the Data Availability Section is restricted to new primary data that are part of this study.
*** Note - All links should resolve to a page where the data can be accessed. ***

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

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

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

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

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

10) When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

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

Further information is available in our Guide to Authors: https://www.embopress.org/page/journal/14602075/authorguide

Revision to The EMBO Journal should be submitted online within 90 days, unless an extension has been requested and approved by the editor; please click on the link below to submit the revision online before 6th Feb 2022:

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

Referee #1:

To editors:
I think this paper describes a potentially interesting new finding that deserves publication in EMBO J. if remaining issues are resolved during the review process.

To authors:
In this manuscript, Yang et al describe the role of TRIM27, an E3 ligase, in negative regulation of autophagy. By interactome analysis, the authors identified TRIM27 as a novel binding partner of ULK1, a component of ULK complex that is critical for autophagy initiation. The TRIM27-ULK1 interaction occurs at basal conditions and increases during nutrient starvation. The authors found that TRIM27 ubiquitinates and degrades ULK1 in a proteasome-dependent manner at both basal and starvation conditions. Loss of TRIM27 in MEF or mice (kidney) resulted in enhanced autophagic flux, suggesting that TRIM27 negatively regulates autophagy by degrading ULK1. The authors further demonstrated that the TRIM27-ULK1 interaction and subsequent degradation of ULK1 is positively regulated by phosphorylation of ULK1 at S495 by STK38L, a kinase that interacts with and is ubiquitinated by TRIM27 (in this case, non-degradative K6 and K11-linked ubiquitination). As similar to TRIM27-depleted cells, STK38L-depleted cells showed enhanced autophagic flux during starvation due to suppression of TRIM27-mediated degradation of ULK1. Finally, the authors investigated the pathophysiological significance of TRIM27 in vivo. The authors found that the expression of TRIM27 is upregulated in several types of human breast cancers while the amount of ULK1 is downregulated. Using spontaneous mammary tumor mouse models (transgenic PyMT mice) and Trim27-deficient mice, the authors showed that Trim27 enhances tumorigenesis likely by suppressing autophagy (but suppresses metastasis). Collectively, the authors demonstrated a novel regulatory role of TRIM27-STK38L-ULK1 axis in negative regulation of autophagy.

The identification of TRIM27 and STK38L as novel negative regulators of autophagy is novel and will provide important insights for researchers in this field. Most of the data in this manuscript are convincing and presented by well-designed experiments. Thus, I have only a few comments shown below.

1. It is still controversial whether ULK1 and ULK2 are essential for autophagy or not (Alers et al., Autophagy 2011 [PMID: 22024743], Kannangara et al., EMBO Rep. 2021 [PMID: 34369648]). Thus, it is important to experimentally confirm the causal relationship between ULK1 accumulation and autophagy enhancement in cells lacking TRIM27 and STK38L. Does the knockdown of ULK1 can decrease the autophagic flux in wild-type cells and TRIM27- or STK38L-deficient cells? This experiment is also important to exclude the involvement of other potential substrates of TRIM27 and STK38L in autophagy regulation.
2. ULK1 and ULK2 are highly homologous. Can ULK2 also be targeted by TRIM27?
3. The authors showed that ULK1 is accumulated in the kidney of systemic Trim27-deficient mice. How about other tissues such as liver and brain, in which autophagy has very important functions?

Referee #2:

In the manuscript the authors described the role of TRIM27 as a negative regulator of autophagy during starvation. In particular, authors showed how TRIM27 ubiquitinates ULK1 thus promoting its degradation via proteasome. Moreover, TRIM27 at the same time promotes STK38L activation via K6-K11 Ub and STK38L phosphorylates ULK1 activating it. It is also interesting the link between TRIM27 and tumorigenesis. The manuscript is well organised and experiments properly done. The authors also well combined in vitro and in vivo data. The manuscript is mature, however this referee has few comment that the authors should consider:
- Does TRIM27 ubiquitinate ATG13 and FIP200 too? Similarly, does STK38L phosphorylate ATG13 and FIP200?
- NEDD4 also regulates ULK1 via Ub. The authors poorly discuss this point and never interrogated their biological system to investigate if TRIM27 and NEDD4 cooperate or compete for ULK1. Authors has the tools to investigate the role of NEDD4 especially in a context of TRIM27 deficiency.
- Fig.3E and Fig.3K will benefit of a higher resolution
- Is TRIM27 protein and/or function upregulated in autophagy deficient cells?

Referee #3:

In the manuscript « TRIM27 Cooperates with STK38L to Inhibit ULK1-mediated Autophagy and Promotes Tumorigenesis » Yang and colleagues study the crosstalk between the E3 ubiquitin ligase TRIM27 and ULK1. The authors identify TRIM27 as ULK1 binding partner and convincingly show that ULK1 is getting ubiquitinated by TRIM27 leading to its proteasomal degradation. This degradation is increased under starvation conditions, catalyzed by a TRIM27- and STK38L-dependent mechanism. TRIM27 activates STK38L by K6 ubiquitination, which in turn phosphorylates ULK1 supporting its TRIM27-dependent degradation. Thus, TRIM27 and STK38L act as negative regulators of ULK1 protein abundance, restricting functional autophagy. Finally, the authors analyze TRIM27 knockout mice and identify a reduced tumor but a higher metastasis burden in the absence of TRIM27. The paper is well written and presents a wealth of data highlighting the regulation of ULK1 by TRIM27 and STK38L. The

proposed mechanism is very interesting, but also raises additional questions which should still be addressed. Whereas biochemical and cell culture experiments explain causal links, the work done in mice is purely correlative and will have to be strengthened to clearly show the role of autophagy in the observed tumor phenotype.

Major points:
(1) Table S1 and Table S5: the quality of the proteomic hits cannot be judged as only lists of proteins are presented. Quantitative data should be shown. Numbers of peptides, intensity of enrichment, etc. should be listed, so that interested readers can try to filter the listed hits based on quantitative criteria. Currently, one can only use the list qualitatively.
(2) Many different western blots are presented. However, almost no quantitative data is shown. At least the most critical blots should be repeated minimally three times, quantified and respective bar diagrams with error bars and statistical evaluations should be shown. This should be done minimally for: Figures 1H, 2D, 2E, 2L, 2O, 4E, 4H, 5A, 5M, 5N
(3) K6 ubiquitination of STK38L, Figure 5J and S6: it is difficult to envision how the same E3 ligase catalyzes different ubiquitin linkages on different target proteins leading to different biological outcomes, especially as data presented in Figure S6A shows gradual differences. As an additional control authors should use BafA1 and MG132 and show that inhibition of the lysosome and proteasome does not affect STK38L protein level. This would further strengthen their argument that its ubiquitination affects activity and not abundance.
(4) Compared to the work done in vitro and in vivo in cell culture, the data presented in mice is not conclusive. Of course, these experiments are more demanding. However, in the current manuscript the presented data is "only" correlative and the role of autophagy in tumorigenesis is not clear (as also stated by the authors in the discussion). Like this the title of the manuscript is also misleading. Whereas the effects of loss of TRIM27 are clear, it is not clear if this is mediated by autophagy or any other process affected by TRIM27. This is rather confusing given data presented in Figures 1-5. The authors have to cross their mice to autophagy incompetent mice such as Atg5-/- or Atg7-/- mice, or at least use an ULK1 inhibitor (if this is feasible) and analyze if this alters the effects of TRIM27 knockout. If this is too time demanding, I would rather suggest to restructure the manuscript and remove data presented in Figure 6.

Minor points:
(1) Page 4, line 63: the yeast orthologs of ULK1 and mTOR are Atg1 and Tor1. The nomenclature should be adapted when yeast counterparts are discussed.
(2) Page4, line 70: autophagy is not only induced by AMPK activation but also by mTOR inhibition which leads to the removal of inhibitory mTOR sites on ULK1. This should be mentioned.
(3) Page 5, line 85/86; "Some relationships between TRIM family proteins and autophagy are known". This is an understatement. More than 70 papers link TRIM proteins to autophagy. A brief summary should be given.
(4) Page 11, line 207: the western blot analyses LC3-II abundance not "expression".
(5) Page 12, line 231: "nutrient starvation and is responsible for the accelerated degradation of ULK1". Accelerated compared to what? I would suggest to either delete "accelerated" or to exchange it with "stimulus-specific".
(6) How come ULK1 was not detected in STK38L IPs (Table S5)? This should at least be discussed.
(7) Page 14, line 257: "indicative of inhibition of autophagic flux." This is a bit misleadingly formulated. An increase in LC3 conversion is indicative of an increased flux. Please rewrite.
(8) Page 18, line 349, Figure 6B: numbering in text and in figure do not match.

Thank you again for the submission of your manuscript (EMBOJ-2021-109777) to The EMBO Journal, as well as for your patience with our response. Your study has been sent to three experts, and we have received reports from all of them, which I enclose below.

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

Best regards,

Daniel Klimmeck

Daniel Klimmeck, PhD
Senior Editor
The EMBO Journal

Responses to reviewers' comments:
We thank the reviewers for contributing their valuable time and for the helpful suggestions provided.

Reviewer #1

Response: We thank the Reviewer for contributing their time to evaluate our manuscript and their encouraging comments. We trust the revisions including the new experiments undertaken meet with the Reviewer's expectations.

To editors:
I think this paper describes a potentially interesting new finding that deserves publication in EMBO J. if remaining issues are resolved during the review process.

To authors:
In this manuscript, Yang et al describe the role of TRIM27, an E3 ligase, in negative regulation of autophagy. By interactome analysis, the authors identified TRIM27 as a novel binding partner of ULK1, a component of ULK complex that is critical for autophagy initiation. The TRIM27-ULK1 interaction occurs at basal conditions and increases during nutrient starvation. The authors found that TRIM27 ubiquitinates and degrades ULK1 in a proteasome-dependent manner at both basal and starvation conditions. Loss of TRIM27 in MEF or mice (kidney) resulted in enhanced autophagic flux, suggesting that TRIM27 negatively regulates autophagy by degrading ULK1. The authors further demonstrated that the TRIM27-ULK1 interaction and subsequent degradation of ULK1 is positively regulated by phosphorylation of ULK1 at S495 by STK38L, a kinase that interacts with and is ubiquitinated by TRIM27 (in this case, non-degradative K6 and K11-linked ubiquitination). As similar to TRIM27-depleted cells, STK38L-depleted cells showed enhanced autophagic flux during starvation due to suppression of TRIM27-mediated degradation of ULK1. Finally, the authors investigated the pathophysiological significance of TRIM27 in vivo. The authors found that the expression of TRIM27 is upregulated in several types of human breast cancers while the amount of ULK1 is downregulated. Using spontaneous mammary tumor mouse models (transgenic PyMT mice) and Trim27-deficient mice, the authors showed that TRIM27enhances tumorigenesis likely by suppressing autophagy (but suppresses metastasis). Collectively, the authors demonstrated a novel regulatory role of TRIM27-STK38L-ULK1 axis in negative regulation of autophagy.

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experiment is also important to exclude the involvement of other potential substrates of TRIM27 and STK38L in autophagy regulation.

Response: A very evocative point. In the two papers mentioned by the reviewer these studies showed ULK1-independent regulation of autophagy in specific cell lines such as DT40 (chicken-derived) cells. However, we would argue that most researchers believe that ULK1 is mostly essential for autophagy. As intimated, the best way to resolve this point is to investigate whether knockdown of ULK1 affects autophagic flux in wild-type, TRIM27- or STK38L-deficient cells. Notably, execution of these experiments showed that knockdown using two independent shRNA significantly inhibited the autophagic flux in wildtype HeLa cells than knockdown of ULK2 (Figure R1A). In addition, we found individual knockdown of ULK1 in STL38L and TRIM27 knockout HeLa cells inhibited LC3II accumulation in the presence of BafA1 (Figs. R1B and R1C) indicating the inhibition of autophagic flux. These data therefore reinforce the importance of ULK1 in autophagy induction in HeLa cells. Moreover, the effects observed in cells lacking TRIM27 and STK38L strengthen our interpretation that ULK1 is the substrate in their respective reactions.

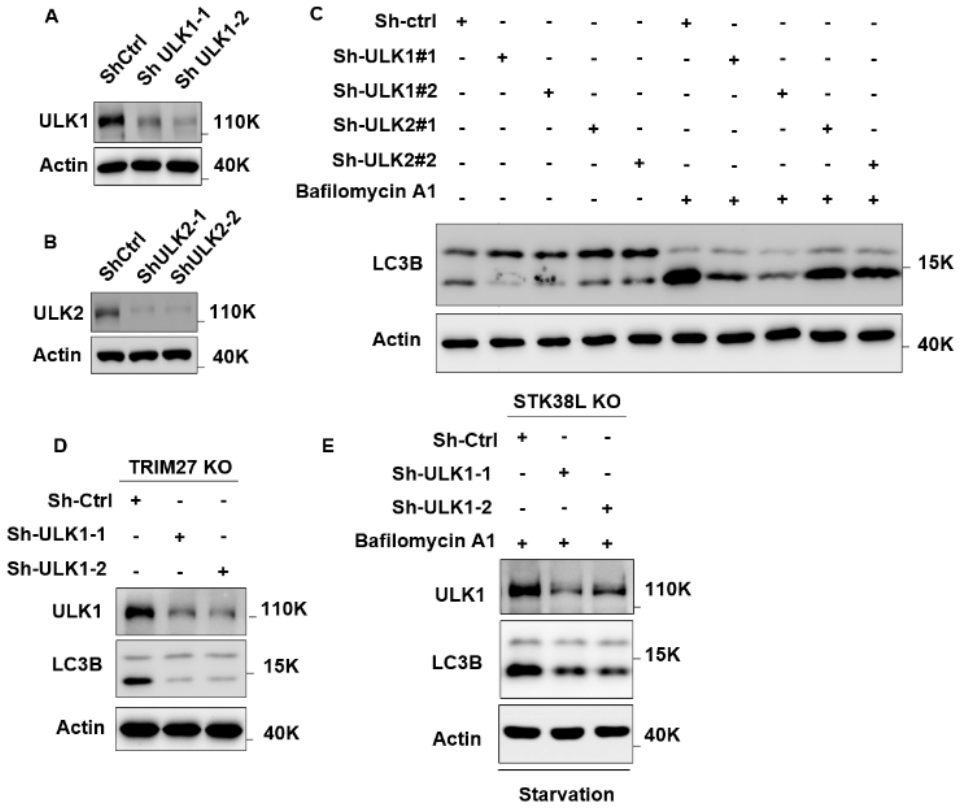


Figure R1: Knockdown of ULK1 can decrease the autophagic flux in wildtype, TRIM27- and STK38L-deficient HeLa cells. (A&B). Western blot analysis of shRNA mediated knockdown efficiency of ULK1 (A) and ULK2 (B) in HeLa cells. (C) Western blot analysis of autophagic flux marker LC3II abundance in the presence of Bafilomycin A treatment for 2 hours for HeLa cells with depletion of ULK1 or ULK2

compared to control. (D) Western blot analysis of LC3II abundance after depletion of ULK1 or ULK2 in TRIM27 (D) or STK38L (E) knockout HeLa cells.

2. ULK1 and ULK2 are highly homologous. Can ULK2 also be targeted by TRIM27?
Response: Indeed, ULK1 and ULK2 are highly homologous, however, it was also reported that ULK1 and ULK2 may be less redundant than previously thought (Demeter et al., Sci Rep. 2020 [PMID: 32616830]). Thus, it is plausible that ULK1 and ULK2 show differences in their autophagy-related interactors and their post-translational regulators. Nevertheless, we agree ULK2 cannot be ignored for this reason alone. In response, we conducted two experiments which provide compelling evidence that TRIM27 targets only ULK1 and not ULK2. As we have shown here in Figure R2A, TRIM27 cannot promote the ubiquitination of ULK2. In particular, we found overexpression of TRIM27 did not downregulate the ULK2 protein levels while it indeed downregulated co-expressed ULK1 (Figure R2B).

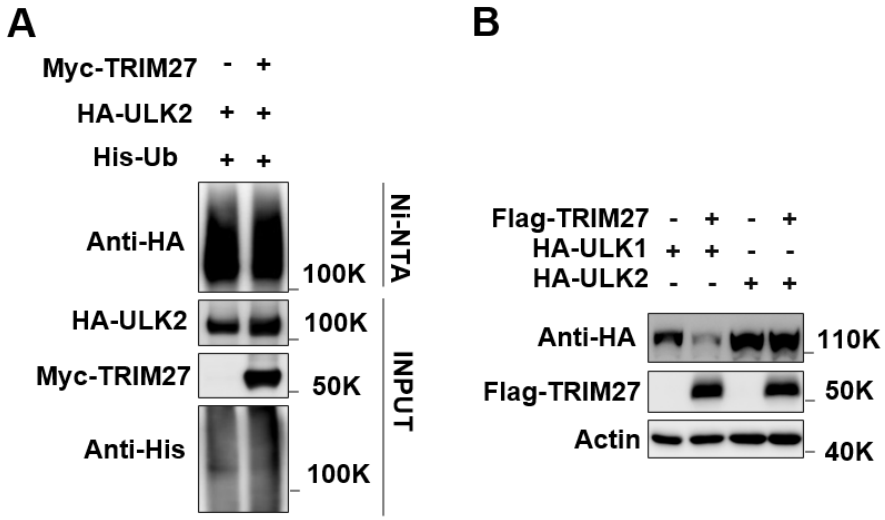
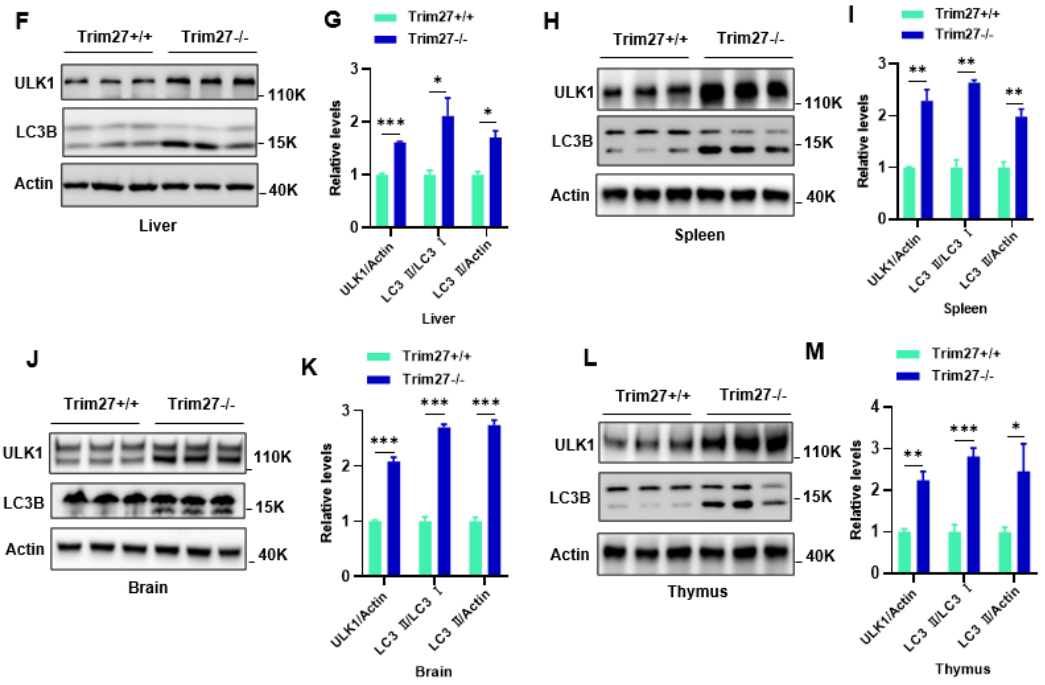


Figure R2: Overexpression of TRIM27 does not promote the ubiquitination of ULK2 nor downregulate ULK2 expression. (A&B).

3. The authors showed that ULK1 is accumulated in the kidney of systemic Trim27-deficient mice. How about other tissues such as liver and brain, in which autophagy has very important functions?
Response: A very interesting question. We surveyed the expression of ULK1 in major organs. In addition to kidney as originally presented, we certainly noticed a clear trend showing that TRIM27 knockout resulted in significantly increased accumulation of ULK1 protein levels in liver, brain, spleen and thymus. Not unexpectedly, there were organ-to-organ variations in the degree of ULK1 accumulation with the strongest effects seen in immune-related organs (thymus and spleen). We report these additional data in the revised manuscript as Fig EV2.

Fig EV2



Referee #2

Response: We thank the Reviewer for contributing their time to evaluate our manuscript and their kind assessment. We hope the explanations and modifications meet with the Reviewer's approval.

In the manuscript the authors described the role of TRIM27 as a negative regulator of autophagy during starvation. In particular, authors showed how TRIM27 ubiquitinates ULK1 thus promoting its degradation via proteasome. Moreover, TRIM27 at the same time promotes STK38L activation via K6-K11 Ub and STK38L phosphorylates ULK1 activating it. It is also interesting the link between TRIM27 and tumorigenesis. The manuscript is well organised and experiments properly done. The authors also well combined in vitro and in vivo data. The manuscript is mature, however this referee has few comment that the authors should consider:

- Does TRIM27 ubiquitinate ATG13 and FIP200 too? Similarly, does STK38L phosphorylate ATG13 and FIP200?

Response: We agree that it is important to carefully elaborate the effector-substrate relationships when describing a mechanism of this nature. The first part of the question was evaluated in the manuscript where we showed using co-expression studies that TRIM27 does not ubiquitinate ATG13 nor FIP200 (Fig. EV1 A and B, respectively). Secondly, to evaluate if STK38L phosphorylates ATG13 and FIP200

we again used co-transfection assays. Reproduced below for review purposes (Fig R3A and B), these experiments clearly showed that ectopically expressed STK38L did not alter the phosphorylation levels of ATG13 and FIP200. Together these data with other results presented in the manuscript provide strong supporting evidence for the proposed mechanism.

Fig EV1

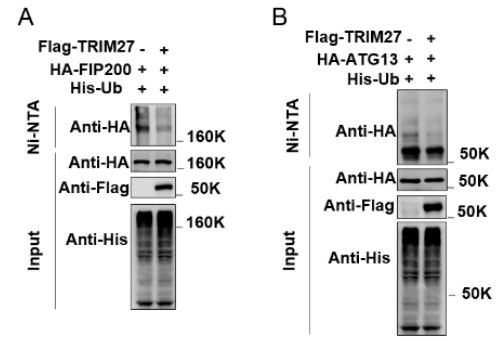
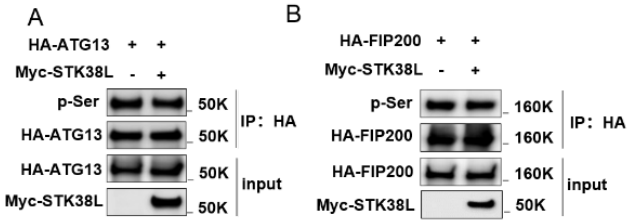


Fig R3



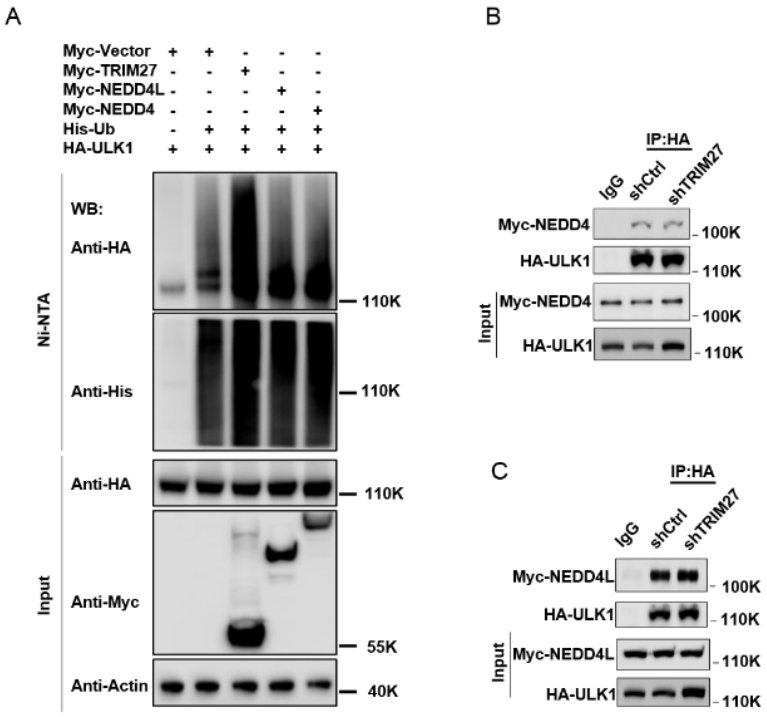
- NEDD4 also regulates ULK1 via Ub. The authors poorly discuss this point and never interrogated their biological system to investigate if TRIM27 and NEDD4 cooperate or compete for ULK1. Authors has the tools to investigate the role of NEDD4 especially in a context of TRIM27 deficiency.

Response: A very interesting question. We did originally mention that a small number of other E3 ligases have been shown to direct the degradation of ULK1 but clearly further clarification is warranted. As intimated, it is expected that there would be multiple negative regulators of ULK1, and such factors could cooperate or compete, or otherwise operate independently in different contexts. Indeed, we identified NEDD4 in our screen presumably because it was reported to bind to several autophagy-related proteins such as Beclin 1, LC3, SQSTM1. On the other hand, NEDD4L (like) that was previously shown to ubiquitinate ULK1, was not recovered in the screen.

As suggested, we compared the relative ability of TRIM27, Nedd4, and Nedd4l to ubiquitinate ULK1 in our experimental systems. Comparative results comparing the ability of ectopically expressed TRIM27, Nedd4 and Nedd4l to ubiquitinate ULK1 showed a clear functional advantage for TRIM27 (Appendix Fig S2A). However, perhaps not unexpectedly, Nedd4 and Nedd4l could recognize ULK1 as a ubiquitination substrate to a lesser degree, at least as ectopically expressed proteins (Appendix Fig S2A). Furthermore, knocking down TRIM27 had no bearing on the interactions between either Nedd4 or Nedd4l and ULK1 (Appendix Fig S2B and C).

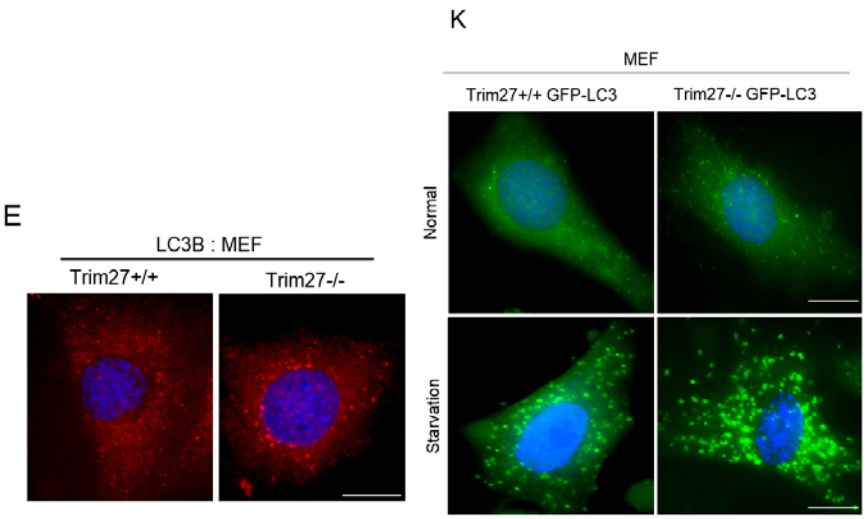
Together this suggests that Nedd4 and Nedd4l would likely cooperate and not compete with TRIM27 to control ULK1 levels (assuming each protein is co-expressed in context). (line 161-168)

Appendix Fig S2



- Fig.3E and Fig.3K will benefit of a higher resolution

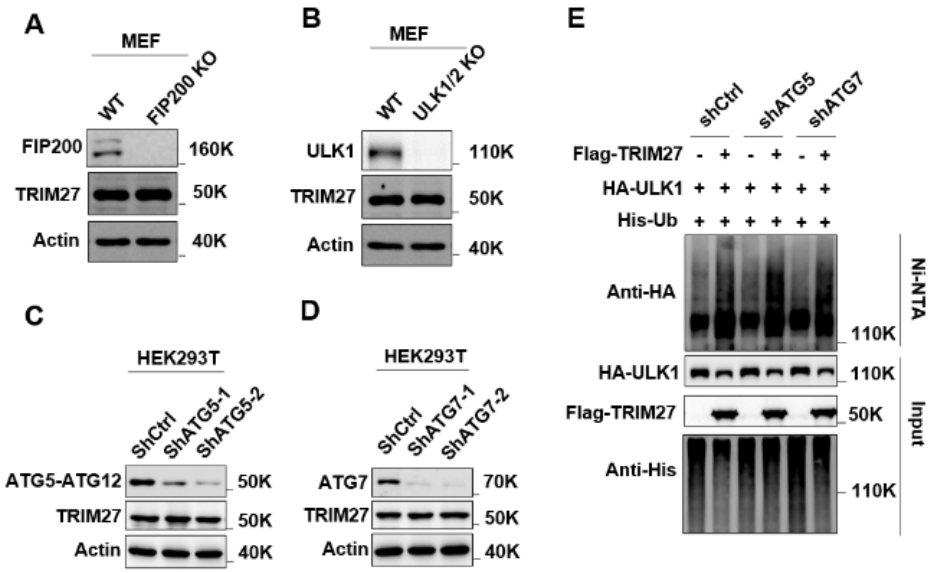
Response: We provided new images with improved resolution as requested.



- Is TRIM27 protein and/or function upregulated in autophagy deficient cells?

Response: In answer to the first part of the question, no, we found TRIM27 protein levels did not significantly change in MEF cells with knockout of different autophagy complex initiation components (FIP200 or ULK1/2 combined) (Fig R4A and B). Furthermore, knockdown of either ATG5 or ATG7 involved autophagosome maturation in HEK293T cells also failed to affect TRIM27 levels (Fig R3C and D). All such cells are considered autophagy deficient. With regard to TRIM27 function, we can only answer this for ULK1 as the target but similarly, its ubiquitination was marginally affected in HEK293T cells after knockdown of ATG5 or ATG7 (Fig R4E).

Figure R4



Referee #3:

Response: We thank the Reviewer for contributing their time and expertise to assess our work. We hope the explanations and responses provide satisfactory solutions to the outstanding issues raised.

In the manuscript « TRIM27 Cooperates with STK38L to Inhibit ULK1-mediated Autophagy and Promotes Tumorigenesis » Yang and colleagues study the crosstalk between the E3 ubiquitin ligase TRIM27 and ULK1. The authors identify TRIM27 as ULK1 binding partner and convincingly show that ULK1 is getting ubiquitinated by TRIM27 leading to its proteasomal degradation. This degradation is increased under starvation conditions, catalyzed by a TRIM27- and STK38L-dependent mechanism. TRIM27 activates STK38L by K6 ubiquitination, which in turn phosphorylates ULK1 supporting its TRIM27-dependent degradation. Thus, TRIM27 and STK38L act as negative regulators of ULK1 protein abundance, restricting functional autophagy. Finally, the authors analyze TRIM27 knockout mice and identify a reduced tumor but a higher metastasis burden in the absence of TRIM27.

The paper is well written and presents a wealth of data highlighting the regulation of ULK1 by TRIM27 and STK38L. The proposed mechanism is very interesting, but also raises additional questions which should still be addressed. Whereas biochemical and cell culture experiments explain causal links, the work done in mice is purely correlative and will have to be strengthened to clearly show the role of autophagy in the observed tumor phenotype.

Major points:

(1) Table S1 and Table S5: the quality of the proteomic hits cannot be judged as only lists of proteins are presented. Quantitative data should be shown. Numbers of peptides, intensity of enrichment, etc. should be listed, so that interested readers can try to filter the listed hits based on quantitative criteria. Currently, one can only use the list qualitatively.

Response: As requested, we added protein mass, number of peptides, percent of coverage to these data as showing in the new Appendix Table S1 and the Score, Matched Peptide Sequences and the Protein Abundance Index (emPAI) in Appendix Table S5 for reader's reference.

Appendix Table S1. FIP200 interaction proteins identified by mass spectrometry					
Accession Number	Molecular Weight	Peptide number	Peptide number	Percent coverage	Percent coverage
		Normal Condition	Starved condition	Normal Condition	Starved condition
RBCC1_MOUSE	182 kDa	108	99	55.00%	53.10%
ATG13_MOUSE	56 kDa	19	19	42.40%	39.30%
ULK1_MOUSE	112 kDa	33	26	39.50%	30.00%
VTI1B_MOUSE	27 kDa	4	6	19.40%	29.30%
ATGA1_MOUSE	25 kDa	4	2	18.80%	6.88%
ULK2_MOUSE	113 kDa	15	14	17.80%	16.40%
DREB_MOUSE	77 kDa	9	14	17.30%	14.40%
PRDX1_MOUSE	22 kDa	3	5	15.60%	24.60%
M2OM_MOUSE	34 kDa	4	20	14.00%	49.00%
DPM1_MOUSE	29 kDa	2	2	12.70%	12.70%
CDC42_MOUSE	21 kDa	2	2	12.60%	11.00%
CHCH3_MOUSE	26 kDa	3	1	12.30%	3.96%
RAC1_MOUSE	21 kDa	2	3	8.85%	16.10%
CCPG1_MOUSE	86 kDa	6	6	7.84%	7.44%
IRGM1_MOUSE	47 kDa	3	2	6.60%	4.89%
CY1_MOUSE	35 kDa	2	2	5.85%	8.62%
RPN2_MOUSE	69 kDa	2	6	5.07%	15.70%
MFGM_MOUSE	51 kDa	2	4	4.75%	9.72%
GRB10_MOUSE	71 kDa	2	2	3.70%	3.70%
CC127_MOUSE	31 kDa	1	3	3.08%	12.30%
PP1B_MOUSE	37 kDa	1	5	3.06%	19.90%
UN45A_MOUSE	103 kDa	2	7	2.75%	9.22%
TAXB1_MOUSE	94 kDa	2	0	2.70%	0
FIL1L_MOUSE	130 kDa	2	0	2.56%	0
OST48_MOUSE	49 kDa	1	3	2.04%	4.99%
RPN1_MOUSE	69 kDa	1	2	1.97%	3.62%
SDPR_MOUSE	47 kDa	1	3	1.91%	8.37%
K1199_MOUSE	153 kDa	2	5	1.76%	3.38%
DPYL3_MOUSE	62 kDa	1	2	1.75%	4.04%
LONP2_MOUSE	95 kDa	1	3	1.41%	3.76%
SYNPO_MOUSE	100 kDa	1	3	1.40%	4.31%
RGRF1_MOUSE	144 kDa	2	1	1.03%	0.56%
MYPT1_MOUSE	115 kDa	0	8	0.00%	7.19%
PHLB2_MOUSE	141 kDa	0	3	0.00%	1.60%
ECHA_MOUSE	83 kDa	0	6	0	9.04%
MOSC2_MOUSE	38 kDa	0	5	0	11.80%
EHD2_MOUSE	61 kDa	0	6	0	11.20%
PP12C_MOUSE	85 kDa	0	4	0	3.45%
LETM1_MOUSE	83 kDa	0	2	0.00%	4.88%
SRPRB_MOUSE	30 kDa	0	4	0	20.80%
SSRA_MOUSE	32 kDa	0	3	0	11.90%
TRI27_MOUSE	59 kDa	0	2	0	4.29%
PALLD_MOUSE	152 kDa	0	2	0.00%	1.99%
NEDD4_MOUSE	103 kDa	0	3	0	2.59%
ILVBL_MOUSE	68 kDa	0	3	0	10.30%
PM34_MOUSE	34 kDa	0	3	0	11.40%
PP1A_MOUSE	38 kDa	0	3	0	20.00%
ARM10_MOUSE	33 kDa	0	2	0	9.15%
DPYL2_MOUSE	62 kDa	0	2	0	4.02%
EHD4_MOUSE	61 kDa	0	2	0	3.51%
KC1A_MOUSE	39 kDa	0	2	0	6.53%
PHB_MOUSE	30 kDa	0	2	0	7.72%
SGPL1_MOUSE	64 kDa	0	2	0	4.05%
SRC8_MOUSE	61 kDa	0	2	0	3.66%
STML2_MOUSE	38 kDa	0	2	0	7.93%
SUN2_MOUSE	82 kDa	0	2	0	2.74%

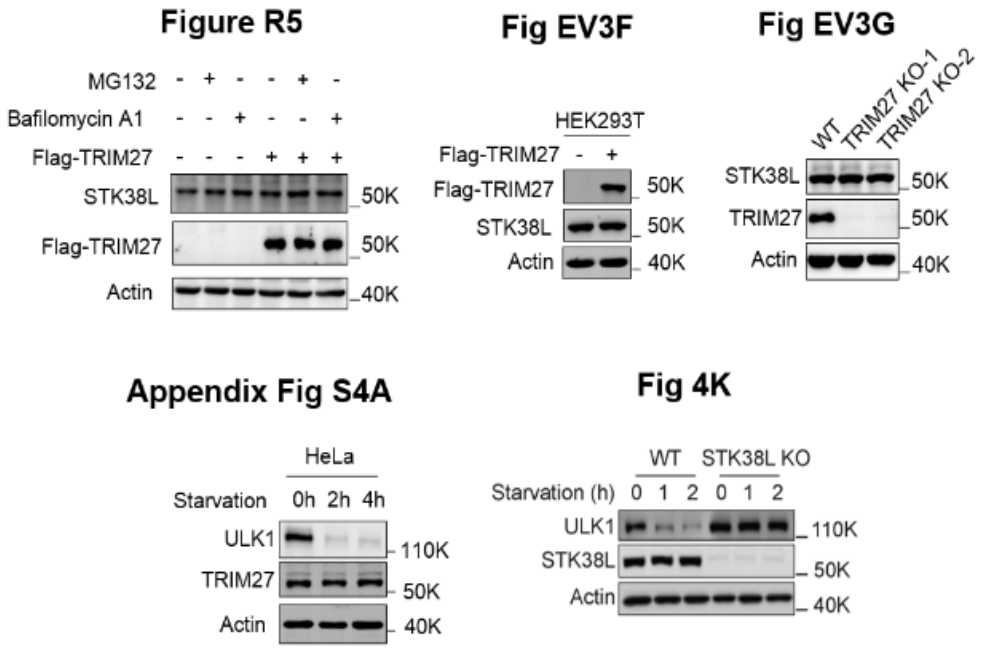
(2) Many different western blots are presented. However, almost no quantitative data is shown. At least the most critical blots should be repeated minimally three times, quantified and respective bar diagrams with error bars and statistical evaluations should be shown. This should be done minimally for: Figures 1H, 2D, 2E, 2L, 2O, 4E, 4H, 5A, 5M, 5N

Response: As requested, we conducted the quantitation of these key experiments and provide the companion bar graphs in the revision.

(3) K6 ubiquitination of STK38L, Figure 5J and S6: it is difficult to envision how the same E3 ligase catalyzes different ubiquitin linkages on different target proteins leading to different biological outcomes, especially as data presented in Figure S6A shows gradual differences. As an additional control authors should use BafA1 and MG132 and show that inhibition of the lysosome and proteasome does not affect STK38L protein level. This would further strengthen their argument that its ubiquitination affects activity and not abundance.

Response: Naturally this is one of the more interesting points concerning our study. Importantly, there are some precedents of the phenomenon that one E3 ligase can ubiquitinate different substates with different ubiquitin linkages. In one study, TRIM25 was shown to ubiquitinate RIP3 with K48-linkages to promote proteasomal degradation of RIP3 whereas another study showed that TRIM25 ubiquitinates TRAF2 via K63 linkages to enhance NF-kB signaling via stabilization of the TRAF2–TRAF5 complex (Mei,P el al. Cell Death Differ. 2021, PMID 33953350; Liu,Y et al. J Immunol. 2020, PMID: 32024699]). Nevertheless, we agree the reviewer raises an important point, although the proposed experiments with proteasomal/lysosomal inhibition are difficult to interpret because the half-life of STK38L along with TRIM27 and ULK1 are relatively long. As shown, we treated cells with and without overexpression ofTRIM27 with MG132 for 8 hours and Bafilomycin A for 4 hours, but there was little accumulation (Figure R5). Nevertheless, we believe a satisfactory answer to this question are provided by other experiments.

First as presented in the manuscript, Fig EV3F showed the level of STK38L remain unchanged in cells with overexpression of TRIM27. Furthermore, knockout of TRIM27 did not result in STK38L protein level changes (Fig EV3G). Importantly, under the physiological challenge of starvation which results in ULK1 degradation, neither TRIM27 nor STK38L are degraded (Appendix Fig S4A and Fig 4K). Since STK38L activity is increased during starvation without changes in its protein levels, this clearly shows that TRIM27-mediated ubiquitination of STK38L regulates its activity not its protein stability.



(4) Compared to the work done in vitro and in vivo in cell culture, the data presented in mice is not conclusive. Of course, these experiments are more demanding. However, in the current manuscript the presented data is "only" correlative and the role of

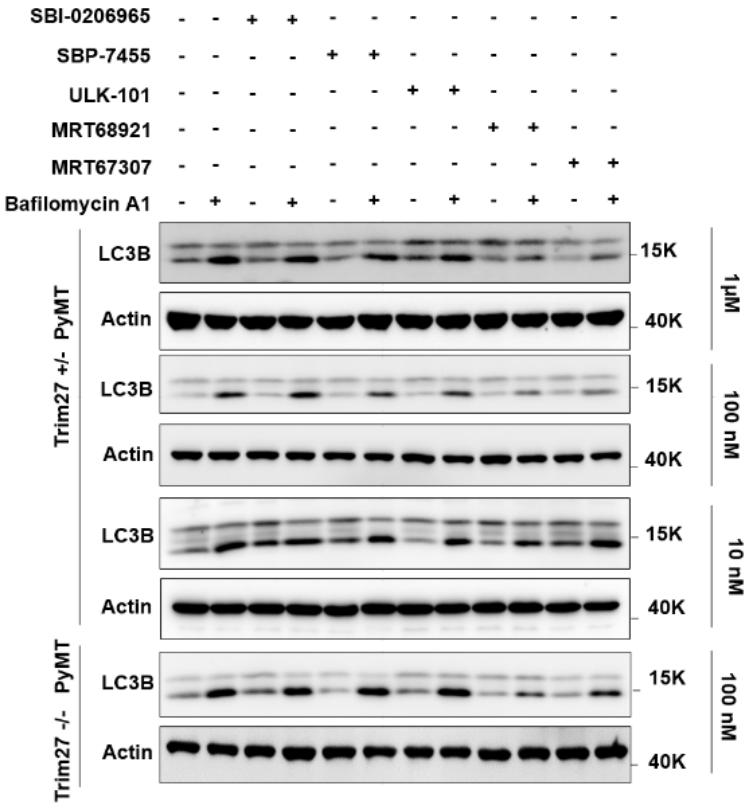
autophagy in tumorigenesis is not clear (as also stated by the authors in the discussion). Like this the title of the manuscript is also misleading. Whereas the effects of loss of TRIM27 are clear, it is not clear if this is mediated by autophagy or any other process affected by TRIM27. This is rather confusing given data presented in Figures 1-5. The authors have to cross their mice to autophagy incompetent mice such as Atg5-/- or Atg7-/- mice, or at least use an ULK1 inhibitor (if this is feasible) and analyze if this alters the effects of TRIM27 knockout. If this is too time demanding, I would rather suggest to restructure the manuscript and remove data presented in Figure 6.

Response: We fully agree with the Reviewer's sentiment that our conclusion regarding the mouse studies is based on associations with tumor growth/metastasis rather than fully demonstrating the causal role of autophagy. However, this issue is shared by all genetic approaches dealing with autophagy-related genes and for example, this issue was raised in our previous collaboration looking at the role of the autophagy adapter FIP200 and tumorigenesis (Wei H, et al. Genes Dev 2011, PMID: 21764854, Chen S et al. Genes Dev 2016, PMID: 27013233).

Since reviewer 1 and 2 didn't criticize these data, removing these data could cause problems. Also as suggested, the animal experiments proposed by the Reviewer not trivial. Specifically, to implement a genetic approach would require conditional knockout mice since germline deletion of Atg5 and Atg7 are both perinatal lethal. Thus, we would need to breed mice combining 4 alleles [Atg5 Flox/Flox (or Atg7), Trim27-/-, MMTV-Cre and MMTV PyMT]. This approach would require considerable time to generate solid data (between 1-2 years) and be complicated by the expected frequency of only 1/64 pups being the desired genotype. So, we rather explored the use of ULK1 inhibitors, namely specific inhibitors of ULK1 phosphorylation reported to inhibit autophagy.

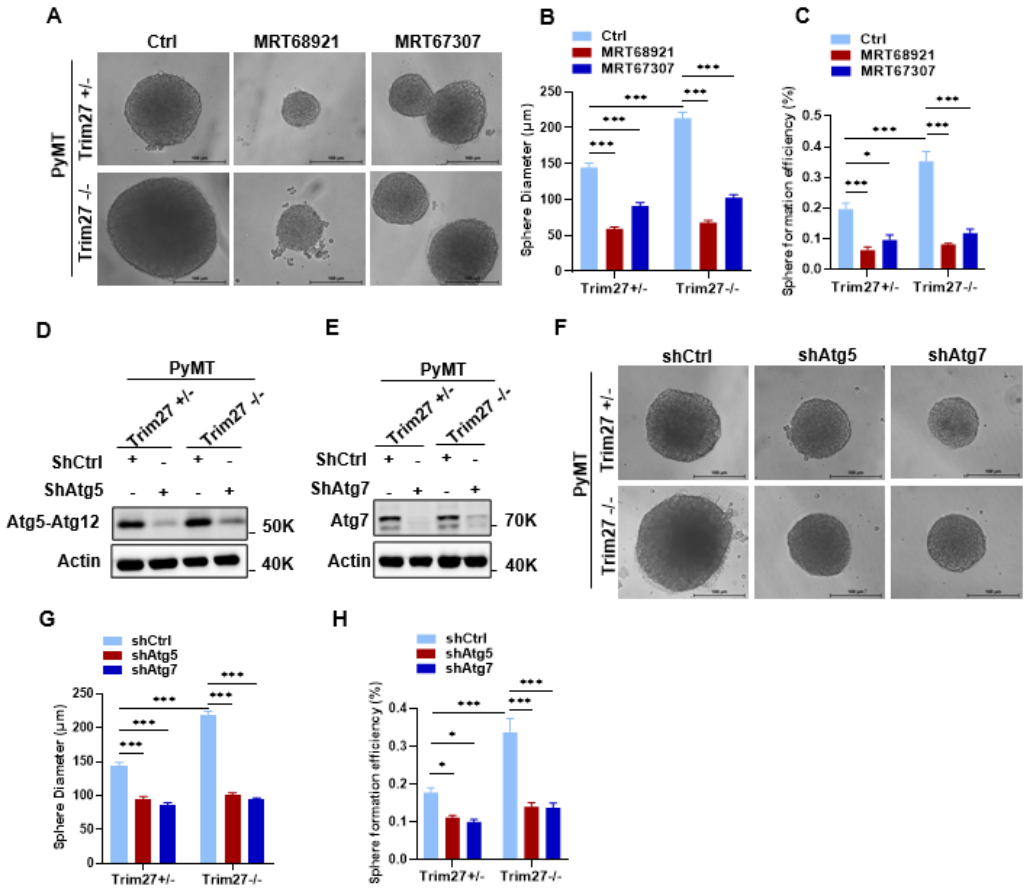
We started out with 5 commercially available ULK1 inhibitors and titrated these against mammary lines derived from the PyMT tumors (derived from the Trim27+/- and Trim27-/- lesions). On this basis, we selected MRT68921 and MRT67307 for further experiments since these were most effective at nanomolar levels as judged by autophagy inhibition in LC3 assays (Appendix Fig S7).

Appendix Fig S7



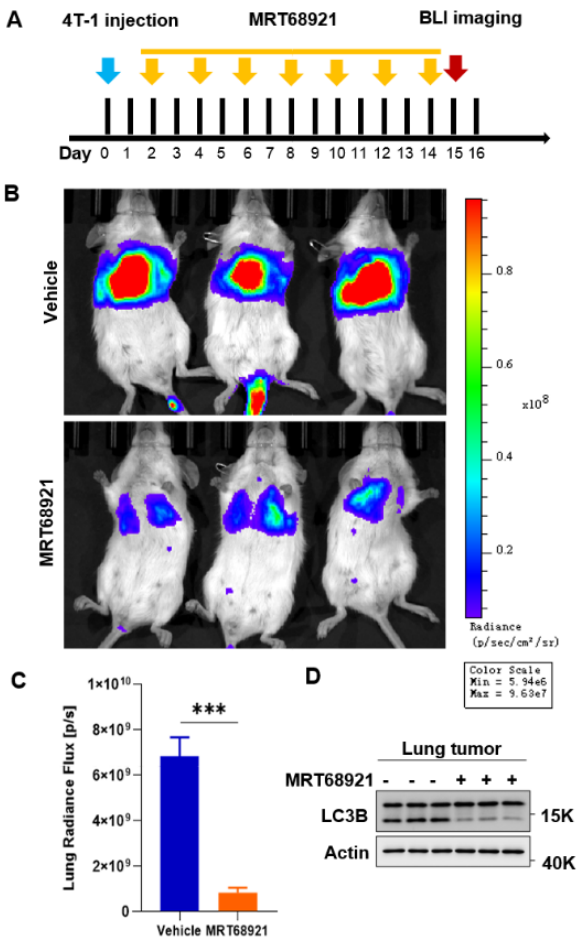
We then adapted the PyMT model to 3D organoid culture to examine the effects of TRIM27 knockout and autophagy inhibition. Interestingly, we found that organoid growth, as judged by organoid size and clonogenicity, was increased for the Trim27-/- mammary tumor cells (Fig. EV5A-C). This indicates that the in vivo latency did not inherently result from a growth deficit in these cells. Moreover, the growth advantage was nullified by ULK1 inhibition, rendering the growth/clonogenicity of Trim27+/- and Trim27-/- cells to the same basal growth state. Furthermore, knockdown of either Atg5 or Atg7 resulted in more significant growth inhibitory effects in Trim27-/- cells than in Trim27+/- organoids (Fig. EV5D-H), again reflecting that growth differences are mediated though differences in autophagy.

Fig EV5



Lastly, given that 4T1 cells are highly metastatic and strongly form lung metastases in vivo, and PyMT cells have relatively low metastatic potential of primary in transplant assays, as also indicated from the low sphere formation efficiency in vitro; Fig. EV5 A-C), we turned to using 4T1 for further metastasis studies. Consistent with the reports in the literature that autophagy inhibition inhibits metastasis (Mowers et al. oncogene, 2017, PMID: 27593926), we found that in vivo lung metastasis of 4T1 cells could be substantially blocked by ULK1 inhibition using MRT68921 (Fig. Appendix Figure 8 A-D). This further supports our original thesis that increases in autophagy in TRIM27 knockout cells could contribute to the increased metastasis. However, reconciling why there is latency in tumorigenesis still remains an open question, but these additional data strengthen the association between the effects of TRIM27 on autophagy and tumorigenesis in the PyMT model.

Appendix Figure S8



Minor points:

(1) Page 4, line 63: the yeast orthologs of ULK1 and mTOR are Atg1 and Tor1. The nomenclature should be adapted when yeast counterparts are discussed.

Response: We adjusted the nomenclature as suggested.

(2) Page4, line 70: autophagy is not only induced by AMPK activation but also by mTOR inhibition which leads to the removal of inhibitory mTOR sites on ULK1. This should be mentioned.

Response: As requested we modified the text to include the Reviewer's point that mTOR inhibition leads to the activation of ULK1.

(3) Page 5, line 85/86; "Some relationships between TRIM family proteins and autophagy are known". This is an understatement. More than 70 papers link TRIM proteins to autophagy. A brief summary should be given.

Response: Apologies, we did not intend to downplay this point. In our Introduction we did cite the most recent review on the subject that describes how TRIM proteins employ a variety of strategies to regulate autophagy (Rienzo et al., Cell Death Differ. 2020 [PMID:31969691]). As suggested, we expanded the information in this

paragraph to include a brief summary and provide some key examples that help illustrate the main points linking TRIM proteins with autophagy (line 85-98).

(4) Page 11, line 207: the western blot analyses LC3-II abundance not "expression".
Response: We have corrected this statement.

(5) Page 12, line 231: "nutrient starvation and is responsible for the accelerated degradation of ULK1". Accelerated compared to what? I would suggest to either delete "accelerated" or to exchange it with "stimulus-specific".
Response: We changed “accelerated” to“stimulus-specific” as suggested.

(6) How come ULK1 was not detected in STK38L IPs (Table S5)? This should at least be discussed.
Response: Table S5 lists the proteins found associated with TRIM27 including STK38L but in any event, we also noticed the absence of ULK1 in this screen. It is not so uncommon that mass spectrometry experiments do not reveal all positive hits that could otherwise be detected by other means. We can only speculate as to the reasons, but we highly suspect this relates to technical limitations since other methods, i.e., Western blot, clearly show that TRIM27 binds with ULK1 (Fig. 1F and 1J). Certainly, characteristics such as hydrophobicity or post-translational modifications are known to affect peptide identification in MS but the fact that ULK1 is a relatively large protein (150kDa) also inherently provides a challenge for MS. Apologies that a more definitive answer cannot provided but we trust the explanation is reasonable.

(7) Page 14, line 257: "indicative of inhibition of autophagic flux." This is a bit misleadingly formulated. An increase in LC3 conversion is indicative of an increased flux. Please rewrite.
Response: We changed the original text to “indicating that STK38L exerts an inhibitory effect on autophagy flux” which is a more precise statement.

(8) Page 18, line 349, Figure 6B: numbering in text and in figure do not match.
Response: Thanks for your careful attention. We corrected these inconsistencies in the revised manuscript.

Dear Dr Wu,

Thank you for submitting your revised manuscript (EMBOJ-2021-109777R) to The EMBO Journal, as well as for your patience with our response. Your amended study was sent back to the three referees for re-evaluation, and we have received comments from two of them, which I enclose below. Please note that we have editorially assessed your response to the concerns pointed to earlier by referee #2 and found these to be adequately responded to. As you will see, the other referees stated that their issues have been comprehensively resolved and they are now broadly in favour of publication, pending minor revision.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal.

Please consider the remaining points of referee #3 carefully, and address these by revising text and data presentation where appropriate.

In addition, we need you to take care of a number of issues related to formatting as detailed below, which should be addressed at re-submission.

Please contact me at any time if you have additional questions related to below points.

Thank you for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to your final revision.

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

Kind regards,

Daniel Klimmeck

Daniel Klimmeck PhD
Senior Editor
The EMBO Journal

Formatting changes required for the revised version of the manuscript:

>> Please move the 'Discussion' section after the 'Results' and before the 'Material and Methods' part of the manuscript.

>> Consider additional changes and comments from our production team as indicated by the .doc file enclosed and leave changes in track mode.

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (28th Jul 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

Link Not Available

Referee #1:

The authors have addressed all my concerns and therefore I support the publication.

Referee #3:

In the revised manuscript « TRIM27 Cooperates with STK38L to Inhibit ULK1-mediated Autophagy and Promotes Tumorigenesis » Yang and colleagues adequately addressed many of the raised concerns. I would like to especially highlight the new Figure EV5 in which a causal link between loss of TRIM27, autophagy, and tumor cell growth is highlighted using cell spheroids ! This is much appreciated.

However, I was not able to find the quantification of blots raised as major point 2 in the first round of revision.
(2) Many different western blots are presented. However, almost no quantitative data is shown. At least the most critical blots should be repeated minimally three times, quantified and respective bar diagrams with error bars and statistical evaluations should be shown. This should be done minimally for: Figures 1H, 2D, 2E, 2L, 2O, 4E, 4H, 5A, 5M, 5N.

In the point-by-point response the authors claim
"Response: As requested, we conducted the quantitation of these key experiments and provide the companion bar graphs in the revision."

Did they forget to include these data? I could also not find it in the accompanying source data. This point has still to be addressed. Right now, one could get the impression that many of the blots were only performed once.

We appreciate the reviewers' constructive contributions to our manuscript.

Referee #1:

The authors have addressed all my concerns and therefore I support the publication.

Referee #3:

In the revised manuscript « TRIM27 Cooperates with STK38L to Inhibit ULK1-mediated Autophagy and Promotes Tumorigenesis » Yang and colleagues
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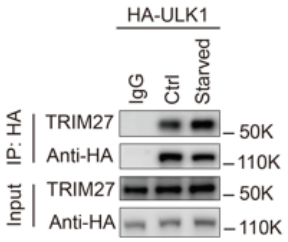
"Response: As requested, we conducted the quantitation of these key experiments and provide the companion bar graphs in the revision."

Did they forget to include these data? I could also not find it in the accompanying source data. This point has still to be addressed. Right now, one could get the impression that many of the blots were only performed once.

Response: Apologies, we forgot to highlight the quantification data requested which were appended to the EV figures. We can assure the reviewer that all of the key experiments were repeated at least three times with similar findings. These data were placed in the supplemental data due to the space limitations of the main figures. Specifically, the corresponding figures are as follows:
Appendix Fig S1E is the statistical evaluation for Fig 1H;
Fig EV1D is for Fig 2D;
Fig EV1G is for Fig 2E;
Fig EV1J is for Fig 2L;
Fig EV1K is for Fig 2O;
Appendix Fig S4E is for Fig 4E;
Appendix Fig S4G Fig 4H;
Fig EV3A is for Fig 5A;
Fig EV3H is for Fig 5M;
Fig EV3I is for Fig 5N.
For the reviewer's convenience, we included the comparison files below.

Fig 1H



Appendix Fig S1E

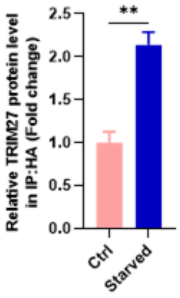


Fig 2D

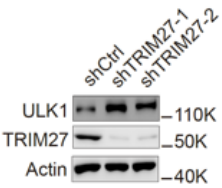


Fig EV1D

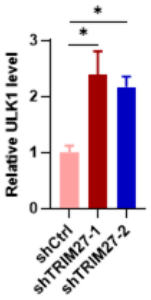


Fig 2E

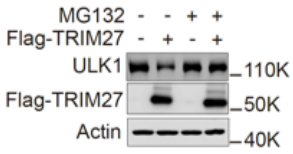


Fig EV1G

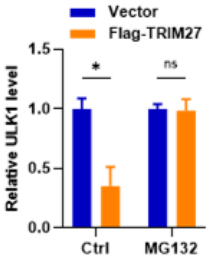


Fig 2O

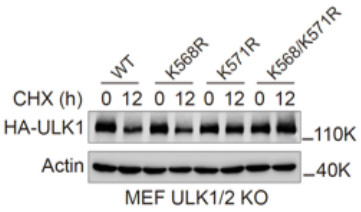


Fig 2L

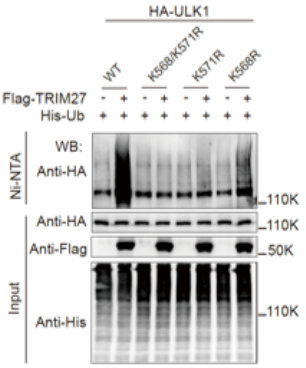


Fig EV1J

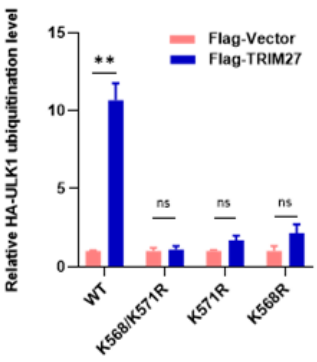


Fig EV1K

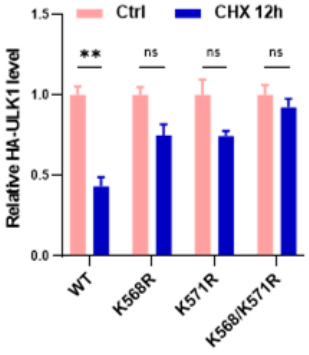
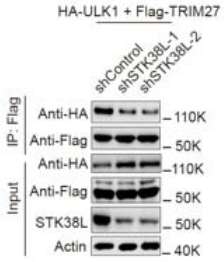


Fig 4E



Appendix Fig S4E

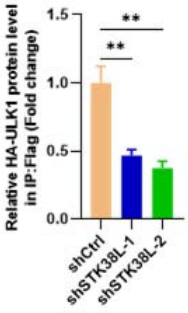
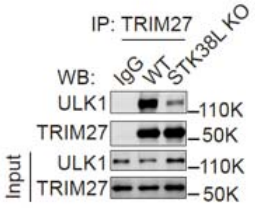


Fig 4H



Appendix Fig S4G

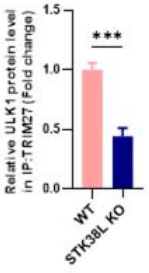


Fig 5A

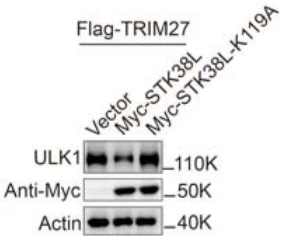


Fig EV3A

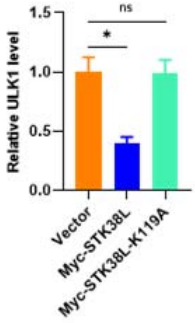


Fig 5N

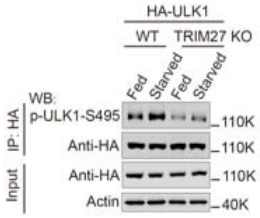


Fig EV3I

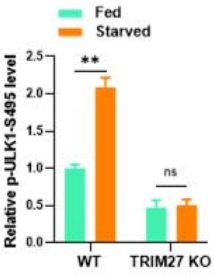


Fig 5M

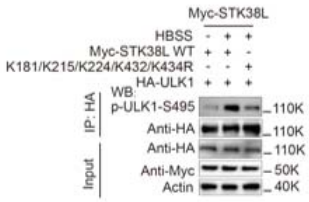
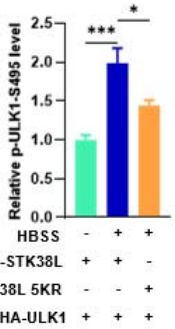


Fig EV3H



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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.
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- plots 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. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship 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.
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- 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.
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  - 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;
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Please complete ALL of the questions below.
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Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Yes	Materials and Methods
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