

MITOL promotes cell survival by degrading Parkin during mitophagy

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DOI: [10.15252/embr.202051903](https://doi.org/10.15252/embr.202051903)

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Review Timeline:

Submission Date:	19th Aug 19
Editorial Decision:	20th Sep 19
Revision Received:	15th Jan 20
Editorial Decision:	24th Feb 20
Revision Received:	16th Oct 20
Editorial Decision:	2nd Dec 20
Revision Received:	10th Dec 20
Accepted:	18th Dec 20

Editor: Deniz Senyilmaz Tiebe

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.)

Dear Prof. Yanagi

Thank you for submitting your manuscript entitled 'MITOL degrades Parkin to protect FKBP38 for cell survival in mitophagy' to EMBO Reports. We have now received four referee reports, which are included below.

The referees express interest in the proposed role of MITOL in regulation of apoptosis in mitophagy. However, they also raise concerns that preclude publication of the manuscript in this journal. Referees point out a number of concerns regarding the conclusiveness of the dataset and the methodologies used; therefore do not recommend publication here. Given these comments from recognized experts in the field who are also experienced reviewers, we cannot offer to publish your manuscript.

However, in case you feel that you can address the referee concerns in a timely and thorough manner, and can obtain data that would considerably strengthen the study as in the referee reports, we would have no objection to consider a revised manuscript (along with a point-by-point response to the referee concerns) in the future. Please note that if you were to send a new manuscript this would be assessed again with respect to the literature and the novelty of your findings at the time of resubmission and in case of a positive editorial evaluation, the manuscript would be sent back to the original referees. I would like to emphasize that we need strong support from the referees to consider publication here.

Please note that referee #4 contacted us separately to point out additional issues that he/she also summarizes in the general comment #6 of his/her report. He/she pointed out the absence of background bands in some lanes of some blots and thus finds that it is essential to supply loading controls that are clearly reprobes of the initial blots to support the data. Please note that we did not base our decision on these additional concerns. However, in our view these issues ought to be resolved before publication irrespective of the journal venue.

Thank you in any case for the opportunity to consider this manuscript. I am sorry that I cannot communicate more positive news, but nevertheless hope that you will find our referees' comments helpful.

Yours sincerely,

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

The manuscript by Shiiba I et al, 2019 suggests a possible mechanistic explanation for the dual role of Parkin during Parkin-mediated mitophagy and cell death through its interaction with a mitochondrial ubiquitin ligase MITOL. The authors proposed that MITOL functions to ubiquitinate K220 of Parkin to add K48-linked Ub chains on Parkin, targeting Parkin for degradation during early stage of PINK1/Parkin mitophagy and therefore inhibiting mitophagy. In the later stage of

PINK1/Parkin mitophagy, MITOL translocates to ER in a FKBP38-dependent manner to degrade phosphorylated Parkin on ER. As a result, ER-localised FKBP38 is protected from degradation and able to protect cells from Parkin-mediated cell death. In general, the manuscript presents some interesting data and is easy to follow. The involvement of FKBP38 in apoptosis during mitophagy has been shown before (DOI: 10.1038/ncomms2400), what's new in this manuscript is that MITOL can control FKBP38 fate to regulate this process. However, there are limitations in both parts of the proposed model which need to be carefully addressed prior to publication. Critically, the authors should at least discuss a recent publication regarding MITOL's role in mitophagy (doi: 10.1074/jbc.RA118.006302) which is in contrast to the author's findings.

Major comments:

1. MITOL overexpression was shown throughout the manuscript to be able to degrade Parkin. However, it was unknown whether Parkin is a specific or non-specific substrate of MITOL due to its overexpression. Parkin itself non-specifically ubiquitinates mitochondrial proteins upon depolarisation (DOI: 10.1074/jbc.RA118.006302). The authors should test if overexpression of MITOL also leads to ubiquitination and degradation of MtK27R-GFP and Mt-MBP-HA as employed in the suggested publication. This possibility might contribute to the fact that degradation of Parkin and the delayed mitophagy by endogenous MITOL were limited and only evident in the presence of CHX as pointed out by the authors (Fig 4C, D and EV2B). Therefore, it is important to confirm if endogenous MITOL can function in the same way as the overexpressed MITOL. To this end, the authors can: 1) Compare Parkin ubiquitination/degradation status in WT and MITOL KO cells without any overexpression of MITOL and Ubiquitin in the presence of proteasome inhibitors; 2) Carefully analyse PINK1/Parkin mitophagy efficiency in WT vs MITOL KO cells because it's likely that the depletion of MITOL doesn't block mitophagy but affect the efficiency of the process. Given the data from this manuscript suggests that overexpression of MITOL inhibited mitophagy, it can be expected that mitophagy will be faster in the absence of MITOL. However, measuring PINK1/Parkin via Tom20 degradation is not an appropriate method since Tom20 is a substrate of Parkin and one of the first proteins to be degraded by the proteasome (DOI: 10.1074/jbc.P110.209338). Mt-Keima assay is a more reliable method to determine mitophagy rate during PINK1/Parkin mitophagy (DOI: 10.1038/nature14893; DOI: 10.1038/s41467-019-08335-6; DOI: 10.1016/j.molcel.2019.02.010) and should be used instead. In addition, it should be considered when using CCCP to induce mitophagy as it inhibits lysosomal degradation of mitochondria also can affect the results. Please take a look at the most recent Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition).

Fig. 1B and 1D: There are no "Input" included.

Fig. 3B: the authors should make sure that expression levels of WT and CS MITOL are comparable.

2. It is claimed by the authors that "the Ubiquitination of MITOL was not affected by Parkin overexpression" in Fig. 2D but a control expressing MITOL-Flag but not Parkin with and without CCCP is missing. Alternatively, the authors could examine endogenous MITOL in the presence and absence of Parkin under mitophagy treatment. Additionally, in contrast to the idea that Parkin doesn't promote MITOL degradation, MITOL seemed to be degraded upon CCCP treatment in the presence of K220R Parkin (Fig. 3G). Could the authors explain this further? K220R mutation within Parkin is essential for its ubiquitination by overexpressed MITOL (Fig. 2F). Does this mutation affect Parkin translocation because K211N Parkin that was unable to be recruited to mitochondria was not ubiquitinated by MITOL (Fig. 2E)? It's also worthwhile mentioning that a recent publication by Matsuda's group (DOI: 10.1074/jbc.RA118.006302) suggests that MITOL promotes Parkin ubiquitination and translocation. The authors should discuss this in relation to their observations.

3. In the second part, the authors investigated the role of MITOL in regulating Parkin-mediated cell death upon mitophagy induction through interaction with both Parkin and FKBP38 on ER. FKBP38 has been shown to recruit Bcl-2 and Bcl-xL to mitochondria to inhibit intrinsic apoptosis (DOI: 10.1038/ncb894). During later stage of mitophagy, FKBP38 and Bcl-2 translocate to ER to inhibit

apoptosis (DOI: 10.1038/ncomms2400) whose mechanisms are not clear. Nevertheless, in this manuscript, using the same reagent CCCP, the authors showed that MITOL also translocated to ER with FKBP38 to degrade phosphorylated Parkin on ER to protect FKBP38. Therefore, the loss of MITOL resulted in more phosphorylated Parkin and less FKBP38 on ER, rendering MITOL KO cells more sensitive to CCCP-induced cell death (Fig. 5). Does the loss of MITOL affect cell death through regulating translocation/degradation of Bcl-2 on ER i.e. mitochondrial Bcl-2 vs ER Bcl-2 or whether ER Bcl-2 is degraded when ER FKBP38 is degraded during mitophagy? In the rest of the experiments where FKBP38 are affected, the effect of FKBP38 degradation on Bcl-2 should be examined. The authors did not mention the sole publication (DOI: 10.1038/ncomms2400) that demonstrates the anti-apoptotic activity of FKBP38 via Bcl-2.

Fig. 5C: Can the authors include WB for the total and mitochondrial fractions in WT and MITOL KO cells to show what fraction of phosphorylated Parkin is accumulated on ER?

Fig. 5B: This doesn't add much to the story. Imaging to show phosphorylated Parkin on the ER in WT vs MITOL KO cells would be more useful.

Fig. 5E: "In the MITOL KO cells, ER targeted FKBP38 was markedly degraded after CCCP treatment compared to WT cells". This conclusion cannot be assessed due to the missing WB panel.

Fig. 5G: Although overexpression of FKBP38 was not able to rescue cell death level in the MITOL KO cells, it should be taken into consideration the expression level of FKBP38 to that of Parkin. Because if excessive Parkin is available, FKBP38 will always be degraded according to the authors' model. How much of FKBP38 was expressed compared to KR FKBP38? Because the latter cannot be ubiquitinated, does it stay the same under mitophagy treatment in the absence of proteasome inhibitor. How does Bcl-2 level/translocation appear in cells expressing this mutant?

Fig. EV3A: "the amount of FKBP38 on the ER was found to be markedly decreased by MITOL". Could the authors include the total fraction to show the amount of FKBP38 in WT vs MITOL KO? The less ER amount could be due to the less total FKBP38 in MITOL KO to start with.

4. There are missing panels in a number of figures: Fig. 1E, Fig. 5E (as mentioned before), Fig. 4B (MITOL "IP" panel and tubulin "Input" panel) and Fig EV2.

5. There are quite a few occasions where things were mislabelled such as in Fig. 2 lane 6 at the top it says "-" HA-Parkin but the WB shows a signal for Parkin or in Fig. 4I the top panel should be Green and the bottom should be Red instead of being Red and Green respectively. The authors should carefully go through the manuscript and correct all the mislabelled figures.

Minor points:

1. It's not sure in some of the blots, endogenous MITOL, untagged MITOL or tagged was used. All of these seem to be marked at the same molecular weight. This should be clearly clarified in all MITOL blots.

Referee #2:

In this manuscript the authors propose a novel pathway in which the key regulator of mitophagy Parkin regulates cell death during the late stages of mitophagy via control of FKBP38 levels at the ER. Moreover, the authors propose that the E3 ubiquitin ligase MITOL negatively regulates this process by promoting degradation of phosphorylated Parkin at the ER, which in turn stabilizes FKBP38. Overall, the authors identify a potentially new and exciting mechanism for Parkin regulation. However, the manuscript relies heavily on MITOL overexpression for phenotypes, and contains a large amount of missing controls, errors, and inconsistencies throughout the data. These problems call into question the strength and rigor of the conclusions, and ultimately limit the impact of the authors' findings. Issues needing attention are listed below.

1. The authors demonstrate that overexpressed MITOL interacts with and ubiquitinylates Parkin, and regulates the extent of mitophagy in Figures 1-3. However, the use of overexpression constructs throughout the paper calls into question how much endogenous MITOL really regulates Parkin and mitophagy. The authors examine this in Figure 4, and the data is inconclusive. In Fig 4A, the IP showing interaction between overexpressed Parkin and endogenous MITOL contains no untagged controls to verify the specificity of the interaction. In 4B, the same IP shows no MITOL coming down with Parkin at all. Moreover, MITOL levels are not reduced by siMITOL in Figure 4C, and the lack of impact of MITOL knockdown on mitophagy in general suggests that endogenous MITOL may have no role in regulating mitophagy. These issues all need to be clarified to determine the extent to which endogenous MITOL regulates endogenous Parkin and mitophagy.

2. The data showing that MITOL regulates levels of phosphorylated, activated Parkin is nice in Figure 5. However, conclusion that this degradation occurs at the ER is premature, based on fractionation data in Figure 5C. 5C does not include any controls to demonstrate the effectiveness of ER and mitochondria separation in the fractionations. The authors need to include the mitochondrial fraction for 5C as they did in Fig 4H. The authors also need to examine Parkin localization via microscopy and determine whether a portion colocalizes with known ER markers in the absence of MITOL.

3. Along the same lines, the authors conclude that Parkin degrades FKBP38 at the ER. However, it is just as likely that Parkin degraded mitochondrial associated FKBP38, and that failure to do so allows for more of it to translocate to the ER upon CCCP. The authors should consider this conclusion.

4. The effect of FKBP38 mutations on the extent of cell death in Fig. 5G is not robust, and no significance values are included for the modest effects. This calls into question the authors model that destruction of FKBP38 by Parkin at the ER promotes cell death. The authors need to strengthen this data or dampen the conclusion that the point of all of this is to regulate cell death. It seems that MITOL may negatively regulate Parkin based on the presented data, but the impact that has on the cell is unclear.

5. Numerous blots and experiments throughout the manuscript are lacking controls, have missing bands, or are mislabeled. This is true throughout the manuscript, and makes interpretations of the data difficult. It also calls into question the rigor of the overall set of experiments. Specific issues noted that need correcting are:

a. Figure 1 should include data demonstrating that the GST tag does not interfere with MITOL localization or activity. Also, why is HA-Parkin detected in the GST minus sample? The authors state this is a GST pull down assay so if there is no GST tag present, there shouldn't be any pull down of HA-Parkin.

b. In 1F, the GFP signal is missing from lanes 3 and 4 of the input blot. Why is this?

c. What is the meaning of the R48K experiment in Figure 2C? It looks like the K63 mutation impacts ubiquitylation, and the 48K does not. Is this correct? This seems inconsistent with the authors' conclusion that K48 ubiquitylation is involved in the MITOL Parkin interaction.

d. Lacking citation for UbPred. In addition, several other statements are also lacking proper citation throughout the manuscript. For example, the authors need to either show or give a citation for the

statement "PINK1 is essential for the Parkin translocation to depolarized mitochondria". Later the authors state "In the PINK1 knockdown cells, the binding between MITOL and Parkin was decreased due to reduced Parkin recruitment to mitochondria" but the authors never cite or show this. Furthermore, the authors should show or cite data for the statement "We noticed that the protein level of Parkin was reduced earlier than the degradation of the OMM protein Tom20 in MITOL overexpression cells"

e. In 2D, the authors conclude that ubiquitylation of MITOL is not impacted by Parkin. However, all samples contain HA Parkin. How do the authors reach this conclusion?

f. In EV2A the authors blot for Parkin and MITOL with respective antibodies, and claim that MITOL is more lowly expressed than Parkin. These are two different antibodies, so the authors are not able to actually make this conclusion from this experiment. Moreover, there is nothing detectable in the MITOL lanes at all.

g. In 4B why is the alpha-tubulin blot in the input empty?

h. As stated in point 1 above, no MITOL comes down with Parkin-HA in the presence of CCCP in Fig 4B. This contradicts conclusion of 4A.

i. In 4C, why is there MITOL in all lanes? The label says that the last two lanes include siRNA against MITOL.

j. The authors need to include colocalization image with known ER marker in Fig 4F.

j. In addition to 4H, microscopy, similar to 4F, would really strengthen the claim "FKBP38 knockdown inhibited the translocation of MITOL to the ER" since fractionation assays can be messy when looking at ER vs. mitochondria and since mitophagy is decreasing the amount of mitochondria under these conditions.

k. The red and green labels appear completely switched in Figure 4I. Also, an ER marker should be included as a control.

l. For 5E the authors target FKBP38 to either the ER or the mitochondria to see where it is targeted for degradation by MITOL. They say that in MITOL KO cells, FKBP38 is markedly degraded when it is targeted to the ER but there is no change when it is targeted to the mitochondria. However, there is no FKBP38-IYFFT on the blot at all.

m. The authors need to verify that FKBP38-IYFFT is targeted to the ER and that FKBP38-ActA to the mitochondria.

n. Figure EV3 needs more controls for the fractionation, including other mitochondrial proteins.

Other more minor issues:

1. For 2A/2B/2C/2E, I would like to see the IP interaction with MITOL overexpression in these blots. The authors clearly have a MITOL antibody so showing an IP:HA MITOL probed blot would not be much work. Would be interesting to see if this interaction changes in the CS E3 ligase mutant or the KR/KN mutants as well.

2. Loading control for 3A is missing

3. Quantification of 3F and 3G. Especially in 3G, it looks like the levels of MITOL in the K220R MITOL overexpression samples are going down. Is it possible the authors are seeing a decrease in Tom20/increase in mitophagy because of this?

4. Show levels of HA-Parkin in 4E.

Referee #3:

In this study by Shiiba et al, the authors suggest that the mitochondrial ubiquitin ligase MITOL ubiquitinates and degrades the E3 ubiquitin ligase Parkin, and protect the antiapoptotic factor FKBP38 from Parkin-mediated degradation, thus promoting cell survival in mitophagy. Overall this is an interesting, and relatively well-designed study. However, I have raised a number of concerns below, that I feel should be addressed before publication in EMJO Rep. In particular, it is unclear where the MITOL-dependent and the Parkin dependent ubiquitination and degradation of Parkin and FKBP38, respectively, occur? And how Parkin recruits to ER?

- Overall, the data is clear. However, a vast majority is using biochemistry. While the biochemistry data are convincing, when possible, the authors should repeat the experiments with alternative methods (E.g. Immunofluorescence, IF).
- In Figure 2D, the authors claim that MITOL ubiquitination is "not affected by Parkin overexpression". Do they mean "by mitochondrial depolarisation"? In order to claim that Parkin overexpression doesn't affect MITOL ubiquitination, the experiment should be repeated with or without Parkin over-expression.
- In Figure 2E, the authors state that "Parkin is ubiquitinated by MITOL after its translocation onto mitochondria". Have they checked whether K211 is a target lysine for MITOL (E.g. using a mitochondrially-targeted Parkin K211R mutant)?
- In figure 3D, the authors state that "MITOL ubiquitinates and subsequently degrades Parkin on mitochondria". The authors should assess the purity of their "mitochondria-rich membrane fraction" by probing with ER markers. In fact, in the last paragraph of the introduction, the authors write that "MITOL translocates to the ER... and degrades Parkin". So where does MITOL-dependent ubiquitination and degradation of Parkin occur? In the mitochondria? In the ER? This should be determined using microscopy.
- In figure 3E, the authors should use an alternative method to assess mitophagy (E.g. mitoKeima/QC).
- In Figure 4F, the authors should perform a CCCP time course and include both mitochondria and ER markers.
- Figure 4G should be repeated with endogenous MITOL (and FKBP38 if possible).
- Figure 4H should be repeated using IF.
- Figure 4I should be repeated with mito and ER markers, and with FKBP38 siRNA. Also, the principle of the Kik-GR is not very well explained for non-specialist readers.
- Figure 5A should be repeated using a Ser65 Parkin antibody
- The blot in Figure 5C should be probed with a mitochondrial marker to assess to purity of the fraction
- In Figure 5D, what is the effect of the Parkin C431S and S65A mutants?
- In Figure 5E, the authors claim that "ER-targeted FKBP38 was markedly degraded after CCCP treatment compared to WT cells". But it is not detected, even in non-CCCP treated fractions? Is it

even expressed? The experiment should be repeated in WB and in IF. Minor comment: in the next sentence, it should be "in contrast", not "in constant"

- Where does the interaction between Parkin and FKBP38 occur? Where does the Parkin-dependent ubiquitination and degradation occur? In the mito and/or in the ER?

- How is Parkin recruited to the ER? Is it dependent on FKBP38? There is an attempt to discuss it in the discussion but this is not very clear. This should be addressed with experiments.

Referee #4:

Understanding the molecular control of Parkin activity in mitophagy in cells receiving mitochondrial damage is important to elucidate its disfunction as causal in certain cases of early onset Parkinson's disease. The current manuscript finds that Parkin itself is controlled following reception of mitochondrial insult by the mitochondrial E3 ubiquitin ligase MITOL (also known as MARCH5). Based largely on overexpression studies, the authors find a direct interaction between these proteins during mitophagy and that MITOL promotes proteasomal degradation of Parkin by K48-linked ubiquitination to negatively regulate mitophagy. They finally provide some data to suggest that MITOL itself shuttles from the mitochondria to ER in the final stages of mitophagy to limit apoptosis involving FKBP38, although I found this aspect of the manuscript less compelling. Overall, the paper reports an interesting finding into the regulation of Parkin.

General comments

1. The paper is almost entirely based on over-expression of either MITOL, Parkin or both. In order to support the potential importance of this mechanism in the control of mitophagy it is essential that they confirm at least some of their findings (i.e interaction, ubiquitination and mitophagy) in a cell line that endogenously expresses both Parkin and MITOL (e.g. SH-SY5Y). The evidence that Parkin was ubiquitinated is limited to IP of the over-expressed protein followed by Western blotting for ubiquitin. However, there is no direct evidence of Parkin being modified either by mass spec to identify diGly motifs on Parkin peptides or detection of modified forms of the protein following Western blotting even in the presence of proteasomal inhibition when ubiquitinated forms might be expected to accumulate. A more quantitative assay to support potential defects in mitophagy would be FACS based assessment of mt-Keima. Together, it is very difficult to gauge how much of the Parkin is actually modified by MITOL, and how significant this mechanism is.

Furthermore, several groups have published a role for MITOL/MARCH5 in controlling mitochondrial dynamics. Could the influence on mitophagy here be a consequence of MITOL's role in regulating mitochondrial form/function rather than a direct influence on Parkin?

2. The authors over-express mutant ubiquitin to attempt to define the linkages catalysed by MITOL on Parkin. These assays are confounded by the endogenous ubiquitin expressed in the cells. I would recommend a more definitive approach would be mass spectrometry or a de-ubiquitinase assay using specific DUBs of a Parkin IP.

3. The authors do report that endogenous MITOL could only mediate Parkin degradation in the presence of CHX (Figure 4D). The justification and conclusions from this experiment are not clear, but it implies that new protein translation (presumably Parkin?) masks any decrease in an endogenous (MITOL) setting. The authors should provide more detailed discussion of this data and its difference from when MITOL is ectopically over-expressed.

4. In Figure 4C they switch to using MITOL siRNA when in other data they utilise KO cells. However, the level of MITOL knockdown with siRNA is minimal. The reason for switching to siRNA for this context was not clear, but given the inefficient knock-down they should use the ko cells.

5. In general, I did not find the data suggesting redistribution of MITOL to the ER in late stage

mitophagy to regulate apoptosis compelling. In Figure 4F they only show an image of a single cell to concluded colocalization of MITOL with FKBP after CCCP. The authors need to include mitochondrial and ER markers together with image quantification to support this redistribution. Figure 4I would be strengthened by showing a DAPI/Hoechst stain of the cells. The authors should confirm that CCCP is driving apoptosis by blocking with caspase inhibitors. Also, the death that the authors observe following CCCP may be independent of Parkin. To confirm Parkin's role the authors should show the cell death of MITOL KO HeLa cells that do not express Parkin.

6. Much of the conclusions of the study hinge on the loss of Parkin protein following activation of mitophagy in the presence of MITOL (e.g Fig3C, F, and H). However, for some of these blots that can be expanded, there is indication that the loading may not be equivalent (see HA blots in Fig3C, F, and H). The authors should provide evidence that the actin loading controls are reprobes of the same blot to remove any such concern. Likewise for the subcellular fractionation blots in 3D and 4H, I recommend that the authors provide evidence to show that the loading controls are reprobes of the same blot rather than separate blots.

Specific comments

1. Figure 1B, not clear what the "-" samples are. Presumably Input?
2. Figure 1E, there is almost no signal for input HA
3. Figure 2B and C- authors should reprobe input fractions with anti-FLAG.
4. Figure 2D- authors should reprobe input fractions with anti-Myc
5. Figures 1F and 2E use Parkin K211N mutant to conclude that interaction and ubiquitination is dependent on mitochondrial localisation. If possible this data could be shown together. Also, binding of Parkin to phospho-Ub precedes the phosphorylation of Parkin as described in the text (page 12).
6. Figure 5C MITOL was detected at the ER fraction after CCCP treatment. Authors need to probe for mitochondrial markers to show the purity of their ER fractions.
7. Figure 1E, the HA blot is blank.
8. Figure 4B, the MITOL IP blot is blank, tubulin input blot is blank.
9. Figure EV2, the MITOL blot is blank.
10. Figure 5E the FKBP38YFFT blot is blank.
11. In addition, the authors attempt to express Parkin to the same level as that observed in the striatum. However, given this is mouse tissue and they are ectopically expression human Parkin, they cannot draw this conclusion as the antibody may detect human and mouse Parkin differently.
12. In general the manuscript is well-written and easy to follow. However, it would benefit from being carefully proof-read for grammatical and spelling errors.

We would like to thank the four Referees for their comments as their suggestions allowed us to obtain new insights and results in the manuscript which needs greater clarification. We have performed additional experiments and corrected numerous mistakes.

The point-by-point responses to the Referee comments are listed below.

Referee #1:

Major comments:

1. MITOL overexpression was shown throughout the manuscript to be able to degrade Parkin. However, it was unknown whether Parkin is a specific or non-specific substrate of MITOL due to its overexpression. Parkin itself non-specifically ubiquitinates mitochondrial proteins upon depolarisation (DOI: 10.1074/jbc.RA118.006302). The authors should test if overexpression of MITOL also leads to ubiquitination and degradation of MtK27R-GFP and Mt-MBP-HA as employed in the suggested publication. This possibility might contribute to the fact that degradation of Parkin and the delayed mitophagy by endogenous MITOL were limited and only evident in the presence of CHX as pointed out by the authors (Fig 4C, D and EV2B). Therefore, it is important to confirm if endogenous MITOL can function in the same way as the overexpressed MITOL. To this end, the authors can: 1) Compare Parkin ubiquitination/degradation status in WT and MITOL KO cells without any overexpression of MITOL and Ubiquitin in the presence of proteasome inhibitors; 2) Carefully analyse PINK1/Parkin mitophagy efficiency in WT vs MITOL KO cells because it's likely that the depletion of MITOL doesn't block mitophagy but affect the efficiency of the process. Given the data from this manuscript suggests that overexpression of MITOL inhibited mitophagy, it can be expected that mitophagy will be faster in the absence of MITOL. However, measuring PINK1/Parkin via Tom20 degradation is not an appropriate method since Tom20 is a substrate of Parkin and one of the first proteins to be degraded by the proteasome (DOI: 10.1074/jbc.P110.209338). Mt-Keima assay is a more reliable method to determine mitophagy rate during PINK1/Parkin mitophagy (DOI: 10.1038/nature14893; DOI: 10.1038/s41467-019-08335-6; DOI: 0.1016/j.molcel.2019.02.010) and should be used instead. In addition, it should be considered when using CCCP to induce mitophagy as it inhibits lysosomal degradation of mitochondria also can affect the results. Please take a look at the most recent Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition).

Our reply:

We appreciate your kind comments. To determine whether Parkin is a specific or non-specific substrate of MITOL, we performed a similar experiment using Mito-GFP. As previously reported, Mito-GFP ubiquitylation by Parkin was reconfirmed, whereas GFP-mito ubiquitylation by MITOL was not observed (Figure EV3B), suggesting that distinct from Parkin, MITOL displays strict substrate-specificity. Actually, we have previously demonstrated that MITOL specifically interacts with and ubiquitinates Mfn2, but not Mfn1 [1]. Similarly, we identified each specific interaction domain between MITOL and Parkin. Thus, we conclude that Parkin is a specific substrate of MITOL. 1) According to this comment, we compared Parkin ubiquitination status in WT and MITOL KO cells without any overexpression of MITOL and ubiquitin in the presence of proteasome inhibitors (Figure 1G). Parkin ubiquitination is reduced in MITOL KO cells. 2) Using Mitophagy Dye (Dojindo), mitophagy was quantified in MITOL overexpression and WT/KO cells (Figure 2E and F). Mitophagy was strongly suppressed by MITOL overexpression, whereas mitophagy was slightly enhanced in MITOL KO cells. These results suggest that endogenous MITOL plays a slight inhibitory role in mitophagy by parkin ubiquitylation on mitochondria.

Fig. 1B and 1D: There are no "Input" included. Fig. 3B: the authors should make sure that expression levels of WT and CS MITOL are comparable.

Our reply:

I am sorry for this careless mistake. The notation (-) in our previous manuscript was input. We corrected this error. MITOL degrades itself by auto-ubiquitination. Since auto-ubiquitination of CS is inhibited, CS is more accumulated than WT. This is the reason why the protein amounts of WT and CS are differently detected (Figure 2B).

2. It is claimed by the authors that "the Ubiquitination of MITOL was not affected by Parkin overexpression" in Fig. 2D but a control expressing MITOL-Flag but not Parkin with and without CCCP is missing. Alternatively, the authors could examine endogenous MITOL in the presence and absence of Parkin under mitophagy treatment. Additionally, in contrast to the idea that Parkin doesn't promote MITOL degradation, MITOL seemed to be degraded upon CCCP treatment in the presence of K220R Parkin (Fig. 3G). Could the authors explain this

further? K220R mutation within Parkin is essential for its ubiquitination by overexpressed MITOL (Fig. 2F). Does this mutation affect Parkin translocation because K211N Parkin that was unable to be recruited to mitochondria was not ubiquitinated by MITOL (Fig. 2E)? It's also worthwhile mentioning that a recent publication by Matsuda's group (DOI: 10.1074/jbc.RA118.006302) suggests that MITOL promotes Parkin ubiquitination and translocation. The authors should discuss this in relation to their observations.

Our reply:

I apologize for my lack of explanation in the text. Here, we tried to insist that MITOL is not regulated by ubiquitin signaling in mitophagy, because no increase or decrease in CCCP-dependent ubiquitination of MITOL was observed. We rewrote the text to avoid misleading. Next, we explain the reason that MITOL expression is reduced in the experiment using Parkin K220R mutant (Figure 2C). The amount of FKBP38 determines the amount of MITOL that can translocate to the ER. Therefore, the overexpressed MITOL that fails to interact with endogenous FKBP38 remains in mitochondria and undergoes degradation in mitophagy with mitochondria. Since K220R mutant can normally execute mitophagy, overexpressed MITOL is degraded in a mitochondrial degradation-dependent manner. We therefore speculate that a small part of MITOL, which can escape from mitochondria and translocate to the ER with FKBP38 during mitophagy, remains in the ER. As a reviewer pointed out, it was possible that the K220R mutant could not translocate to mitochondria. Therefore, we demonstrated that mitochondrial translocation of K220R mutant was the same as that of the wild type Parkin (Figure EV3E).

3. In the second part, the authors investigated the role of MITOL in regulating Parkin-mediated cell death upon mitophagy induction through interaction with both Parkin and FKBP38 on ER. FKBP38 has been shown to recruit Bcl-2 and Bcl-xL to mitochondria to inhibit intrinsic apoptosis (DOI: 10.1038/ncb894). During later stage of mitophagy, FKBP38 and Bcl-2 translocate to ER to inhibit apoptosis (DOI: 10.1038/ncomms2400) whose mechanisms are not clear. Nevertheless, in this manuscript, using the same reagent CCCP, the authors showed that MITOL also translocated to ER with FKBP38 to degrade phosphorylated Parkin on ER to protect FKBP38. Therefore, the loss of MITOL resulted in more phosphorylated Parkin and less FKBP38 on ER, rendering MITOL KO cells more sensitive to CCCP-induced cell death (Fig. 5). Does the loss of MITOL affect cell death through regulating translocation/degradation of Bcl-2

on ER i.e. mitochondrial Bcl-2 vs ER Bcl-2 or whether ER Bcl-2 is degraded when ER FKBP38 is degraded during mitophagy? In the rest of the experiments where FKBP38 are affected, the effect of FKBP38 degradation on Bcl-2 should be examined. The authors did not mention the sole publication (DOI: 10.1038/ncomms2400) that demonstrates the anti-apoptotic activity of FKBP38 via Bcl-2.

Our reply:

According to this kind comment, we examined the intracellular localization of Bcl-2 in MITOL WT / KO during mitophagy by immunofluorescence staining. Interestingly, Bcl-2 was able to translocate from mitochondria to the ER during mitophagy in MITOL WT cells, but it diffusely localized in the cytosol in MITOL KO cells (Figure EV5C). Cell organelle fractionations experiment also revealed that a cytosolic localization of Bcl-2 were increased in MITOL KO cells (Figure 4C) although the total expression level of Bcl-2 was not changed. This result suggests that reduced FKBP38 by MITOL deletion impaired Bcl-2 function at the ER, leading to cell death.

Fig. 5C: Can the authors include WB for the total and mitochondrial fractions in WT and MITOL KO cells to show what fraction of phosphorylated Parkin is accumulated on ER?

Our reply:

We performed WB for each organelle fraction in WT and MITOL KO cells and showed that phosphorylated Parkin is accumulated on ER (Figure 4C).

Fig. 5B: This doesn't add much to the story. Imaging to show phosphorylated Parkin on the ER in WT vs MITOL KO cells would be more useful.

Our reply:

Since PLA signal was much weaker than ER marker, it was not suitable for double staining with ER marker in Figure 4B. Instead, we showed the distribution of Parkin in the ER by using Parkin-KikGR and ER marker in Figure 4D.

Fig. 5E: "In the MITOL KO cells, ER targeted FKBP38 was markedly degraded after CCCP treatment compared to WT cells". This conclusion cannot be assessed due to the missing WB panel.

Our reply:

We are very sorry for this careless mistake. The figure was missing when the resolution of the figure was decreased. The figure was shown in Figure 5B.

Fig. 5G: Although overexpression of FKBP38 was not able to rescue cell death level in the MITOL KO cells, it should be taken into consideration the expression level of FKBP38 to that of Parkin. Because if excessive Parkin is available, FKBP38 will always be degraded according to the authors' model. How much of FKBP38 was expressed compared to KR FKBP38? Because the latter cannot be ubiquitinated, does it stay the same under mitophagy treatment in the absence of proteasome inhibitor. How does Bcl-2 level/translocation appear in cells expressing this mutant?

Our reply:

We compared the amounts of WT-FKBP38 and KR-FKBP38 in Figure EV5A. We showed that a major part of WT-FKBP38 disappeared by degradation during mitophagy. That is the reason that WT-FKBP38 has a low efficiency to rescue cell death.

Fig. EV3A: "the amount of FKBP38 on the ER was found to be markedly decreased by MITOL". Could the authors include the total fraction to show the amount of FKBP38 in WT vs MITOL KO? The less ER amount could be due to the less total FKBP38 in MITOL KO to start with.

Our reply:

We are sorry for this fuzzy figure. We isolated each fraction and demonstrated that there is no change in the amount of FKBP38 between MITOL WT and KO cells at the resting condition (Figure 4C). Expectedly, there is a difference in the amount of FKBP38 depending on mitophagy.

4. There are missing panels in a number of figures: Fig. 1E, Fig. 5E (as mentioned before), Fig. 4B (MITOL "IP" panel and tubulin "Input" panel) and Fig EV2.

Our reply:

We are sorry for our careless mistakes. Figures were appropriately improved.

5. There are quite a few occasions where things were mislabelled such as in Fig. 2 lane 6 at the top it says "-" HA-Parkin but the WB shows a signal for Parkin or in Fig. 4I the top panel should be Green and the bottom should be Red instead of being Red and Green respectively. The authors should carefully go through the manuscript and correct all the mislabelled figures.

Our reply:

We are sorry for our careless mistakes. They were appropriately improved.

Minor points:

1. It's not sure in some of the blots, endogenous MITOL, untagged MITOL or tagged was used. All of these seem to be marked at the same molecular weight. This should be clearly clarified in all MITOL blots.

Our reply:

We clearly clarified them in all MITOL blots.

Referee #2:

In this manuscript the authors propose a novel pathway in which the key regulator of mitophagy Parkin regulates cell death during the late stages of mitophagy via control of FKBP38 levels at the ER. Moreover, the authors propose that the E3 ubiquitin ligase MITOL negatively regulates this process by promoting degradation of phosphorylated Parkin at the ER, which in turn stabilizes FKBP38. Overall, the authors identify a potentially new and exciting mechanism for Parkin regulation. However, the manuscript relies heavily on MITOL overexpression for phenotypes, and contains a large amount of missing controls, errors, and inconsistencies throughout the data. These problems call into question the strength and rigor of the conclusions, and ultimately limit the impact of the authors' findings. Issues needing attention are listed below.

1. The authors demonstrate that overexpressed MITOL interacts with and ubiquitinylates Parkin, and regulates the extent of mitophagy in Figures 1-3. However, the use of overexpression constructs throughout the paper calls into question how much endogenous MITOL really regulates Parkin and mitophagy. The authors examine this in Figure 4, and the

data is inconclusive. In Fig 4A, the IP showing interaction between overexpressed Parkin and endogenous MITOL contains no untagged controls to verify the specificity of the interaction. In 4B, the same IP shows no MITOL coming down with Parkin at all. Moreover, MITOL levels are not reduced by siMITOL in Figure 4C, and the lack of impact of MITOL knockdown on mitophagy in general suggests that endogenous MITOL may have no role in regulating mitophagy. These issues all need to be clarified to determine the extent to which endogenous MITOL regulates endogenous Parkin and mitophagy.

Our reply:

Thank you for your indications. The difference of MITOL I.P. data between previous Figures 4A and 4B was caused by the difference in the composition of the lysis buffer. If Parkin ubiquitylation was detected using NP-40 buffer as same as previous Figure 4A is detected, it is possible that auto-ubiquitinated MITOL bound to Parkin is contaminated with Parkin ubiquitylation. To avoid this possibility, we used RIPA buffer and sonicated samples in previous Figure 4B to dissociate MITOL from Parkin. In order to strongly suggest a direct regulation of Parkin by endogenous MITOL, we compared Parkin ubiquitylation in each organelle fraction of MITOL WT/KO cells (Figure 1G). Furthermore, we examined the effect of endogenous MITOL in mitophagy by using mtphagy Dye, and found that mitophagy was slightly up-regulated by MITOL knockout (Figure 2E and F), suggesting a limited role of endogenous MITOL in Parkin-mediated mitophagy. In addition, when the amount of phosphorylated Parkin in each organelle fraction was compared, the phosphorylated parkin was remarkably accumulated in MITOL KO cells (Figure 4C). These results suggest that endogenous MITOL regulates the amount of phosphorylated Parkin so as not to affect mitophagy, and prevents cell death caused by phosphorylated Parkin.

2. The data showing that MITOL regulates levels of phosphorylated, activated Parkin is nice in Figure 5. However, conclusion that this degradation occurs at the ER is premature, based on fractionation data in Figure 5C. 5C does not include any controls to demonstrate the effectiveness of ER and mitochondria separation in the fractionations. The authors need to include the mitochondrial fraction for 5C as they did in Fig 4H. The authors also need to examine Parkin localization via microscopy and determine whether a portion colocalizes with known ER markers in the absence of MITOL.

Our reply:

Following your kind advice, we measured the amount of Parkin and phosphorylated Parkin in each organelle fraction (Figure 4C). In addition, Parkin-KikGR was used to observe the localization of Parkin in MITOL KO cells (Figure 4D). From these data, it can be seen that when mitophagy is induced in MITOL KO cells, Parkin is localized and accumulated in the ER.

3. Along the same lines, the authors conclude that Parkin degrades FKBP38 at the ER. However, it is just as likely that Parkin degraded mitochondrial associated FKBP38, and that failure to do so allows for more of it to translocate to the ER upon CCCP. The authors should consider this conclusion.

Our reply:

Thank you for your comment. It is difficult to deny that possibility. We could not completely determine whether Parkin degrades FKBP38 on mitochondria or FKBP38 that has translocated to the ER. However, we confirmed that the degradation rate of FKBP38 in MITOL KO cells is not significantly different from that of WT cells at the beginning of mitophagy. We speculate that Parkin regulates FKBP38 not only on mitochondria but also on the ER during late phase of mitophagy.

4. The effect of FKBP38 mutations on the extent of cell death in Fig. 5G is not robust, and no significance values are included for the modest effects. This calls into question the authors model that destruction of FKBP38 by Parkin at the ER promotes cell death. The authors need to strengthen this data or dampen the conclusion that the point of all of this is to regulate cell death. It seems that MITOL may negatively regulate Parkin based on the presented data, but the impact that has on the cell is unclear.

Our reply:

To further understand the role of MITOL-induced degradation of Parkin on cell death, we examined the effect of Parkin K220R mutant on cell death and found that Parkin K220R mutant caused the same degree of cell death as in MITOL KO cells (Figure 5E). However, in the rescue experiment of KR mutant of FKBP38, the rescue efficiency was almost the same as that of MITOL KO cells. As I mentioned in the discussion, it is possible that not only FKBP38 Parkin randomly degrades other substrates on the ER and cause cell death in mitophagy.

5. Numerous blots and experiments throughout the manuscript are lacking controls, have missing bands, or are mislabeled. This is true throughout the manuscript, and makes interpretations of the data difficult. It also calls into question the rigor of the overall set of experiments. Specific issues noted that need correcting are:

a. Figure 1 should include data demonstrating that the GST tag does not interfere with MITOL localization or activity. Also, why is HA-Parkin detected in the GST minus sample? The authors state this is a GST pull down assay so if there is no GST tag present, there shouldn't be any pull down of HA-Parkin.

Our reply:

We are sorry for these careless mistakes. We improved this Figure and sentence.

b. In 1F, the GFP signal is missing from lanes 3 and 4 of the input blot. Why is this?

Our reply:

We are sorry for this careless mistake. We improved this Figure.

c. What is the meaning of the R48K experiment in Figure 2C? It looks like the K63 mutation impacts ubiquitylation, and the 48K does not. Is this correct? This seems inconsistent with the authors' conclusion that K48 ubiquitination is involved in the MITOL Parkin interaction.

Our reply:

I was sorry for not being clear enough. Since the KR mutant changes lysine at positions 48 and 63 of ubiquitin to arginine, mutants that cannot form ubiquitin chains are the relevant ubiquitin species (Figure EV2C). On the other hand, the RK mutant changes all ubiquitin lysines to arginine and then changes arginine at positions 48 and 63 to lysine (Figure EV2D). Therefore, a mutant that can form a ubiquitin chain is the relevant ubiquitin species.

d. Lacking citation for UbPred. In addition, several other statements are also lacking proper citation throughout the manuscript. For example, the authors need to either show or give a citation for the statement "PINK1 is essential for the Parkin translocation to depolarized

mitochondria". Later the authors state "In the PINK1 knockdown cells, the binding between MITOL and Parkin was decreased due to reduced Parkin recruitment to mitochondria" but the authors never cite or show this. Furthermore, the authors should show or cite data for the statement "We noticed that the protein level of Parkin was reduced earlier than the degradation of the OMM protein Tom20 in MITOL overexpression cells"

Our reply:

We added several references and corrected the inappropriate expressions.

e. In 2D, the authors conclude that ubiquitinylation of MITOL is not impacted by Parkin. However, all samples contain HA Parkin. How do the authors reach this conclusion?

Our reply:

I apologize for misleading you. What we wanted to mention here is that MITOL is not regulated by ubiquitin signaling in mitophagy, since no increase or decrease in CCCP-dependent ubiquitylation of MITOL was observed (Figure EV2A). The text was rewritten to avoid the misunderstanding.

f. In EV2A the authors blot for Parkin and MITOL with respective antibodies, and claim that MITOL is more lowly expressed than Parkin. These are two different antibodies, so the authors are not able to actually make this conclusion from this experiment. Moreover, there is nothing detectable in the MITOL lanes at all.

Our reply:

I agree with your opinion. We performed various experiments, but withdrew relevant text and data because we were unable to prove what we wanted to say.

g. In 4B why is the alpha-tubulin blot in the input empty?

Our reply:

We corrected the mistake that was pointed out.

h. As stated in point 1 above, no MITOL comes down with Parkin-HA in the presence of CCCP in Fig 4B. This contradicts conclusion of 4A.

Our reply:

We explained this in 1. This is due to the difference of buffer.

i. In 4C, why is there MITOL in all lanes? The label says that the last two lanes include siRNA against MITOL.

Our reply:

We removed a result of siRNA and added new results using MITOL WT / KO cells (Figure EV3F).

j. The authors need to include colocalization image with known ER marker in Fig 4F.

Our reply:

We included colocalization image with known ER and mitochondria markers (Figure 3B and 3C).

j. In addition to 4H, microscopy, similar to 4F, would really strengthen the claim "FKBP38 knockdown inhibited the translocation of MITOL to the ER" since fractionation assays can be messy when looking at ER vs. mitochondria and since mitophagy is decreasing the amount of mitochondria under these conditions.

Our reply:

Following your comment, immunostaining images were added because fraction assay alone is not convincing (Figure EV4C).

k. The red and green labels appear completely switched in Figure 4I. Also, an ER marker should be included as a control.

Our reply:

We corrected the mistake that was pointed out.

l. For 5E the authors target FKBP38 to either the ER or the mitochondria to see where it is targeted for degradation by MITOL. They say that in MITOL KO cells, FKBP38 is markedly degraded when it is targeted to the ER but there is no change when it is targeted to the mitochondria. However, there is no FKBP38-IYFFT on the blot at all.

Our reply:

We are very sorry for this careless mistake. The figure was missing when the resolution of the figure was decreased. The figure was shown in Figure 5B.

m. The authors need to verify that FKBP38-IYFFT is targeted to the ER and that FKBP38-ActA to the mitochondria.

Our reply:

We verified that FKBP38-IYFFT is targeted to the ER and that FKBP38-ActA to the mitochondria (Figure EV5B).

n. Figure EV3 needs more controls for the fractionation, including other mitochondrial proteins.

Our reply:

We properly improved Figure 4C.

Other more minor issues:

1. For 2A/2B/2C/2E, I would like to see the IP interaction with MITOL overexpression in these blots. The authors clearly have a MITOL antibody so showing an IP:HA MITOL probed blot would not be much work. Would be interesting to see if this interaction changes in the CS E3 ligase mutant or the KR/KN mutants as well.

Our reply:

Although it is very interesting point, mentioned in 1., we could not detect the IP interaction with MITOL because of using RIPA lysis buffer and sonicated samples.

2. Loading control for 3A is missing

Our reply:

I am sorry for this mistake. Figure 2A was properly improved.

3. Quantification of 3F and 3G. Especially in 3G, it looks like the levels of MITOL in the K220R MITOL overexpression samples are going down. Is it possible the authors are seeing a decrease in Tom20/increase in mitophagy because of this?

Our reply:

The quantity of MITOL translocated to the ER depends on the quantity of FKBP38. Thus, the overexpressed MITOL, which could not bind with FKBP38 remains on mitochondria and undergoes degradation with mitochondria in mitophagy. In case of K220R mutant, mitophagy normally occurs and therefore, the overexpressed MITOL is degraded in a mitochondrial degradation-dependent manner (Figure 2C). We presume that the remaining MITOL here is the MITOL that translocated to the ER with FKBP38 and escaped from mitochondrial degradation.

4. Show levels of HA-Parkin in 4E.

Our reply:

We show the levels of HA-Parkin in whole fraction of Figure 4C. There was no change in the total level of HA-Parkin.

Referee #3:

In this study by Shiiba et al, the authors suggest that the mitochondrial ubiquitin ligase MITOL ubiquitinates and degrades the E3 ubiquitin ligase Parkin, and protect the antiapoptotic factor FKBP38 from Parkin-mediated degradation, thus promoting cell survival in mitophagy.

Overall this is an interesting, and relatively well-designed study. However, I have raised a number of concerns below, that I feel should be addressed before publication in EMJO Rep.

In particular, it is unclear where the MITOL-dependent and the Parkin dependent ubiquitination and degradation of Parkin and FKBP38, respectively, occur? And how Parkin recruits to ER?

- Overall, the data is clear. However, a vast majority is using biochemistry. While the biochemistry data are convincing, when possible, the authors should repeat the experiments with alternative methods (E.g. Immunofluorescence, IF).

Our reply:

We thank for your suggestions. In our revised manuscript, we performed various experiments using Immunofluorescence and obtained convincing results.

- In Figure 2D, the authors claim that MITOL ubiquitination is "not affected by Parkin overexpression". Do they mean "by mitochondrial depolarisation"? In order to claim that Parkin overexpression doesn't affect MITOL ubiquitination, the experiment should be repeated with or without Parkin over-expression.

Our reply:

That is right. We confirmed that the MITOL ubiquitination level did not change under CCCP treatment, especially when Parkin is activated. The text has been changed following your comment.

- In Figure 2E, the authors state that "Parkin is ubiquitinated by MITOL after its translocation onto mitochondria". Have they checked whether K211 is a target lysine for MITOL (E.g. using a mitochondrially-targeted Parkin K211R mutant)?

Our reply:

Most of the ubiquitin signals disappear in the K220R mutant in Figure 1E. Therefore, we conclude that K211 is not a target lysine for MITOL.

- In figure 3D, the authors state that "MITOL ubiquitinates and subsequently degrades Parkin on mitochondria". The authors should assess the purity of their "mitochondria-rich membrane fraction" by probing with ER markers. In fact, in the last paragraph of the introduction, the authors write that "MITOL translocates to the ER... and degrades Parkin". So where does MITOL-dependent ubiquitination and degradation of Parkin occur? In the mitochondria? In the ER? This should be determined using microscopy.

Our reply:

This is a very important point. Since it was very difficult to prove it, we could not determine it completely. However, the ubiquitination assay of each organelle fraction in MITOL WT / KO cells revealed that Parkin ubiquitination by MITOL was observed in both mitochondrial and ER fractions (Figure 1G), suggesting that it occurs on both organelles.

- In figure 3E, the authors should use an alternative method to assess mitophagy (E.g. mitoKeima/QC).

Our reply:

According to your comment, we quantified mitophagy by using mtphagy Dye (Dojindo) in MITOL overexpression cells and in WT / KO cells (Figure 2E and F). When MITOL was overexpressed, mitophagy was strongly suppressed, and in MITOL KO cells, mitophagy was slightly increased.

- In Figure 4F, the authors should perform a CCCP time course and include both mitochondria and ER markers.

Our reply:

Following your advice, co-staining with ER and mitochondria markers and quantitative data were added (Figures 3B and 3C).

- Figure 4G should be repeated with endogenous MITOL (and FKBP38 if possible).

Our reply:

IP assay revealed the binding of endogenous FKBP38 to endogenous MITOL (Figure EV4A).

- Figure 4H should be repeated using IF.

Our reply:

Following this advice, IF image was added in Figure EV4C.

- Figure 4I should be repeated with mito and ER markers, and with FKBP38 siRNA. Also, the principle of the Kik-GR is not very well explained for non-specialist readers.

Our reply:

Co-staining image was added in Figure 3E. Also, a schematic experiment model has been added for easy to follow.

- Figure 5A should be repeated using a Ser65 Parkin antibody

Our reply:

Thank you for your kind advice. We repeated it using a Ser65 Parkin antibody as shown in Figure 4C.

- The blot in Figure 5C should be probed with a mitochondrial marker to assess to purity of the fraction

Our reply:

Following your advice, we improved it in Figure 4C.

- In Figure 5D, what is the effect of the Parkin C431S and S65A mutants?

Our reply:

As you pointed out, we had better check it. However, since Parkin C431S / S65A is known to be impaired translocation to the mitochondria [2, 3], we assumed that they are not suitable for showing the correct effect of MITOL for Parkin in mitophagy. Therefore, we did not perform this experiment.

- In Figure 5E, the authors claim that "ER-targeted FKBP38 was markedly degraded after CCCP treatment compared to WT cells". But it is not detected, even in non-CCCP treated fractions? Is it even expressed? The experiment should be repeated in WB and in IF. Minor comment: in the next sentence, it should be "in contrast", not "in constant"

Our reply:

We are very sorry for this careless mistake. The figure was missing when the resolution of the figure was decreased. The figure was shown in Figure 5B.

- Where does the interaction between Parkin and FKBP38 occur? Where does the Parkin-dependent ubiquitination and degradation occur? In the mito and/or in the ER?

Our reply:

This is a very important question. However, it is very difficult to identify it. We predict that binding and ubiquitination occur in both mitochondria and ER judging from fractionation assay (Figure 1G), but it is difficult to prove it completely. We mention it in the Discussion.

- How is Parkin recruited to the ER? Is it dependent on FKBP38? There is an attempt to discuss it in the discussion but this is not very clear. This should be addressed with experiments.

Our reply:

This is an important question. Since Parkin recruitment to the ER was observed even in the FKBP38 knockdown, Parkin translocates to the ER in an FKBP38-independent manner. We mention this in the Discussion.

Referee #4:

Understanding the molecular control of Parkin activity in mitophagy in cells receiving mitochondrial damage is important to elucidate its dysfunction as causal in certain cases of early onset Parkinson's disease. The current manuscript finds that Parkin itself is controlled following reception of mitochondrial insult by the mitochondrial the E3 ubiquitin ligase MITOL (also known as MARCH5). Based largely on overexpression studies, the authors an direct interaction between these proteins during mitophagy and that MITOL promotes proteasomal degradation of Parkin by K48-linked ubiquitination to negatively regulate mitophagy. They finally provide some data to suggest that MITOL itself shuttles from the mitochondria to ER in the final stages of mitophagy to limit apoptosis involving FKBP38, although I found this aspect of the manuscript less compelling.

Overall, the paper reports an interesting finding into the regulation of Parkin.

General comments

1. The paper is almost entirely based on over-expression of either MITOL, Parkin or both. In order to support the potential importance of this mechanism in the control of mitophagy it is essential that they confirm at least some of their findings (i.e interaction, ubiquitination and mitophagy) in a cell line that endogenously expresses both Parkin and MITOL (e.g. SH-SY5Y). The evidence that Parkin was ubiquitinated is limited to IP of the over-expressed protein followed by Western blotting for ubiquitin. However, there is no direct evidence of Parkin being modified either by mass spec to identify diGly motifs on Parkin peptides or detection of modified forms of the protein following Western blotting even in the presence of proteasomal inhibition when ubiquitinated forms might be expected to accumulate. A more quantitative assay to support potential defects in mitophagy would be FACS based assessment of mt-Keima. Together, it is very difficult to gauge how much of the Parkin is actually modified by MITOL, and how significant this mechanism is. Furthermore, several groups have published a role for MITOL/MARCH5 in controlling mitochondrial dynamics. Could the influence on mitophagy here be a consequence of MITOL's role in regulating mitochondrial form/function rather than a direct influence on Parkin?

Our reply:

Thank you for your comment. First, to confirm the endogenous Parkin-MITOL interaction, we used SH-SY5Y cells and found that they interacted each other in a CCCP-dependent manner (Figure EV1A). In addition, when we evaluated Parkin ubiquitination by endogenous MITOL in MITOL WT / KO cells, Parkin ubiquitination was strongly enhanced in MITOL WT cells when proteasome was inhibited (Figure 1G). This result suggests that endogenous MITOL regulates Parkin. Using mtphagy Dye (Dojindo), mitophagy was quantified in MITOL overexpression and WT / KO cells (Figure 2E and F). Mitophagy was strongly suppressed by MITOL overexpression, whereas mitophagy was slightly enhanced in MITOL KO cells, suggesting that endogenous MITOL plays only a slight inhibitory role in mitophagy by Parkin ubiquitylation on mitochondria. Thus, we conclude that MITOL regulates the level of Parkin not to disturb mitochondrial quality control by Parkin. About possibility that MITOL affects mitophagy through regulation of mitochondrial dynamics. This is certainly a very important point. It has been reported that mitochondrial fission occurs independently of fission factor (Drp1) under CCCP conditions [4]. In addition, fusion factors Mfn1/2 were reported to be rapidly degraded early in mitophagy [5]. Considering these reports, it is expected that mitochondrial dynamics-related factors are less likely to be controlled by MITOL under mitophagy.

2. The authors over-express mutant ubiquitin to attempt to define the linkages catalysed by MITOL on Parkin. These assays are confounded by the endogenous ubiquitin expressed in the cells. I would recommend a more definitive approach would be mass spectrometry or a de-ubiquitinase assay using specific DUBs of a Parkin IP.

Our reply:

According to this comment, we identified the ubiquitin species added to Parkin using deubiquitinating enzymes (DUBs) (Figure 1F). We found that the Parkin ubiquitination was markedly attenuated by OTUB1 treatment, a K48-specific deubiquitinase. Therefore, we conclude that the ubiquitin species added to Parkin is the K48-linked ubiquitin chain in mitophagy.

3. The authors do report that endogenous MITOL could only mediate Parkin degradation in the presence of CHX (Figure 4D). The justification and conclusions from this experiment are not clear, but it implies that new protein translation (presumably Parkin?) masks any decrease in an endogenous (MITOL) setting. The authors should provide more detailed discussion of this data and its difference from when MITOL is ectopically over-expressed.

Our reply:

We apologize for the lack of explanation. The reason that the degradation of Parkin by endogenous MITOL detected in the presence of CHX is considered to be due to the low amount of Parkin. Although endogenous MITOL slowly degrades phosphorylated Parkin (Figure 4A), Parkin is newly synthesized immediately (Figure 4D), so the amount of Parkin is uniformed in mitophagy. Therefore, Parkin degradation can be seen when inhibiting the newly synthesized Parkin by CHX treatment (Figure EV3F). On the other hand, by MITOL overexpression, Parkin degradation progresses rapidly, so a large-scale Parkin degradation is observed even without CHX treatment (Figure 2B).

4. In Figure 4C they switch to using MITOL siRNA when in other data they utilise KO cells. However, the level of MITOL knockdown with siRNA is minimal. The reason for switching to siRNA for this context was not clear, but given the inefficient knock-down they should use the ko cells.

Our reply:

Instead of the knockdown experiment, we performed a similar experiment focusing on KO cells (Figure EV3F).

5. In general, I did not find the data suggesting redistribution of MITOL to the ER in late stage mitophagy to regulate apoptosis compelling. In Figure 4F they only show an image of a single cell to concluded colocalization of MITOL with FKBP after CCCP. The authors need to include mitochondrial and ER markers together with image quantification to support this redistribution. Figure 4I would be strengthened by showing a DAPI/Hoechst stain of the cells. The authors should confirm that CCCP is driving apoptosis by blocking with caspase inhibitors. Also, the death that the authors observe following CCCP may be independent of Parkin. To confirm Parkin's role the authors should show the cell death of MITOL KO HeLa cells that do not express Parkin.

Our reply:

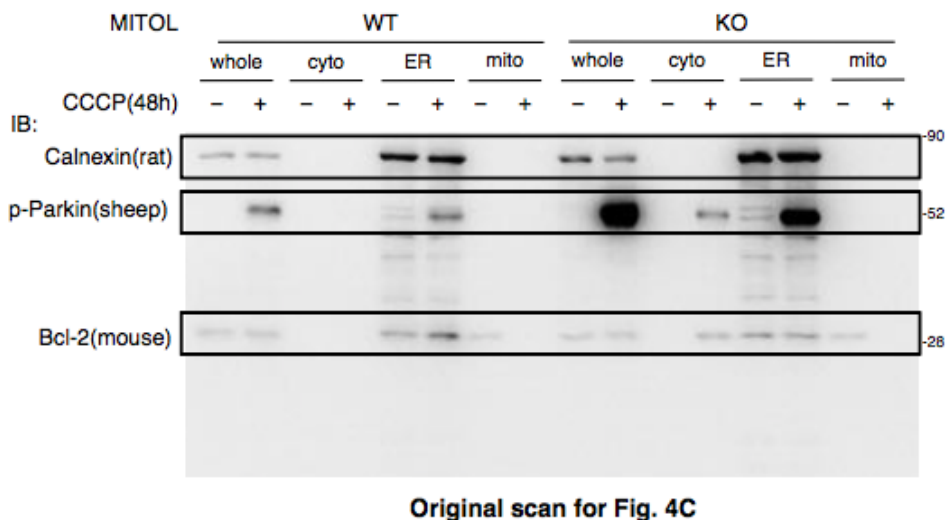
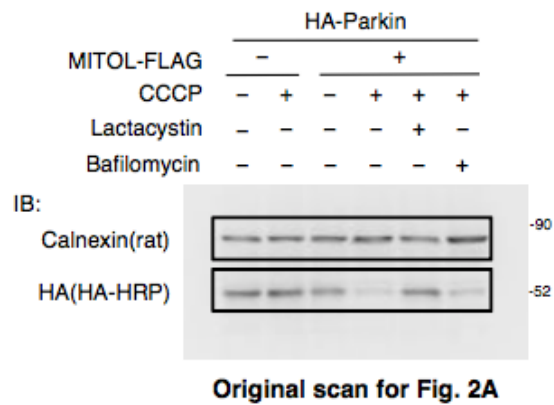
For Figure 4F, co-staining images with ER and mitochondria, and quantitative data were added (Figure 3B and C). For Figure 4I, we re-show a co-staining experiment with the ER marker (Figure 3E). In order to determine the mechanism of cell death in MITOL KO, we used the pan-Caspase inhibitor Z-VAD-fmk, and found that cell death was suppressed by Z-VAD-fmk (Figure EV5D). As previously reported, we confirmed that Z-VAD-fmk does not suppress cell death during mitophagy in MITOL WT cells, suggesting that this is mitophagic cell death. Furthermore, to show the importance of Parkin regulation by MITOL on the cell death, we examined the effect of Parkin K220R on cell death and found that it was similar to that of MITOL KO cells (Figure 5E). We therefore conclude that Parkin degradation by MITOL is essential for suppressing cell death.

6. Much of the conclusions of the study hinge on the loss of Parkin protein following activation of mitophagy in the presence of MITOL (e.g Fig3C, F, and H). However, for some of these blots that can be expanded, there is indication that the loading may not be equivalent (see HA blots in Fig3C, F, and H). The authors should provide evidence that the actin loading controls are reprobes of the same blot to remove any such concern. Likewise for the subcellular

fractionation blots in 3D and 4H, I recommend that the authors provide evidence to show that the loading controls are reprobes of the same blot rather than separate blots.

Our reply:

We have reconfirmed several experiments on the same membrane as shown in partly below.



Specific comments

- 1. Figure 1B, not clear what the "-" samples are. Presumably Input?*
- 2. Figure 1E, there is almost no signal for input HA*
- 3. Figure 2B and C- authors should reprobe input fractions with anti-FLAG.*

4. Figure 2D- authors should reprobe input fractions with anti-Myc
5. Figures 1F ad 2E use Parkin K211N mutant to conclude that interaction and ubiquitination is dependent on mitochondrial localisation. If possible this data could be shown together. Also, binding of Parkin to phospho-Ub precedes the phosphorylation of Parkin as described in the text (page 12).
6. Figure 5C MITOL was detected at the ER fraction after CCCP treatment. Authors need to probe for mitochondrial markers to show the purity of their ER fractions.
7. Figure 1E, the HA blot is blank.
8. Figure 4B, the MITOL IP blot is blank, tubulin input blot is blank.
9. Figure EV2, the MITOL blot is blank.
10. Figure 5E the FKBP38IYFFT blot is blank.
11. In addition, the authors attempt to express Parkin to the same level as that observed in the striatum. However, given this is mouse tissue and they are ectopically expression human Parkin, they cannot draw this conclusion as the antibody may detect human and mouse Parkin differently.
12. In general the manuscript is well-written and easy to follow. However, it would benefit from being carefully proof-read for grammatical and spelling errors.

Our reply:

Thanks for your kind comments. We responded appropriately to all comments.

References

1. Sugiura A, Yonashiro R, Fukuda T, Matsushita N, Nagashima S, Inatome R, Yanagi S (2011) A mitochondrial ubiquitin ligase MITOL controls cell toxicity of polyglutamine-expanded protein. *Mitochondrion* **11**: 139-146
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5. Gegg ME, Cooper JM, Chau KY, Rojo M, Schapira AH, Taanman JW (2010) Mitofusin 1 and mitofusin 2 are ubiquitinated in a PINK1/parkin-dependent manner upon induction of mitophagy. *Hum Mol Genet* **19**: 4861-4870

Dear Prof. Yanagi,

Thank you for submitting a revised version of your manuscript, which was rejected post-review last year. We have now received comments from three of the original referees, which can be seen below.

Referees acknowledge that the manuscript has been improved during the revision. However, they have outstanding concerns. In particular they find that

1. Assessment of mitophagy is not conclusive (ref #1 point 1, ref #2 point 2, ref #3 point 3)
2. Whether MITOL is ubiquitinated in response to CCCP is unclear (ref #1 point 2, ref #3 point 1)
3. The data showing Parkin and MITOL relocalization to ER are not strong enough (ref 1 point 3, ref #2 point 3, ref #3 point 6).

Given that these concerns affect the main findings of the manuscript, we cannot offer to publish your manuscript in its current form.

I am sorry that I cannot convey more positive news, but I hope that the referee comments will be helpful in your continued work in this area.

Kind regards,

Deniz Senyilmaz Tiebe

-

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

The manuscript has been significantly improved after the revision. In general, the authors have made an excellent effort to address the comments. However, the following comments/concerns should also be attended to:

1. Previous comment 1:

The authors used Mitophagy Detection Kit which uses mitochondrial dye and lyso dye to measure mitophagy. However, the combination of these dyes and CCCP have been found to be problematic (DOI: 10.4161/auto.26557). Could the authors do the same experiments using Oligomycin and Antimycin A to show the same effect of MITOL on mitophagy?

"I am sorry for this careless mistake. The notation (-) in our previous manuscript was input. We corrected this error. MITOL degrades itself by auto-ubiquitination. Since auto-ubiquitination of CS is inhibited, CS is more accumulated than WT. This is the reason why the protein amounts of WT and CS are differently detected (Figure 2B)." : Could the authors show this experimentally or discuss this in relation to previous publications?

In addition, on page 10 and occasionally throughout the manuscript, the authors still refer to mitophagy through TOM20 degradation. As mentioned in the previous comment, this is not

accurate and should be avoided. The authors can immunoblot for proteins within mitochondria such as COXII rather than those on the mitochondrial outer membrane like TOM20 to show mitochondrial degradation. Otherwise, the conclusions about mitophagy occurring cannot be drawn.

2. Previous comment 2:

"Here, we tried to insist that MITOL is not regulated by ubiquitin signaling in mitophagy, because no increase or decrease in CCCP-dependent ubiquitination of MITOL was observed.": CCCP may have induced additional ubiquitination and degradation of MITOL that cannot be seen in the absence of inhibitors. Indeed, the recent publication (DOI: 10.15252/embr.201947728) showed that Parkin can ubiquitinate MITOL upon CCCP treatment. Could the authors discuss this in relation to their results or do the authors have additional data to support their argument?

3. Previous comment 3:

The recent publication (DOI: 10.15252/embr.201947728) showed that MITOL translocate to peroxisomes rather than ER under the exact same conditions using HeLa cells. The only difference is that while this publication stained for endogenous ER marker in cells expressing Parkin and MITOL, the authors either co-expressed an ER construct (Figure 3B) or stained for endogenous ER marker in cells expressing Parkin, MITOL and FKBP38 (Figure 3E). Could the authors discuss this in relation to the finding? Is that possible to stain with Calnexin and MITOL in cells without FKBP38 and mCherry-Sec61 to make sure that overexpression of FKBP38 or an ER construct does not affect the result? Do ER fractions from subcellular fractionations also contain peroxisomes?

Referee #2:

Overall, the authors have done a nice job addressing concerns raised in the previous review, especially in terms of showing the effects of MITOL on Parkin are not resulting from MITOL overexpression. The manuscript is much improved, and outlines an important advance in the regulation of Parkin-mediated mitophagy. There are three issues still remaining that should be addressed.

1. The addition of cycloheximide in Figure 2D now shows that steady state levels of Parkin are modestly elevated in MITOL KO cells. The rigor of this result would be boosted with a CHX-chase experiment to determine whether the half-life of Parkin is changed MITOL KO cells in the presence of CCCP.
2. The incorporation of the mitophagy dye utilized in Figure 2E and F is nice, but it would be beneficial to see controls using bafilomycin or autophagy mutants to demonstrate the specificity of the assay for measuring mitophagy.
3. In Figure EV4C, the authors show that MITOL is absent in cells lacking FKBP38 after treatment of cells with CCCP for 48 hours. However, in the EV4B fractionation experiment, MITOL is present in the mitochondrial fraction in CCCP-treated FKBP38 knockdown cells. Why is there a discrepancy between these results? Based on EV4B, I would expect to see MITOL-myc localized in the mitochondria in the 48 hr CCCP treated cells shown in E4VC but this is not the case.

Referee #3:

In the revised version of their manuscript, Shiiba et al have performed a number of additional experiments. However, their answers to some of my initial comments are elusive while some others are simply not addressed. Additionally, inaccuracies/overstatements remain throughout the text, making the revision of the manuscript difficult and calling into questions some of the overall conclusions.

Re the answers to the reviewer's comments, to give few examples:

- Re my comment for Figure 2D, the authors have not repeated the experiment with or without Parkin over-expression. In Figure EV2A, the authors check that MITOL ubiquitination isn't affected by CCCP treatment. But they don't check whether it is affected in Parkin KO or by Parkin over-expression. This experiment is needed to conclude that "MITOL unilaterally ubiquitinates Parkin in a CCCP-dependent manner", as stated page 7.
- Re my comment for Figure 3D, no IF experiment was performed.
- Re my comment for Figure 3E, the authors have decided to use the mtphagy Dye instead of using MtKeima or MtQC to detect mitophagy in live cells over time. Unfortunately, mitophagy was only assessed at 24hrs, no time course was performed, and no representative images were presented.
- Re my comment on Figure 4F, no CCCP time course was performed.
- Re my comment for Figure 5D, the authors have decided to not perform the experiment because they "assumed" that since Parkin C431S / S65A translocation are known to be impaired, it was not suitable for showing the correct effect of MITOL for Parkin in mitophagy. It would have been a good control experiment though.
- Re my last comment, the authors say "This is an important question. Since Parkin recruitment to the ER was observed even in the FKBP38 knockdown, Parkin translocates to the ER in an FKBP38-independent manner. We mention this in the Discussion". Which experiments are they referring to?

Other comments:

- The first sentence of the results section is incorrect: "It is well known that Parkin is recruited to depolarize the mitochondrial membranes after treatment with the mitochondrial uncoupler, carbonyl cyanide-m-chlorophenylhydrazone (CCCP)". The reviewer is hoping that this is "just" a typo, as opposed to a misunderstanding of the mitophagy process.
- In Figure EV2B, the authors use the K211N Parkin mutant to claim that "Parkin is ubiquitinated by MITOL after its translocation to the mitochondria". They refer to PMID 20098416 to claim that this mutant doesn't relocate to the mitochondria. This is not strictly true. In this paper, Geisler et al show that the relocation of this mutant to the mitochondria is delayed, as opposed to abolished (it is not localised at mitochondria after 2hrs of CCCP, but it is localised with clustered mitochondria after 24hrs). In their experiment, the authors treat the cells with CCCP for 8hrs, and it is very possible that K211N Parkin mutant is localised at mitochondria after 8hrs. Their conclusion isn't valid without showing that K211N Parkin isn't localised at the mitochondria after 8hrs CCCP treatment.
- Despite additional experiments, where the MITOL-dependent ubiquitination of Parkin and the Parkin dependent degradation of FKBP38 occur, remains unclear.
- Whether/how Parkin relocates to the ER in their model remains unclear.
- Many of the experiments remain over-expression (E.g why over-express FKBP38 in Figure EV5?)

Minor comments

- I would delete "directly" in the title "MITOL directly ubiquitinates Parkin in Parkin-mediated mitophagy". In vitro ubiquitination assays with recombinant MITOL and Parkin would be required to determine whether the ubiquitination is direct. For the same reason, page 9, I would rephrase the sentence "MITOL adds the K48-linked polyubiquitin chain to Parkin".

- When describing Figure 1A, the authors should precise that they refer to MITOL-Myc (as opposed to endogenous Myc)
- The authors should indicate (in legends and in results) that they refer to Ser65-Parkin when mentioning phospho-Parkin
- The manuscript would benefit from further proof-read for grammatical and spelling errors. For example, page 11, the sentence "Therefore, it is possible that regardless of the fact that the effect of endogenous MITOL on mitophagy might be milder compared to that of the overexpressed one, it can regulate the quantity of Parkin" isn't very unclear.

We thank all referees for their helpful and constructive comments. In response to their comments, we changed the detection method of mitophagy, and reexamined the localization of endogenous MITOL and MITOL ubiquitination during the induction of mitophagy. Regarding the quantitative measurement of mitophagy, we have generated and examined MITOL WT and KO cells stably expressing mt-keima, which has a high sensitivity to detect mitophagy. In addition, in order to investigate the translocation of endogenous MITOL in more detail and precise, we have established a cell line which is knocked in EGFP tag at the N-terminal of endogenous MITOL. Furthermore, we confirmed ubiquitination of MITOL with or without Parkin in time-course experiments of CCCP-treated cells. Again, we appreciate that the quality of this paper has been improved by all comments of the referees. Our comments are written point-by-point below.

Referee #1:

The manuscript has been significantly improved after the revision. In general, the authors have made an excellent effort to address the comments. However, the following comments/concerns should also be attended to:

1. Previous comment 1:

The authors used Mitophagy Detection Kit which uses mitochondrial dye and lyso dye to measure mitophagy. However, the combination of these dyes and CCCP have been found to be problematic (DOI: 10.4161/auto.26557). Could the authors do the same experiments using Oligomycin and Antimycin A to show the same effect of MITOL on mitophagy?

Our reply:

We thank the referee#1 for the critical comments. As you pointed out, the combination of mitophagy detection kit and CCCP was reported to be problematic, so we are reexamined using mt-keima instead (Fig 2E and Fig EV3E). As a result, we found that mitophagy was significantly enhanced in MITOL KO cells, and overexpression of MITOL suppressed mitophagy. Consistently, Parkin K220R, a ubiquitination-defective mutant by MITOL, revealed a significant increase in mitophagy. Parkin K220R-dependent enhancement of mitophagy was not inhibited by MITOL overexpression.

"I am sorry for this careless mistake. The notation (-) in our previous manuscript was input. We corrected this error. MITOL degrades itself by auto-ubiquitination. Since auto-ubiquitination of CS is inhibited, CS is more accumulated than WT. This is the reason

why the protein amounts of WT and CS are differently detected (Figure 2B)." : Could the authors show this experimentally or discuss this in relation to previous publications?

Our reply:

MITOL regulates or maintains its quantitative level by its auto-ubiquitination activity (DOI: 10.1111/febs.13568). Therefore, the MITOL CS mutant lacking E3 ubiquitin ligase activity usually tends to be more expressed than that of WT.

In addition, on page 10 and occasionally throughout the manuscript, the authors still refer to mitophagy through TOM20 degradation. As mentioned in the previous comment, this is not accurate and should be avoided. The authors can immunoblot for proteins within mitochondria such as COXII rather than those on the mitochondrial outer membrane like TOM20 to show mitochondrial degradation. Otherwise, the conclusions about mitophagy occurring cannot be drawn.

Our reply:

According to your comment, the part of mitophagy mentioned with Tom20 was deleted from the text.

2. Previous comment 2:

"Here, we tried to insist that MITOL is not regulated by ubiquitin signaling in mitophagy, because no increase or decrease in CCCP-dependent ubiquitination of MITOL was observed.": CCCP may have induced additional ubiquitination and degradation of MITOL that cannot be seen in the absence of inhibitors. Indeed, the recent publication (DOI: 10.15252/embr.201947728) showed that Parkin can ubiquitinate MITOL upon CCCP treatment. Could the authors discuss this in relation to their results or do the authors have additional data to support their argument?

Our reply:

We thank the reviewer for the critical comments. Since the time of CCCP stimulation in the recent publication (DOI: 10.15252 / embr.201947728) was different from our experiments, we reexamined the MITOL ubiquitination after CCCP treatment with time courses. As a result, ubiquitination of CS mutant, a ligase activity dead mutant, was observed at 3 hours after CCCP stimulation (Fig EV2A), indicating that MITOL is ubiquitinated by Parkin only at early stage in a CCCP-dependent manner. The referee's indication was correct. Therefore, the sentences of "we tried to insist that MITOL is not regulated by ubiquitin signaling in mitophagy, because no increase or decrease in

CCCP-dependent ubiquitination of MITOL was observed.” has been modified and the explanation was added in the results section (lines 19-22 on page 8).

3. Previous comment 3:

The recent publication (DOI: 10.15252/embr.201947728) showed that MITOL translocate to peroxisomes rather than ER under the exact same conditions using HeLa cells. The only difference is that while this publication stained for endogenous ER marker in cells expressing Parkin and MITOL, the authors either co-expressed an ER construct (Figure 3B) or stained for endogenous ER marker in cells expressing Parkin, MITOL and FKBP38 (Figure 3E). Could the authors discuss this in relation to the finding? Is that possible to stain with Calnexin and MITOL in cells without FKBP38 and mCherry-Sec61 to make sure that overexpression of FKBP38 or an ER construct does not affect the result? Do ER fractions from subcellular fractionations also contain peroxisomes?

Our reply:

We thank the reviewer for the critical comments. In order to eliminate the possibility that the overexpression of FKBP38, Sec61, and MITOL affect the localization of MITOL, we generated EGFP-MITOL knock-in cells that can detect the localization of endogenous MITOL without overexpression. Localization of MITOL using EGFP-MITOL knock-in cells revealed that MITOL was co-localized with ER marker calnexin in both CCCP 12h and 24h treatments (Fig 3C). From this result, we conclude that MITOL translocates to the ER during mitophagy.

Referee #2:

Overall, the authors have done a nice job addressing concerns raised in the previous review, especially in terms of showing the effects of MITOL on Parkin are not resulting from MITOL overexpression. The manuscript is much improved, and outlines an important advance in the regulation of Parkin-mediated mitophagy. There are three issues still remaining that should be addressed.

1. The addition of cycloheximide in Figure 2D now shows that steady state levels of Parkin are modestly elevated in MITOL KO cells. The rigor of this result would be boosted with a CHX-chase experiment to determine whether the half-life of Parkin is changed MITOL KO cells in the presence of CCCP.

Our reply:

We performed an additional CHX assay in Figure EV3 to quantify HA-Parkin accumulation, but there was no increase in HA-Parkin accumulation in MITOL KO in the steady state (Figure EV3).

2. The incorporation of the mitophagy dye utilized in Figure 2E and F is nice, but it would be beneficial to see controls using bafilomycin or autophagy mutants to demonstrate the specificity of the assay for measuring mitophagy.

Our reply:

We thank the reviewer for the critical comments. According to the referee#1 and referee #3's comment, since mitophagy dye and CCCP are not compatible with each other, mitophagy was requantified using various cells expressing mt-keima (Fig 2E and Fig EV3E).

3. In Figure EV4C, the authors show that MITOL is absent in cells lacking FKBP38 after treatment of cells with CCCP for 48 hours. However, in the EV4B fractionation experiment, MITOL is present in the mitochondrial fraction in CCCP-treated FKBP38 knockdown cells. Why is there a discrepancy between these results? Based on EV4B, I would expect to see MITOL-myc localized in the mitochondria in the 48 hr CCCP treated cells shown in E4VC but this is not the case.

Our reply:

We thank the reviewer for a careful assessment of the figures. The notation 48h of figure was incorrect, and the notation 30h of legends was correct. I am sorry for this careless mistake. The presence or absence of MITOL has changed due to the CCCP treated time.

Referee #3:

In the revised version of their manuscript, Shiiba et al have performed a number of additional experiments. However, their answers to some of my initial comments are elusive while some others are simply not addressed. Additionally, inaccuracies/overstatements remain throughout the text, making the revision of the manuscript difficult and calling into questions some of the overall conclusions.

Re the answers to the reviewer's comments, to give few examples:

- Re my comment for Figure 2D, the authors have not repeated the experiment with or without Parkin over-expression. In Figure EV2A, the authors check that MITOL ubiquitination isn't affected by CCCP treatment. But they don't check whether it is affected in Parkin KO or by Parkin over-expression. This experiment is needed to conclude that "MITOL unilaterally ubiquitinates Parkin in a CCCP-dependent manner", as stated page 7.

Our reply:

We thank the reviewer for the constructive suggestion. According to the comment, we examined MITOL ubiquitination after CCCP treatment with or without Parkin with time courses. We found that ubiquitination of MITOL CS mutant, which lacks auto-ubiquitination activity, was observed at 3 hours after CCCP stimulation, suggesting that MITOL is ubiquitinated by Parkin at early stage in a CCCP-dependent manner. On the other hand, MITOL ubiquitination is attenuated in the late stage of mitophagy. It is speculated that unknown mechanism underlying downregulation of MITOL ubiquitination exists during mitophagy. It is assumed that this attenuation of MITOL ubiquitination affects the localization change of MITOL, and we would like to investigate it in the next project about these mechanisms in detail. Based on these results, we corrected the following sentence "MITOL unilaterally ubiquitinates Parkin in a CCCP-dependent manner". I should have done additional experiments according to your previous comment. I am deeply grateful for your insightful comments.

- Re my comment for Figure 3D, no IF experiment was performed.

Our reply:

We appreciate the reviewer's comments. We attempted to observe where endogenous MITOL degrades Parkin in EGFP-MITOL knock in cells using a fluorescence microscope, however as described in the text, the degradation of Parkin by endogenous MITOL was very mild and difficult to detect it.

- Re my comment for Figure 3E, the authors have decided to use the mitophagy Dye instead of using MtKeima or MtQC to detect mitophagy in live cells over time. Unfortunately, mitophagy was only assessed at 24hrs, no time course was performed, and no representative images were presented.

Our reply:

We thank the reviewer for the critical comments. As pointed out by other referees, mitophagy dye is not compatible with CCCP, so experimental results using mitophagy dye

were excluded. Instead, we generated various cell lines expressing mt-keima and re-experimented (Fig 2E and Fig EV3E). The result showed mitophagy was significantly enhanced in MITOL KO cells, and overexpression of MITOL suppressed mitophagy. Moreover, Parkin K220R mutant, a ubiquitination-defective mutant by MITOL, revealed a significant increase in mitophagy consistent with MITOL KO, and inhibition of mitophagy by MITOL overexpression was not observed.

- Re my comment on Figure 4F, no CCCP time course was performed.

Our reply:

We thank the reviewer for the constructive suggestion. We established a cell line in which EGFP is knocked in at the N-terminus of the endogenous MITOL, and observed changes in the localization of MITOL with time courses (Fig 3C). We confirmed the translocation of MITOL to the ER as in the previous result.

- Re my comment for Figure 5D, the authors have decided to not perform the experiment because they "assumed" that since Parkin C431S / S65A translocation are known to be impaired, it was not suitable for showing the correct effect of MITOL for Parkin in mitophagy. It would have been a good control experiment though.

Our reply:

We thank the reviewer for the critical comments. As you pointed out, we generated C431S mutant, an inactive form of Parkin, and S65A mutant, a phosphorylation-defective Parkin mutants, and examined whether these Parkin mutants could ubiquitinate FKBP38. It was found that ubiquitination of FKBP38 by S65A mutant was significantly attenuated, and ubiquitination of FKBP38 by C431S was not detected (Fig EV5B). From these results, we conclude that an E3 ubiquitin ligase activity of Parkin is essential for FKBP38 ubiquitination.

- Re my last comment, the authors say "This is an important question. Since Parkin recruitment to the ER was observed even in the FKBP38 knockdown, Parkin translocates to the ER in an FKBP38-independent manner. We mention this in the Discussion". Which experiments are they referring to?

Our reply:

We apologize to you for the confusing description. In our previous text, we only mentioned it in the discussion and did not add the data. In this time, we add the data about the effect of Parkin accumulation in FKBP38 knock down condition. It is found that there is no difference in the amount of Parkin in the ER fraction even in the

FKBP38-knockdown cells. We therefore conclude that Parkin accumulates in the ER in a FKBP38-independent manner (Fig EV5A).

Other comments:

- The first sentence of the results section is incorrect: "It is well known that Parkin is recruited to depolarize the mitochondrial membranes after treatment with the mitochondrial uncoupler, carbonyl cyanide-m-chlorophenylhydrazone (CCCP)". The reviewer is hoping that this is "just" a typo, as opposed to a misunderstanding of the mitophagy process.

Our reply:

I am sorry for this mistake. According to the referee's suggestion we have changed the discription: "It is well-known that Parkin is selectively recruited to mitochondria with low membrane potential that is induced by an uncoupler, carbonyl cyanide m-chlorophenylhydrazone (CCCP)".

- In Figure EV2B, the authors use the K211N Parkin mutant to claim that "Parkin is ubiquitinated by MITOL after its translocation to the mitochondria". They refer to PMID 20098416 to claim that this mutant doesn't relocalise to the mitochondria. This is not strictly true. In this paper, Geisler et al show that the relocation of this mutant to the mitochondria is delayed, as opposed to abolished (it is not localised at mitochondria after 2hrs of CCCP, but it is localised with clustered mitochondria after 24hrs). In their experiment, the authors treat the cells with CCCP for 8hrs, and it is very possible that K211N Parkin mutant is localised at mitochondria after 8hrs. Their conclusion isn't valid without showing that K211N Parkin isn't localised at the mitochondria after 8hrs CCCP treatment.

Our reply:

We appreciate the referee's critical comments. According to the referee's comments, we checked the localization of Parkin K211N mutant 8 hours after CCCP treatment. We confirmed that K211N mutant failed to translocate to mitochondria even at the 8h after CCCP treatment (Fig EV1F).

- Despite additional experiments, where the MITOL-dependent ubiquitination of Parkin and the Parkin dependent degradation of FKBP38 occur, remains unclear.

- Whether/how Parkin relocates to the ER in their model remains unclear.

Our reply:

It is exactly as you said. At present, we could not clarify the above two points in detail. Due to the limitations of the experimental system, it was too difficult to detect where endogenous MITOL ubiquitinates and degraded Parkin and where endogenous FKBP38 is regulated by Parkin. We would like to establish and examine FKBP38 knock-in cells. In addition, the detailed mechanism for the translocation of Parkin to the ER could not be identified. Since Parkin translocates to the ER in a FKBP38-independent manner, we assumed that there may be a novel mechanism of Parkin translocation between mitochondria and ER. We would like to identify it as a future task.

- Many of the experiments remain over-expression (E.g why over-express FKBP38 in Figure EV5?)

Our reply:

Regarding the experiments in Figure EV5, we had to compare it with KR mutant, so we confirmed it in the overexpression (Figure EV6 A). As you pointed out, we generated and examined EGFP-MITOL knock-in cells to eliminate the possibility that overexpression of FKBP38 and MITOL may affect the localization changes of MITOL (Fig 3C). As a result, mitophagy-dependent MITOL translocation to the ER was clearly observed, similar to previous result using overexpression.

Minor comments

- I would delete "directly" in the title "MITOL directly ubiquitinates Parkin in Parkin-mediated mitophagy". In vitro ubiquitination assays with recombinant MITOL and Parkin would be required to determine whether the ubiquitination is direct. For the same reason, page 9, I would rephrase the sentence "MITOL adds the K48-linked polyubiquitin chain to Parkin".

We thank the reviewer for the constructive suggestion. According to the referee's suggestion we have deleted "Directly" in the title and sentences and changed the description of "MITOL adds the K48-linked polyubiquitin chain to Parkin" (lines 10-11 on page 10, lines 14-15 on page 10, lines 14-17 on page 13).

- When describing Figure 1A, the authors should precise that they refer to MITOL-Myc (as opposed to endogenous Myc)

Our reply:

We thank the reviewer for drawing our attention to confusing description. Following referee's comment, the notation of "myc" was changed to "MITOL-myc" in Figures 1A and 1C.

- The authors should indicate (in legends and in results) that they refer to Ser65-Parkin when mentioning phospho-Parkin

Our reply:

According to the reviewer comment, we changed the sentence and notion of "Phospho-Parkin" to "Phospho-Ser65-Parkin" especially the part of using specific antibody (Fig 4C).

- The manuscript would benefit from further proof-read for grammatical and spelling errors. For example, page 11, the sentence "Therefore, it is possible that regardless of the fact that the effect of endogenous MITOL on mitophagy might be milder compared to that of the overexpressed one, it can regulate the quantity of Parkin" isn't very unclear.

Our reply:

We thank the reviewer for drawing our attention to grammatical and spelling errors. According to the reviewer's comment, we deleted the sentence pointed out and had our text checked by a native speaker.

Dear Shigeru,

Thank you for submitting your revised manuscript. It has now been seen by all of the original referees, whose comments are copied below.

As you can see, referees find that the study is significantly improved during revision and recommend publication. However, the referees have some remaining minor concerns. In particular, referee #2 finds that the half-life experiment he/she requested earlier has not been provided. Moreover, both referee #2 and #3 find that additional controls for FACS based analysis on mtKeima are required. I think it would be good to sort these out and I would like to discuss with you what could be done to address these comments in a reasonable timeframe. You might already have good arguments/data at hand regarding these points. Please contact me to discuss this issue further before you embark on the revision.

Thank you again for giving us to consider your manuscript for EMBO Reports, I look forward to your revision.

Kind regards,

Deniz

--

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

In general, the authors have made excellent efforts resolving the raised issues and I appreciate the generation of the EGFP-MITOL knock-in cell line for the clarification of MITOL translocation during CCCP treatment. I have no further comments to add.

Referee #2:

Overall, while the authors attempted to address the concerns raised in the previous round of review, I am afraid that some of the issues I raised are still outstanding. In previous comments, I had requested that the authors perform a cycloheximide-chase analysis to go increase the rigor of the experiment in Figure 2D. While they performed a quantification of this result from 2D, a half-life experiment is still lacking. In addition, while the replacement of the previous dye-based mitophagy assays with the mKieda is a better approach, the new assays still do not include controls such as autophagy mutants showing its effectiveness. Finally, I still have concerns about the large number of mistakes throughout the manuscript at each stage of the revision process, as the authors have now suggested that the discrepancies in data between previous figures EV4B and EV4C raised in the previous review are due to mislabeled time points. Unfortunately, there have been many errors such as this at each stage of the review, which has raised some concerns about the overall rigor and reproducibility of the results.

Referee #3:

The reviewers have done a great job in addressing most of my comments.

I only have one comment: I am surprised that the authors have analysed their mtkeima data by FACS, as opposed to microscopy, as it is usually the case. If using FACS, I would think they need additional controls as it would emit in both red and green channel most of the time.

Point-by point comment for referees

Referee #1:

In general, the authors have made excellent efforts resolving the raised issues and I appreciate the generation of the EGFP-MITOL knock-in cell line for the clarification of MITOL translocation during CCCP treatment. I have no further comments to add.

We greatly appreciate your previous constructive comments that strengthen our manuscript.

Referee #2:

Overall, while the authors attempted to address the concerns raised in the previous round of review, I am afraid that some of the issues I raised are still outstanding. In previous comments, I had requested that the authors perform a cycloheximide-chase analysis to go increase the rigor of the experiment in Figure 2D. While they performed a quantification

of this result from 2D, a half-life experiment is still lacking. In addition, while the replacement of the previous dye-based mitophagy assays with the mKeima is a better approach, the new assays still do not include controls such as autophagy mutants showing its effectiveness. Finally, I still have concerns about the large number of mistakes throughout the manuscript at each stage of the revision process, as the authors have now suggested that the discrepancies in data between previous figures EV4B and EV4C raised in the previous review are due to mislabeled time points. Unfortunately, there have been many errors such as this at each stage of the review, which has raised some concerns about the overall rigor and reproducibility of the results.

We agree with your suggestion about CHX assay and an additional result is included in Figure 2D. As compared to MITOL KO cells, the protein level of Parkin in MITOL WT cells decreased after CCCP stimulation in a time course-dependent manner.

According to your suggestion about the negative control for FACS analysis, we examined the effect of bafilomycin A1, an autophagy inhibitor, on fluorescence of mKeima during mitophagy and demonstrated that FACS analysis was working without any problems (Fig EV3E). Furthermore, cell line stably expressing mt-Keima used in our FACS analysis was generated by our collaborators, Prof. Kanki and Dr. Yamashita, who are experts in the field of mitophagy research. They had already proved that this cell line works reliably in their previous studies (DOI: 10.1083/jcb.201605093 and DOI: 10.1038/s41598-020-58315-w). So, we cite two references in Page;xxx/line;xxxx.

Finally, we apologize our mistake in each stage of review. We will make sure never to do the same mistake ever again.

Referee #3:

The reviewers have done a great job in addressing most of my comments.

I only have one comment: I am surprised that the authors have analysed their mtkeima data by FACS, as opposed to microscopy, as it is usually the case. If using FACS, I would

think they need additional controls as it would emit in both red and green channel most of the time.

Following your advice, we performed the mt-Keima assay using bafilomycin A1 as a negative control (Fig EV3E).

We greatly appreciate your previous constructive comments that strengthen our manuscript.

Dear Shigeru,

Thank you for submitting your revised manuscript. I have now looked at everything and all is fine. Therefore I am very pleased to accept your manuscript for publication in EMBO Reports.

Congratulations on a nice work!

Before we can transfer your manuscript to our production team, we need to sort out the following. As per our guidelines, please add a 'Data Availability Section', where you state that no data were deposited in a public database. You can send the manuscript file per email to me.

Kind regards,

Deniz

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Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

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At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Shigeru Yanagi

Journal Submitted to: EMBO Reports

Manuscript Number: EMBOR-2019-49097V4

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 χ^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?	We performed at least three independent experiments. We chose the sample size routinely used in the field of molecular and cellular biology.
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.	The cells were randomly selected for imaging and analyzing.
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.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, differences between groups were examined for statistical significance using one-way ANOVA followed by the Tukey post hoc test or Student's t test.
Is there an estimate of variation within each group of data?	Yes.

USEFUL LINKS FOR COMPLETING THIS FORM

<http://www.antibodypedia.com>
<http://1degreebio.org>
<http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor>

<http://grants.nih.gov/grants/olaw/olaw.htm>
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<http://biomodels.net/miriam/>
<http://jij.biochem.sun.ac.za>
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
<http://www.selectagents.gov/>

Is the variance similar between the groups that are being statistically compared?	Yes.
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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).	We described these information in the materials and methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All our cell lines have been checked for mycoplasma contermination.

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

D- Animal Models

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

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
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

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'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data 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
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
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

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
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