EMBO reports

Parkin coordinates mitochondrial lipid remodeling to execute mitophagy

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1st Editorial Decision **4th May 2022**

Dear Dr. Lin,

Thank you for the submission of your manuscript to EMBO reports. We have now received the reports from the two referees who agreed to review your manuscript, which I copy below. Both referees think that your manuscript is interesting and their comments are quite positive.

As you will see from their reports, however, while being positive they suggest some additional controls and clarifications. There are significant technical caveats that will need your attentionin a revised version of the manuscript.

Given the referees evaluations and the potential interest of your study, I would like to give you the opportunity to revise your manuscript, with the understanding that the referee concerns must be fully addressed. It is EMBO reports policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses to the main concerns.

Revised manuscripts should be submitted within three months of a request for revision. Please contact us if a 3-months time frame is not sufficient so that we can discuss this further. We can also discuss the revisions in a video chat, if you like.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:
1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.
2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

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

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure). See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

3) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

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

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

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6) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines
<https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>

7) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please remember to provide a reviewer password if the datasets are not yet public. The accession numbers and database should be listed in a formal "Data

Availability" section placed after Materials & Method (see also https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please note that the Data Availability Section is restricted to new primary data that are part of this study. * Note - All links should resolve to a page where the data can be accessed. *
If your study has not produced novel datasets, please mention this fact in the Data Availability Section.

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at <https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

9) Our journal also encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,

- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,

- the nature of the bars and error bars (s.d., s.e.m.),

- If the data are obtained from n {less than or equal to} 2, use scatter blots showing the individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

- Please also include scale bars in all microscopy images.

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As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File (RPF) to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript. You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

I look forward to seeing a revised form of your manuscript when it is ready. Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

David del Alamo, PhD
Editor
EMBO reports

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Referee #1:

Lin and colleagues report that mitochondrial lipid remodeling controls mitophagy induced by mitochondria depolarization in Parkin-overexpressing cells. The authors show that following mitochondrial depolarization, PLD2 is recruited to mitochondria where it hydrolyses an unknown phospholipid to produce PA. PA is then converted to DAG via Lipin-1. These processes are dependent on the presence of Parkin following depolarization. DAG is proposed to be required for further recruitment of the autophagy machinery to drive autophagosome formation and mitophagy. The authors have many interesting and novel findings, which will improve our understanding of how autophagosomes form de novo following Parkin activation. However, as presented, more robust data is needed to support the conclusions proposed by the authors. Given that there are many aspects to this paper, there are quite a lot of comments below. Additionally, the hypothesis that Golgi-derived vesicles deliver EndoB1 to mitochondria needs strengthening significantly if it is to be included in the manuscript (my feeling is this could potentially be removed and placed in a follow-up manuscript, to allow the authors to focus on the exciting initial mechanism they have discovered). Below are some points to hopefully help in this endeavor.

Major comments:
1. A main concern with the paper is that when proteins are knocked down only one siRNA is used to target that protein. This is not sufficient given notorious siRNA off-target effects. In two instances the authors do rescue the siRNA phenotype, which is very good, but the necessary control blots are missing (see below). In the absence of a rescue experiment, the authors need to use multiple targeting siRNAs, or better yet CRISPR. Given the ease of making CRISPR KOs, the authors should at least try to KO one of the key pathway proteins to show it has the same effect as the siRNAs (eg. PLD2 or Lipin1 or EndoB1).
2. The authors only monitor TOM20 in their IF studies and given that this is a degraded by the proteasome following Parkin ubiquitination, the authors should confirm in one instance that other mitochondrial markers co-localize with PA/DAG.
3. In Fig. 1D, the statistical analyses are missing.
4. In Fig. EV1E, the SH-SY5Y cells, with endogenous Parkin, show significant recruitment of PA and DAG binding proteins to all mitochondria - similar to the Parkin over-expressing HeLa. However, wholesale mitophagy does not occur in these cells (otherwise these would be used by the field rather than the more artificial HeLa overexpressing Parkin). If PA and DAG are enriched on mitochondria but not driving mitophagy, what do the authors think is happening? Some explanation would be helpful here.
5. In Fig. 2 the authors show that PLD2 localizes to mitochondria and use of an inhibitor blocks PA production. Given that a small molecule inhibitor likely has off-target effects, does depletion of PLD2 block PA production (bearing in mind point 1)?
6. The siRNA rescue experiments in Fig. 2E and F are welcome, however, a western blot is needed to show the level of depletion of endogenous protein and compared with the level of the rescue exogenously expressed proteins. The IF images in panel E should be replaced with the corresponding ones with the endogenous and siRNA -/+ rescue. The authors also need to show that loss of Lipin does not alter PA-binding staining under control conditions too (they only show CCCP).
7. In Fig. 3D, it would be informative to show the Lipin KD + DPG without CCCP. Does this result in mitochondrial DAG and LC3 recruitment?
8. Related, does the addition of DPG rescue CCCP-induced mitophagy in the Lipin KD cells (LC3 puncta are shown but actual mitophagy is not)?
9. In Fig.4A-C the authors show that Parkin activity is required for DAG but not PA production (via binding proteins). However, they earlier state that Parkin is required to increase PA production, though say this as data not shown. Clarification here is needed and the authors should show that in these panels, cells without Parkin do not recruit either lipid binding protein. If this indeed the case, then some speculation may be needed in the discussion as to how PLD2 is recruited to mitochondria - if it is independent of Parkin ubiquitination.
10. Related to the above, the authors need show a western blot detailing the level of expression of WT Parkin and mutants.
11. Likewise, in Fig 4D-E, the authors need to show western blots of KD and levels of OPTN rescue.
12. Citrate synthetase and alpha-tubulin in Fig. 2C and Fig. 5C appear to be the same. Some clarification is needed here - are these the same blots from the same experiment?
13. In Fig. 5 the authors claim that TGN-derived vesicles traffic to mitochondria, given that TGN38 co-localizes with mitochondria after treatments. Could it be that these are ATG9 positive vesicles (ATG9 can be on the TGN)? This would certainly make sense, given ATG9's autophagy role in lipid transfer. The authors should test this by IF and in their OPTN/NDP52 KD model.
14. The authors go on to suggest that it is TGN-localized EndoB1 that is critical. However, EndoB1 is present on multiple membranes and can also be directly recruited from the cytosol to membranes. If the authors want to propose this model, then more work is needed to show that it is specifically this pool of EndoB1.
15. Is it specifically TGN-derived membranes? As a control the authors should monitor other Golgi markers such as GM130.
16. The current model is that OPTN and related receptors directly recruit the ULK1 kinase complex to ubiquitinated mitochondria to initiate autophagosome formation. Is this still the case upon loss of EndoB1?
Minor comments:
1. Figure legends could be more accurate, such as the EV Figure 1 (B-C), the Oligomycin/Antimycin concentration should be 10/4 μM, rather than mM.
2. The tag position for Parkin needs to be confirmed. Sometimes it is in N-terminal, while the other place shows C-terminal (EV Fig1A: is it mCherry-Parkin or Parkin-mCherry?).
3. The scale bar should be put in Fig. 2C.
4. Some antibodies' catalog numbers are needed, such as MFN1, and LC3.
5. Figure 4 is titled with "....and exogenous DAG restores mitophagy.", but no data is shown about this.
6. Fig. 4C legend: the quantification should be for experiments shown in A and B, rather than "D and E".
7. A technical point about BafA1 concentration in this manuscript. Usually, the BafA1 concentration will be saturated to inhibit

autophagic flux from 20 to 50nM in most cell lines including HeLa cells. Not sure why 1μM is used here?
8. Fig.5 legend title claims more than the data. How OPTN and NDP52 "deliver" the EndoB1 is not shown. No data about the "EndoB1-postive Golgi vesicles" and "ubiquitinated" mitochondria are actually shown.
9. In the introduction, line 42, the authors state that lysosomes are also tagged with ubiquitin to drive autophagy. Ubiquitin tagging is seen as a general mechanism and it is not just mitochondria and lysosomes - peroxisomes, protein aggregates, intracellular pathogens etc. all get ubiquitinated prior to autophagy.

Referee #2:

In this manuscript, the authors found that focal DAG production on mitochondria is necessary for mitophagosome formation and the subsequent clearance of mitochondria during PINK1/Parkin mediated-mitophagy. They identified several factors that are required for the DAG production such as PLD2, Lipin1 and EndoB1. PLD2 was recruited to mitochondria directly by Parkin (but this process does not require E3 ligase activity of Parkin) and contributed to production of PA, a source of DAG, on damaged mitochondria. NDP52 and OPTN, autophagy receptors which play important roles in mitophagosome formation downstream of Parkin, were critical for subsequent DAG production by activating PA phosphatase Lipin1 and by recruiting EndoB1, one of the components of autophagy machineries. Overall, their findings are striking and intriguing. However, some of their arguments are not fully supported by experimental evidence. Also, in Introduction section, the authors should mention recent several important papers which describe how NDP52 and OPTN recruit autophagy machineries during PINK1/Parkin-mediated mitophagy. It is highly recommended to explain what we know so far and what are remaining questions for better understanding of readers who are not familiar with this field. Please see specific comments below.

Major points

1. I recommend the authors to refer to following recent review - it well summarized recent findings regarding NDP52 and OPTN-mediated mitophagosome formation during PINK1/Parkin-mediated mitophagy.

Mechanisms underlying ubiquitin-driven selective mitochondrial and bacterial autophagy.
Goodall EA, Kaus F, Harper JW
Mol. Cell, 2022 Apr 21;82(8):1501-1513

2. In Fig. 1A, it is surprising that the signal of PA reporter is almost exclusively observed on mitochondria within cells after CCCP treatment. To exclude the concern that the overexpression of Parkin somehow disturbs the microscopic analysis of the subcellular localization of PA reporters, the authors should confirm that the signal of PA reporter on mitochondria after OA treatment is cancelled in Parkin knockout (KO) SH-SY5Y cells in Extended Fig. 1E and F.

3. In Fig. 1D, the authors mentioned that the relative abundance of DAG species was normalized by PC, because PC was not affected by CCCP. The authors should also present the actual data that the abundance of PC was not affected by CCCP. Also, they should perform statistical analysis using at least n=3 data sets.

4. Line 120 on page 7; the authors mentioned that no mitochondrial PLD2 was detected in the absence of Parkin (Fig. EV2C). However, Fig. EV2C shows that CCCP-dependent interaction between PLD2 and Parkin. The authors should add appropriate data.

5. In Fig. 2F, the authors should also present representative ICC images. This is also the same for Fig. 4E.

6. In Fig. 3C, the authors should confirm that Lipin1 knockdown attenuates the clearance of damaged mitochondria using another Lipin1 siRNA that targets different region of Lipin1 mRNA. Alternatively, they should perform rescue experiments as performed in Fig. 2F.

7. In Fig. 3D and In Fig. EV3B (Fig. EV3), the authors mentioned that lower expression of YFP-DAGR or DAGR reporter with a lower binding affinity (RFP-DAGR) show LC3-positive, but not mitochondria-positive, signal within cells. It is not clear why this happens.

8. In Fig. 3E and F, the reduction of LC3-positive vesicles in Lipin1 deficient cells are nicely rescued by the external addition of DPG. The authors are highly recommended to examine whether the attenuation of mitochondria clearance in Lipin1 deficient cells in Fig. 3C is also rescued by the external addition of DPG.

9. In Fig. 5, the authors should confirm that the mitochondrial recruitment of EndoB1 after CCCP treatment is not observed under double knockdown of NDP52 and OPTN.

10. Line 64 on page 4; the authors argue that OPTN and NDP52 activates the PA-phosphatase Lipin-1. Does this mean enzymatic activity of Lipin1? How about the subcellular localization of Lipin1 before and after CCCP treatment?

11. It is better to discuss how EndoB1 is involved in DAG formation. What is a relationship between Lipin1 and EndoB1?

Minor points

1. In Extended Fig. 1, the concentration of antimycin A and oligomycin A should be wrong. uM order is usually used, not mM.

2. Fig. EV3B should be just Fig. EV3.

3. In Fig. 4E, "P" indicated in the right corner of graph should be removed.

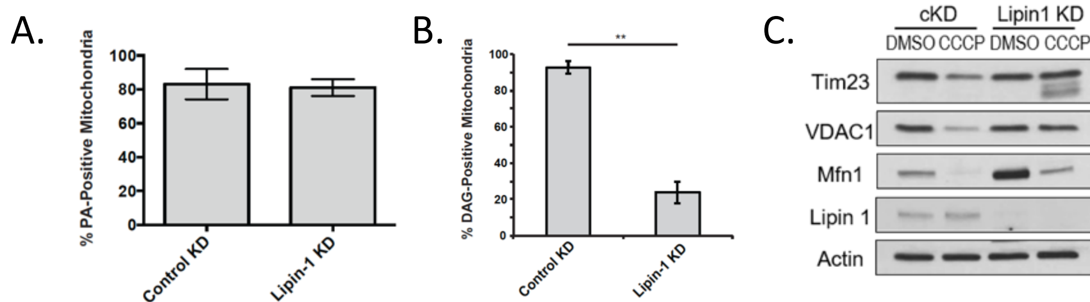
Referee #1:

Lin and colleagues report that mitochondrial lipid remodeling controls mitophagy induced by mitochondria depolarization in Parkin-overexpressing cells. The authors show that following mitochondrial depolarization, PLD2 is recruited to mitochondria where it hydrolyses an unknown phospholipid to produce PA. PA is then converted to DAG via Lipin-1. These processes are dependent on the presence of Parkin following depolarization. DAG is proposed to be required for further recruitment of the autophagy machinery to drive autophagosome formation and mitophagy. The authors have many interesting and novel findings, which will improve our understanding of how autophagosomes form de novo following Parkin activation. However, as presented, more robust data is needed to support the conclusions proposed by the authors. Given that there are many aspects to this paper, there are quite a lot of comments below. Additionally, the hypothesis that Golgi-derived vesicles deliver EndoB1 to mitochondria needs strengthening significantly if it is to be included in the manuscript (my feeling is this could potentially be removed and placed in a follow-up manuscript, to allow the authors to focus on the exciting initial mechanism they have discovered). Below are some points to hopefully help in this endeavor.

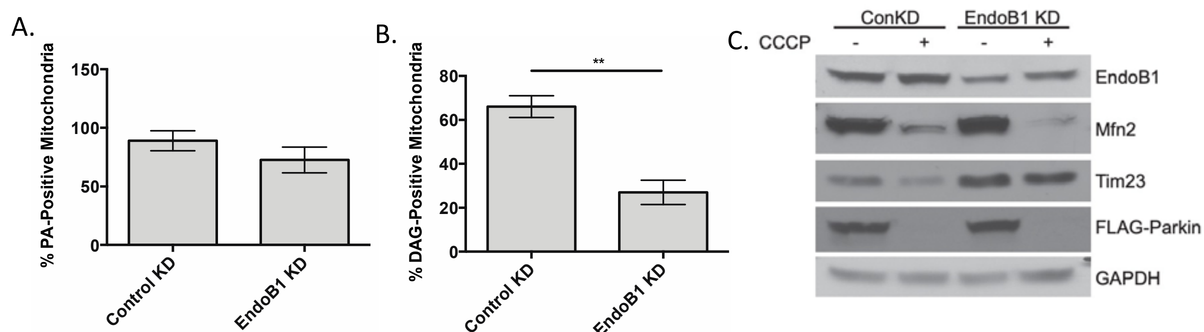
Major comments:

1. *A main concern with the paper is that when proteins are knocked down only one siRNA is used to target that protein. This is not sufficient given notorious siRNA off-target effects. In two instances the authors do rescue the siRNA phenotype, which is very good, but the necessary control blots are missing (see below). In the absence of a rescue experiment, the authors need to use multiple targeting siRNAs, or better yet CRISPR. Given the ease of making CRISPR KOs, the authors should at least try to KO one of the key pathway proteins to show it has the same effect as the siRNAs (eg. PLD2 or Lipin1 or EndoB1).*

→ This report indeed uses more than one targeting event to knock down Lipin 1 (two independent siRNAs) and endoB1 (one siRNA and one shRNA). They produced similar mitochondrial DAG and mitophagy phenotypes. Due to the space limit, we did not include the data in the manuscript. Below, we have presented some of the data using an independent siRNA (Lipin 1, Stealth siRNA, Invitrogen (life technologies); 1299001) and an endoB1 shRNA (provided by Dr. Richard Youle (Karbowski *et al*, 2004)) for reviewers.



Legend: **A second Lipin 1 siRNA also inhibits mitochondrial DAG production and mitophagy.** Control or Lipin-1 siRNA knockdown (KD) HeLa cells were transfected with PA or DAG reporter and Parkin followed by CCCP treatment. (A-B) Note that Lipin 1 KD inhibits DAG, but not PA, reporter accumulation on mitochondria following CCCP treatment. (C). Western blots showed that Lipin 1 KD reduced mitophagy efficiency, as indicated by the retention of mitochondrial Tim23 and VDAC1 after CCCP treatment.



Legend: An independent endoB1 shRNA suppressed mitochondrial DAG production and mitophagy. (A-B) HeLa cells were transfected with control or EndoB1 shRNA plasmids, followed by transfection with lipid reporters and FLAG-Parkin and CCCP treatment (10mM). EndoB1 KD reduced DAG but not PA reporter accumulation on the mitochondria following CCCP treatment. **(C)** The effect of EndoB1 shRNA on Parkin-mediated mitophagy was assessed by immunoblotting for indicated mitochondrial proteins. Like Lipin 1 and PLD2 inhibition, endoB1 shRNA suppressed CCCP-induced Tim23, but not MFN2, degradation.

For PLD2, although we have used only one siRNA (EV Fig 2E-F), its inhibitory effect on mitochondrial PA and DAG production was confirmed independently using a chemical inhibitor (Fig 2D).

2. *The authors only monitor TOM20 in their IF studies and given that this is a degraded by the proteasome following Parkin ubiquitination, the authors should confirm in one instance that other mitochondrial markers co-localize with PA/DAG.*
→ Although TOM20 degradation is thought to be mainly mediated by the proteasome, its kinetics is much slower than that of MFN1 and MFN2- the two best-characterized proteasomal substrates and tracks much better with the loss of mitochondrial mass during mitophagy. Therefore, TOM20 is a reliable marker for mitophagy, as we have previously reported (Lee *et al*, 2010). Furthermore, the TOM20-based IF analyses were independently confirmed by immuno-blotting for mitochondrial proteins localized in the intermediate space (Tim23) and matrix (citrate synthase and Hsp60), as shown in Fig 3C and Fig EV2I. We also have included COX IV as a mitochondrial marker to confirm its colocalization with PA as shown in EV Fig 1G.

3. *In Fig. 1D, the statistical analyses are missing.-*
→ Due to the resource and time limitation to repeat the experiments, we have removed this panel and moved it into the discussion as a preliminary finding. To reflect the fact that it is a single data point, we have replaced the bar graphs with the original mass spectrometry profile (Fig EV5D).

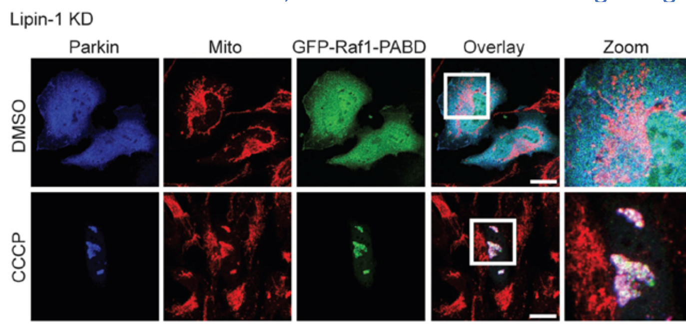
4. *In Fig. EV1E, the SH-SY5Y cells, with endogenous Parkin, show significant recruitment of PA and DAG binding proteins to all mitochondria - similar to the Parkin over-expressing HeLa. However, wholesale mitophagy does not occur in these cells (otherwise these would be used by the field rather than the more artificial HeLa overexpressing Parkin). If PA and DAG are enriched on mitochondria but not driving mitophagy, what do the authors think is happening? Some explanation would be helpful here.*
→ The reviewer is correct that endogenous Parkin is not sufficient to drive a robust mitophagy. The concentration of DAG and PA reporter is much variable in SH-SY5Y cells. We have chosen cells with more prominent DAGR and PA reporter accumulation to provide visual evidence that endogenous Parkin can support DAG and PA production. As these lipid reporters can prevent mitochondrial degradation, we suspect this effect also enhances the accumulation of DAG- and PA-positive mitochondria.
As expected, Parkin KD in SH-SY5Y cells reduced mitochondrial aggregation and the PA-GFP reporter accumulation (Fig EV1G-H).

5. *In Fig. 2 the authors show that PLD2 localizes to mitochondria and use of an inhibitor blocks PA production. Given that a small molecule inhibitor likely has off-target effects, does depletion of PLD2 block PA production (bearing in mind point 1)?*
->Yes, PLD2 siRNA-mediated KD suppressed mitochondrial PA production (EV2D-E). Thus, siRNA and chemical inhibition of PLD2 produced consistent phenotypes.

6. *The siRNA rescue experiments in Fig. 2E and F are welcome, however, a western blot is needed to show the level of depletion of endogenous protein and compared with the level of the rescue exogenously expressed proteins. The IF images in panel E should be replaced with the corresponding ones with the endogenous and siRNA -/+ rescue.*
→ The expression of WT and CD mutant Lipin 1 is presented in Fig EV2K. The representative IF images are shown in Fig EV2J.

The authors also need to show that loss of Lipin does not alter PA-binding staining under control conditions too (they only show CCCP).

-→ There is no mitochondrial PA accumulation without CCCP. Thus, Lipin 1 KD under this condition will not affect PA status, as shown in the following image.

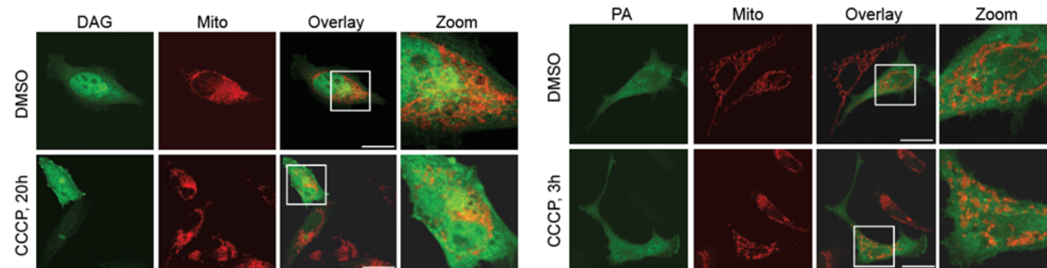


7-8. In Fig. 3D, it would be informative to show the Lipin KD + DPG without CCCP. Related, does the addition of DPG rescue CCCP-induced mitophagy in the Lipin KD cells (LC3 puncta are shown but actual mitophagy is not)? Does this result in mitochondrial DAG and LC3 recruitment?

→ Because DPG has been shown previously to rescue starvation-induced autophagy phenotype in Lipin 1 KO cells (Zhang *et al*, 2014), we decided to investigate the effect of DPG on OPTN/NDP52 double KD cells, which are deficient in DAG production and mitophagy. As shown in the new "Fig4F-G ", exogenous DPG treatment can significantly rescue the mitophagy defects in OPTN/NDP52. The data suggest that a key function of OPTN and NDP52 in mitophagy is to promote mitochondrial DAG production.

9. In Fig.4A-C the authors show that Parkin activity is required for DAG but not PA production (via binding proteins). However, they earlier state that Parkin is required to increase PA production, though say this as data not shown. Clarification here is needed and the authors should show that in these panels, cells without Parkin do not recruit either lipid binding protein. If this indeed the case, then some speculation may be needed in the discussion as to how PLD2 is recruited to mitochondria - if it is independent of Parkin ubiquitination.

→ There is no recruitment of DAG or PA reporter in the absence of Parkin, as shown by the representative images. These data were not included in the report to reduce the number of Figures.



Legend: Hela cells transfected with DAG or PA reporter alone without Parkin were treated with DMSO or CCCP as indicated. Note that there is no accumulation of PA or DAG reporter.

For the potential mechanism by which Parkin recruits PLD2 to mitochondria, we have presented the following evidence: "Co-immunoprecipitation indicates that Parkin interacts with PLD2, suggesting a mechanism by which PLD2 is recruited to Parkin-tagged mitochondria (Fig EV2C)."

10. *Related to the above, the authors need show a western blot detailing the level of expression of WT Parkin and mutants.*

→ Western blots of WT and mutant Parkin are included in Fig 4A.

11. *Likewise, in Fig 4D-E, the authors need to show western blots of KD and levels of OPTN rescue.*

→ Western blots are included in Fig 4E and representative images in Fig EV4D.

12. Citrate synthetase and alpha-tubulin in Fig. 2B and Fig. 5C appear to be the same. Some clarification is needed here - are these the same blots from the same experiment?

→ Yes. To avoid confusion, we have specifically pointed out this fact in the Figure Legend.

"Mitochondrial and cytosolic fractions obtained from control and CCCP treated cells, as was

described and analyzed in Fig 2B".

13. *In Fig. 5 the authors claim that TGN-derived vesicles traffic to mitochondria, given that TGN38 co-localizes with mitochondria after treatments. Could it be that these are ATG9 positive vesicles (ATG9 can be on the TGN)? This would certainly make sense, given ATG9's autophagy role in lipid transfer. The authors should test this by IF and in their OPTN/NDP52 KD model.*
→ The involvement of Golgi-derived ATG9 is indeed probable. Because we have decided to remove the data on Golgi-TGN38, as suggested by the reviewer, the role of ATG9 will be investigated in the future study.

14. *The authors go on to suggest that it is TGN-localized EndoB1 that is critical. However, EndoB1 is present on multiple membranes and can also be directly recruited from the cytosol to membranes. If the authors want to propose this model, then more work is needed to show that it is specifically this pool of EndoB1.*
→ We agree with the reviewer. We have included the following statement in the discussion: " However, the involvement of non-Golgi EndoB1, OPTN, and NDP52 in mitophagy cannot be excluded."

15. *Is it specifically TGN-derived membranes? As a control the authors should monitor other Golgi markers such as GM130.*
-→As suggested by the Reviewer, we have removed the TGN38 study.

16. *The current model is that OPTN and related receptors directly recruit the ULK1 kinase complex to ubiquitinated mitochondria to initiate autophagosome formation. Is this still the case upon loss of EndoB1?*
→ This is indeed an interesting question worthy of further investigation. In the revised discussion, we have included the following statement "Whether OPTN/NDP52 or EndoB1 also utilize DAG to regulate additional factors critical for mitophagy, for example, Ulk1 (Vargas *et al*, 2019) and TBK1 (Richter *et al*, 2016), is a crucial question that requires further investigation."

Minor comments:
1. Figure legends could be more accurate, such as the EV Figure 1 (B-C), the Oligomycin/Antimycin concentration should be 10/4 μM, rather than mM.
→ Corrected.

2. The tag position for Parkin needs to be confirmed. Sometimes it is in N-terminal, while the other place shows C-terminal (EV Fig1A: is it mCherry-Parkin or Parkin-mCherry?).
→ It is mCherry-Parkin, as described in Yang JY, Yang WY (2013) Bit-by-bit autophagic removal of parkin-labelled mitochondria. *Nat Commun* 4: 2428

3. The scale bar should be put in Fig. 2C.
→ Scale bar was included.

4. Some antibodies' catalog numbers are needed, such as MFN1, and LC3.
→ They are included.

5. Figure 4 is titled with "....and exogenous DAG restores mitophagy.", but no data is shown about this.
→ The new data on the effect of exogenous DAG is added (new Fig 4F-G).

6. Fig. 4C legend: the quantification should be for experiments shown in A and B, rather than "D and E".
→ Corrected.

7. A technical point about BafA1 concentration in this manuscript. Usually, the BafA1 concentration will be saturated to inhibit autophagic flux from 20 to 50nM in most cell lines including HeLa cells. Not sure why 1μM is used here?

→ It was an error. Corrected to 0.1 μM.

8. Fig.5 legend title claims more than the data. How OPTN and NDP52 "deliver" the EndoB1 is not shown. No data about the "EndoB1-postive Golgi vesicles" and "ubiquitinated" mitochondria are actually shown.
→ It is modified to "OPTN and NDP52-depedent EndoB1 mitochondrial recruitment is required for mitochondrial DAG production"

9. In the introduction, line 42, the authors state that lysosomes are also tagged with ubiquitin to drive autophagy. Ubiquitin tagging is seen as a general mechanism and it is not just mitochondria and lysosomes - peroxisomes, protein aggregates, intracellular pathogens etc. all get ubiquitinated prior to autophagy.
→ The statement only refers to organelle autophagy.

Referee #2:

In this manuscript, the authors found that focal DAG production on mitochondria is necessary for mitophagosome formation and the subsequent clearance of mitochondria during PINK1/Parkin mediated-mitophagy. They identified several factors that are required for the DAG production such as PLD2, Lipin1 and EndoB1. PLD2 was recruited to mitochondria directly by Parkin (but this process does not require E3 ligase activity of Parkin) and contributed to production of PA, a source of DAG, on damaged mitochondria. NDP52 and OPTN, autophagy receptors which play important roles in mitophagosome formation downstream of Parkin, were critical for subsequent DAG production by activating PA phosphatase Lipin1 and by recruiting EndoB1, one of the components of autophagy machineries. Overall, their findings are striking and intriguing. However, some of their arguments are not fully supported by experimental evidence. Also, in Introduction section, the authors should mention recent several important papers which describe how NDP52 and OPTN recruit autophagy machineries during PINK1/Parkin-mediated mitophagy. It is highly recommended to explain what we know so far and what are remaining questions for better understanding of readers who are not familiar with this field. Please see specific comments below.

Major points

1. I recommend the authors to refer to following recent review - it well summarized recent findings regarding NDP52 and OPTN-mediated mitophagosome formation during PINK1/Parkin-mediated mitophagy. Mechanisms underlying ubiquitin-driven selective mitochondrial and bacterial autophagy. Goodall EA, Kaus F, Harper JW
Mol. Cell, 2022 Apr 21;82(8):1501-1513
-→ The reference is included.

2. In Fig. 1A, it is surprising that the signal of PA reporter is almost exclusively observed on mitochondria within cells after CCCP treatment. To exclude the concern that the overexpression of Parkin somehow disturbs the microscopic analysis of the subcellular localization of PA reporters, the authors should confirm that the signal of PA reporter on mitochondria after OA treatment is cancelled in Parkin knockout (KO) SH-SY5Y cells in Extended Fig. 1E and F.
→ Parkin KD in SH-SY5Y cells reduced mitochondrial aggregation and the PA-GFP reporter accumulation (Fig EV1G-H).

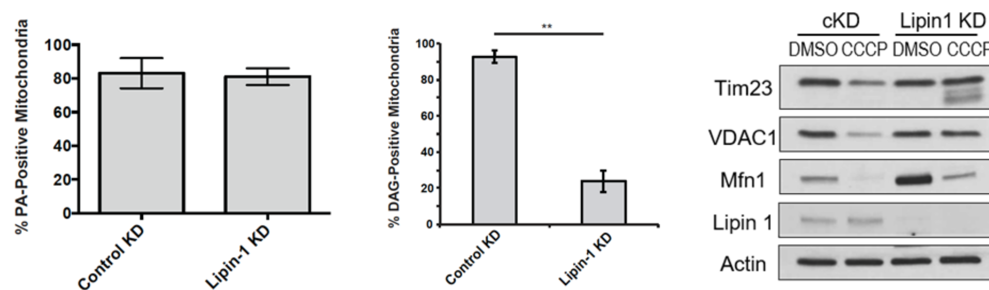
3. In Fig. 1D, the authors mentioned that the relative abundance of DAG species was normalized by PC, because PC was not affected by CCCP. The authors should also present the actual data that the abundance of PC was not affected by CCCP. Also, they should perform statistical analysis using at least n=3 data sets.
→Due to the resource and time limitation to repeat the experiments, we have removed this panel and moved it into the discussion as a preliminary finding. To reflect the fact that it is a single data point, we have replaced the bar graphs with the original mass spectrometry profile (Fig EV5D).

4. Line 120 on page 7; the authors mentioned that no mitochondrial PLD2 was detected in the absence of Parkin (Fig. EV2C). However, Fig. EV2C shows that CCCP-dependent interaction between PLD2 and Parkin. The authors should add appropriate data.
-→ We have corrected the error. The text now reads "Co-immunoprecipitation indicates that Parkin interacts with PLD2, suggesting a mechanism by which PLD2 is recruited to Parkin-tagged mitochondria (Fig EV2C)."

5. In Fig. 2F, the authors should also present representative ICC images. This is also the same for Fig. 4E.----

→ The representative images are now included in Fig EV2J and Fig EV4D.

6. In Fig. 3C, the authors should confirm that Lipin1 knockdown attenuates the clearance of damaged mitochondria using another Lipin1 siRNA that targets different region of Lipin1 mRNA. Alternatively, they should perform rescue experiments as performed in Fig. 2F.
→ Lipin 1 KD phenotypes were assessed by two independent siRNA, and they produced similar results. Due to the space limitation, only one siRNA was shown in the report. The results from a second Lipin 1 siRNA (Stealth siRNA, Invitrogen (life technologies); 1299001) are provided below.



Legend: Control or Lipin-1 siRNA knockdown (KD) HeLa cells were transfected with PA or DAG reporter and Parkin followed by CCCP treatment. Note that Lipin 1 KD inhibits DAG, but not PA, reporter accumulation on mitochondria following CCCP treatment. Western blots showed that Lipin 1 KD reduced mitophagy efficiency, as indicated by the retention of mitochondrial Tim23 and VDAC1 after CCCP treatment.

7. In Fig. 3D and In Fig. EV3B (Fig. EV3), the authors mentioned that lower expression of YFP-DAGR or DAGR reporter with a lower binding affinity (RFP-DAGR) show LC3-positive, but not mitochondria-positive, signal within cells. It is not clear why this happens.
→ The two DAG reporters are made of different DAG-binding domains. The DAGR-YFP version used a DAG-binding domain from PKCδ with a much higher affinity for DAG than that of DAGR-RFP (from PKCβ, estimated to be a 3-fold difference (Giorgione *et al*, 2006)). We suspect that high-affinity binding is required for detectable association with mitochondria. These references were included in the manuscript.

8. In Fig. 3E and F, the reduction of LC3-positive vesicles in Lipin1 deficient cells are nicely rescued by the external addition of DPG. The authors are highly recommended to examine whether the attenuation of mitochondria clearance in Lipin1 deficient cells in Fig. 3C is also rescued by the external addition of DPG.
→ Because DPG has been shown previously to rescue starvation-induced autophagy phenotype in Llipin 1 KO cells (Zhang *et al.*, 2014), we decided to investigate the effect of DPG on OPTN/NDP52 double KD cells, which are deficient in DAG production and mitophagy. As shown in the new "Fig4F-G ", exogenous DPG treatment can significantly rescue the mitophagy defects in OPTN/NDP52. The data suggest that a key function of OPTN and NDP52 in mitophagy is to promote mitochondrial DAG production.

9. In Fig. 5, the authors should confirm that the mitochondrial recruitment of EndoB1 after CCCP treatment is not observed under double knockdown of NDP52 and OPTN.
-→ As shown in new Figure 5C, CCCP-induced EndoB1 mitochondrial recruitment is reduced in OPTN/NDP52 knockdown cells.

10. Line 64 on page 4; the authors argue that OPTN and NDP52 activates the PA-phosphatase Lipin-1. Does this mean enzymatic activity of Lipin1? How about the subcellular localization of Lipin1 before and after CCCP treatment?

-→ Yes, the conclusion was inferred by the requirement of OPTN and NDP52 for Lipin-1-dependent mitochondrial DAG production. However, we did not detect Lipin 1 recruitment to mitochondria; it remains cytosolic. Therefore, we speculate that Lipin 1 affects mitochondrial DAG production via mitochondrial EndoB1, whose recruitment to mitochondria depends on OPTN and NDP52.

11. It is better to discuss how EndoB1 is involved in DAG formation. What is a relationship between Lipin1 and EndoB1?

-→ We have included the following discussion: "As we have not been able to detect Lipin 1 on Parkin-tagged mitochondria, we speculate that Lipin 1 affects mitochondrial DAG production via mitochondrial EndoB1, whose recruitment to mitochondria depends on OPTN and NDP52. Supporting these findings, aberrant accumulation of mitochondria was observed in Lipin 1 and EndoB1 knockout mice (Takahashi *et al*, 2013; Zhang *et al.*, 2014)."

Minor points

1. In Extended Fig. 1, the concentration of antimycin A and oligomycin A should be wrong. uM order is usually used, not mM.

→corrected.

2. Fig. EV3B should be just Fig. EV3.

→corrected.

3. In Fig. 4E, "P" indicated in the right corner of graph should be removed.

→ Corrected.

Reference:

Giorgione JR, Lin JH, McCammon JA, Newton AC (2006) Increased membrane affinity of the C1 domain of protein kinase Cdelta compensates for the lack of involvement of its C2 domain in membrane recruitment. *J Biol Chem* 281: 1660-1669

Karbowski M, Jeong SY, Youle RJ (2004) Endophilin B1 is required for the maintenance of mitochondrial morphology. *J Cell Biol* 166: 1027-1039

Lee JY, Nagano Y, Taylor JP, Lim KL, Yao TP (2010) Disease-causing mutations in Parkin impair mitochondrial ubiquitination, aggregation, and HDAC6-dependent mitophagy. *J Cell Biol* 189: 671-679

Richter B, Sliter DA, Herhaus L, Stolz A, Wang C, Beli P, Zaffagnini G, Wild P, Martens S, Wagner SA *et al* (2016) Phosphorylation of OPTN by TBK1 enhances its binding to Ub chains and promotes selective autophagy of damaged mitochondria. *Proc Natl Acad Sci U S A* 113: 4039-4044

Takahashi Y, Hori T, Cooper TK, Liao J, Desai N, Serfass JM, Young MM, Park S, Izu Y, Wang HG (2013) Bif-1 haploinsufficiency promotes chromosomal instability and accelerates Myc-driven lymphomagenesis via suppression of mitophagy. *Blood* 121: 1622-1632

Vargas JNS, Wang C, Bunker E, Hao L, Maric D, Schiavo G, Randow F, Youle RJ (2019) Spatiotemporal Control of ULK1 Activation by NDP52 and TBK1 during Selective Autophagy. *Mol Cell* 74: 347-362 e346

Zhang P, Verity MA, Reue K (2014) Lipin-1 regulates autophagy clearance and intersects with statin drug effects in skeletal muscle. *Cell Metab* 20: 267-279

1st Revision - Editorial Decision — 31st Aug 2022

Dear Dr. Yao

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

As you will see, all referees are very positive about the study and request only minor changes before it can be published. Please address the remaining concerns from referee 1 experimentally and please provide a point-by-point response. I also agree with the suggestions from referee 2. Please rearrange the figures and please unify the size of the panel letters. You may also want to consult our figure guidelines (see https://www.embopress.org/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf)

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study.

- Your manuscript contains 5 figures and will be published in our Reports section. To make this possible, we kindly ask you to combine the Results and Discussion section and to keep an eye on our character limit for Reports (25,000 plus/minus 2,000 characters including spaces but excluding materials & methods and references)

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- We generally recommend arranging figure panels so that they can be called out in an alphabetical order. In this case, we note that Fig. 1C is called out before 1B.
- A callout to Fig EV4B is missing.

- Please remove the movie legend from the manuscript file and provide it as simple README.txt file . Then zip the movie with its legend and upload the ZIP file.

- Please remove the list of Abbreviations and define each abbreviation when it first occurs in the text.

- In the legend of Figure 2 you mention a Source Data file, which has not been uploaded to our online submission system.

- Please add headings 'References' and 'Expanded View Figure Legends'.

- The Figure legends should follow after the References.

- In the Author Checklist: Please complete the section Materials -Core Facilities.

- I attach to this email a related manuscript file with comments by our data editors. Please address all comments and upload a revised file with tracked changes with your final manuscript submission. I have also taken the liberty to make some changes to the Abstract. Could you please review it?

- Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x300-600 pixels large (width x height) in PNG for JPG format. You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

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

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

This is a re-review of a manuscript by Lin et al., and the authors have done a good job in addressing my concerns. I just have one point.
1) With regards to the response to my previous point 8 about DPG rescuing mitophagy phenotypes - the new data in Fig4F-G are very striking and exciting. The IF images should be much larger and the quantitation must be done in triplicate with statistical analysis performed (especially as the authors claim significance in the legend and discussion). Given that these data suggest OPTN/NDP52 are not critical for mitophagy when excess DAG is present, I also recommend confirming the rescue in mitophagy by other means (e.g. western blot loss of mitochondrial proteins).
This should also perhaps be discussed more in the conclusions as to how DAG can overcome the already known functions of OPTN/NDP52 in recruiting FIP200/binding LC3.

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In the revised version of the manuscript, the authors fully answered to this reviewer's concerns.
As a minor suggestion, I highly recommend the authors to reorganize and finalize figures. Some figure panels are too small (for example, Fig. 4F, EV Fig. 3 etc.), or overlapped with other panels (EV Fig. 2H), or saturation indication is not removed (EV Fig. 2K). Letter size in each panel should be unified for better presentation (for example, EV Fig. 2K). Also, some typos are observed in the body of the manuscript (for example, Line 124 on page 7, Lipid KD should be Lipin-1 KD).

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→ Within the limit.

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→ Information was provided under Acknowledgement.

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Summary of the findings: The study showed that Parkin orchestrates mitophagy by coordinating mitochondrial lipid remodeling and sequential production of mitochondrial phosphatic acid and diacylglycerol.

Bullet Points:

1. Parkin recruits lipid-modifying enzymes to produce mitochondrial phosphatidic acid and diacylglycerol.
2. Parkin-mediated mitochondrial ubiquitination and ubiquitin-binding autophagic receptors, optineurin and NDP52, are required for mitochondrial diacylglycerol production.
3. Mitochondrial diacylglycerol production stimulates autophagosome assembly and mitophagy.

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

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

This is a re-review of a manuscript by Lin et al., and the authors have done a good job in addressing my concerns. I just have one point.
1) With regards to the response to my previous point 8 about DPG rescuing mitophagy phenotypes - the new data in Fig4F-G are very striking and exciting. The IF images should be much larger and the quantitation must be done in triplicate with statistical analysis performed (especially as the authors claim significance in the legend and discussion). Given that these data suggest OPTN/NDP52 are not critical for mitophagy when excess DAG is present, I also recommend confirming the rescue in mitophagy by other means (e.g. western blot loss of mitochondrial proteins).

This should also perhaps be discussed more in the conclusions as to how DAG can overcome the already known functions of OPTN/NDP52 in recruiting FIP200/binding LC3.

Response:
1. We have enlarged the images in Fig 4F.
2. Although the data were based on two independent experiments, the differences among samples are substantial (over 2-fold; e.g., DPG elevates mitophagy from ~20% to over 50% in NDP52 and OPTN KD cells) and reached statistical significance by SEM.

Because of the known pro-autophagic activity of a cell-permeable DAG (DPG) and the large number of experimental data presented in this report, after consulting with the editor, we hope the reviewer would agree that the immunoblotting experiment would not be essential to support the role of DAG in mitophagy for this manuscript.
3. We thank the reviewer for a critical question. We have included the following discussion and a new reference to explain a potential mechanism on how a cell-permeable DAG might enable LC3 recruitment to the mitochondria. It reads:
Line 220, *"Thus, a key function of OPTN/NDP52 in mitophagy is stimulating DAG production. Interestingly, OPTN/NDP52, known as LC3-binding autophagic receptors, can recruit LC3 to damaged mitochondria without directly binding LC3 (Padman et al, 2019). Based on our findings, we speculate that OPTN/NDP52 could indirectly recruit LC3 by activating DAG-*

dependent mitophagosome assembly on ubiquitinated mitochondria- an activity that a cell-permeable DAG can mimic."
Padman BS, Nguyen TN, Uoselis L, Skulsuppaisarn M, Nguyen LK, Lazarou M (2019) LC3/GABARAPs drive ubiquitin-independent recruitment of Optineurin and NDP52 to amplify mitophagy. *Nat Commun* 10: 408

Referee #2:

In the revised version of the manuscript, the authors fully answered to this reviewer's concerns. As a minor suggestion, I highly recommend the authors to reorganize and finalize figures. Some figure panels are too small (for example, Fig. 4F, EV Fig. 3 etc.), or overlapped with other panels (EV Fig. 2H), or saturation indication is not removed (EV Fig. 2K). Letter size in each panel should be unified for better presentation (for example, EV Fig. 2K). Also, some typos are observed in the body of the manuscript (for example, Line 124 on page 7, Lipid KD should be Lipin-1 KD).
Response: We have made all the corrections.

2nd Revision - Editorial Decision 26th Sep 2022

Dr. Tso-Pang Yao
Duke University
Department of Pharmacology and Cancer Biology
LSRC C-330, Research Drive
Durham, NC 27710
United States

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- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

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Each figure caption should contain the following information, for each panel where they are relevant:
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- 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.
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Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

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Include a statement about **sample size** estimate even if no statistical methods were used.	Not Applicable	
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Figure legends

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure legends

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: 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.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
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Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
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.	Not Applicable	
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.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Not Applicable	
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	