SAMM50 acts with p62 in piecemeal basal- and OXPHOS-induced mitophagy of SAM- and MICOS components

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Re: JCB manuscript #202009092

Prof. Terje Johansen
The Arctic University of Norway
Molecular Cancer Research Group, Department of Medical Biology, University of Tromsø
Tromsø 9037
Norway

Dear Prof. Johansen,

Thank you for submitting your manuscript entitled "SAMM50 acts with p62 in piecemeal basal- and OXPHOS-induced mitophagy of SAM- and MICOS components". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that the reviewers are positive about the potential advance provided by your study, however have made constructive comments to ensure that your conclusions are fully supported by your data. In particular, in revising please ensure that you thoroughly address these main concerns:

- Improved validation of the mitochondrial localization of p62 including both higher quality imaging data and additional markers as controls for your subcellular fractionation experiments (Rev 2 and 3).
- Better validate the role of p62 and SAMM50 in OXPHOS-mitophagy (Rev 1 and 2).
- Provide improved evidence for your claims regarding basal mitophagy or refocus the study on OXPHOS-induced mitophagy (Rev 1 and 3). Please also clarify and be consistent with your terminology regarding different types of mitophagy.
- Respond to the concerns regarding the interaction between LAMM50 and ATG8s (Rev 2). We would be happy to discuss your thoughts on how to address this point.

In addition, we hope that you will be able to address all of the remaining reviewer comments in your revised manuscript.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Tamotsu Yoshimori, PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In this manuscript, the authors demonstrated that SAMM50, one of SAM component, directory interacts with Atg8 family proteins via LIR to mediate basal mitophagy which mainly degrades SAM and MICOS complexes. Another SAM component, MTX1, also interacts with Atg8 family to facilitate the basal mitophagy. Furthermore, the authors found that p62 interacts with SAMM50 and interaction promotes the OXPHOS-induced mitophagy. Taken together, the authors concluded that SAMM50 acts as receptor for basal mitophagy and OXPHOS-induced mitophagy and that p62 cooperates with SAMM50.

Overall, the experiments are well-designed with proper controls and the results support most part of the conclusion.
I have the following suggestions for the authors to consider:

(1) The role and importance of p62 on basal mitophagy still unclear. Although the authors clearly demonstrated that p62 is important for the OXPHOS-induced mitophagy in Figure 7 and 8, basal mitophagy and the OXPHOS-induced mitophagy may be different pathway. Because most of SAM and MICOS proteins well accumulated by bafilomycin treatment even in p62KO cells (Figure S4D), this reviewer feels that p62 don't have an important role in basal mitophagy.

(2) The authors explained the SAMM50 dependent basal mitophagy as piecemeal type mitophagy. Piecemeal mitophagy eliminates only a portion of mitochondria network, leaving the rest intact. Thus, morphological observation is important to distinguish piecemeal mitophagy from conventional mitophagy. In this study, most part of basal mitophagy is observed by accumulation of SAM and MICOS proteins after bafilomycin treatment. Although their present several microscopic observations, they are not enough to distinguish piecemeal mitophagy and conventional mitophagy.

(3) Although p62 is essential for the OXPHOS-induced mitophagy and p62 interacts with SAMM50 under the OXPHOS-induced mitophagy inducing condition, it is still unclear whether SAMM50 and its interaction with p62 are required for this mitophagy.

(4) Is the OXPHOS-induced mitophagy also piecemeal mitophagy?

(5) Cox8-EGFP-mCherry is sensitive method to detect mitophagy (Figure 8). Is it possible to detect basal mitophagy using this system? For example, by tagging EGFP-mCherry on SAM or MICOS proteins.

Reviewer #2 (Comments to the Authors (Required)):

The authors show that SAMM50 interacts directly with ATG8 family proteins and p62/SQSTM1 to act as a receptor for a basal mitophagy. Namely, they suggest SAMM50 recruits ATG8 family proteins through a canonical LIR motif and interacts with p62/SQSTM1 to mediate basal mitophagy of SAM- and MICOS components. Upon metabolic switch to oxidative phosphorylation, SAMM50 and p62 cooperate to mediate efficient mitophagy.

The authors have conducted many experiments and obtained huge amounts of data. I really respect the authors' efforts on these experiments. On the other hand, some data are unconvincing, and I think the hypothesis that SAMM50 binds to LC3 via the N-terminal LIR is unreasonable. Specific points are described below.

Major comments
1. Mitochondrial localization of p62
The authors immunoprecipitated endogenous p62 and identified various mitochondrial proteins including SAMM50 as interacting proteins under basal conditions. Given that these data are starting points of their hypothesis, p62 should localize on mitochondria. Subcellular fractionation revealed p62 is recovered in the mitochondrial fraction (Fig. 1B, the ratio of cytosol to mitochondria is almost 1:1). However, although we also examined subcellular localization of endogenous p62, we have never seen such the major distribution of p62 on mitochondria, as suggested by this

fractionation experiments.
Moreover, immunocytochemical data showing co-localization of p62 with SAMM50 (Fig. 1 C) are low quality and not satisfactory when compared with JCB standards. Quality of immunocytochemical data of Fig. 1D is better than Fig. 1C, however, the data still do not support co-localization of mCherry-p62 with EGFP-MIC19 persuasively. The authors should show more convincing data for mitochondrial localization of p62.

2. Lysosomal degradation of MICOS, Metaxin, and SAMM50 components.
Accumulation of SAMM50, MICOS complex proteins (MIC19 and MIC60), Metaxin component MTX1/2, and TOM complex protein TOMM40 upon BafA1 treatment are key data. However, results vary upon experiments. Accumulation of SAMM50 in WT cells following BafA1 treatment is clearly observed in Fig.4A, Fig.4H, and Fig.S4J, whereas not obvious in Fig.4D and Fig.S4G. Similarly, accumulation of MIC19 following BafA1 treatment is clearly observed in Fig.4, whereas not obvious in Fig.5C. Accumulation of MTX1/2 following BafA1 treatment is obvious in Fig.4A and Fig.4D, whereas is not observed in Fig.7J. These variations might be derived from the secondary effects including cell toxicity. To avoid such the secondary effects, the authors may want to consider drug treatment time shorter than 24 hours.

3. Whether SAMM50 interacts with ATG8s via a LIR motif in the NTS to accelerate mitophagy.
The authors speculated that SAMM50 interacts with ATG8s for selective delivery of mitochondrial fragment to the lysosome, and found potential LIR motif in N-terminal of SAMM50. The authors show huge data including co-crystal structure of SAMM50(24-35) peptide bound to GABALAP (Fig.6), and thus I agree that N-term sequence of SAMM50 has a potential to interact with ATG8s. However, interaction between SAMM50 and ATG8s is implausible under basal physiological conditions as following reasons.
Extensive analyses have already been performed on proteins involved in beta-barrel insertion, namely SAMM50 and prokaryotic BamA. The authors described "the orientation of the N-terminal POTRA domain have been debated (Chen 2016; Habib 2007; Sommer 2011)". However, in light of the vast knowledge of SAMM50 and the structure of the BamA, it is reliable and certain that the POTRA domain localizes in the IMS (regarding this topic, the authors would agree that the POTRA domain localizes in IMS, as described in page 10). Then, is it possible that the POTRA domain localizes in IMS but the N-terminus is exposed to cytosol to bind LC3?
One possibility is that the N-terminus penetrates the center of the beta-barrel pore. Indeed, in the case of VDAC1 and FhaC, their N-termini pass through the beta-barrel pore and reach the cytosol side (see Clantin et al., "Structure of the membrane protein FhaC", Science 2007; Ujwal et. al., "The crystal structure of moue VDAC1", PNAS 2008). In the case of FhaC, the N-terminus penetrates the beta-barrel and faces the (topologically) cytosol side. In FhaC, the alpha helix H1 (29 amino acids) is almost embedded in the center of the beta barrel pore, and this length (ca. 30 amno acids) is required to penetrate the beta-barrel and expose N-terminal to cytosol. In the case of VDAC1, the N-terminal enters the beta-barrel pore, and the alpha-helix of about 30 amino acids in length is used.
Another possibility is that the N-terminal side forms an alpha-helix and penetrates the membrane in the vicinity of SAMM50 via the alpha-helix , rather than through the beta-barrel pore of SAMM50. Tom6 and Tom5 near Tom40 (beta-barrel pore) are just like this case (see Araiso et al. "Structure of the mitochondrial import gate", Nature 2019). Also in this case, Tom6 uses about 25 amino acids length to across the membrane.
In any case, to let SAMM50 interact with the cytosolic factor such as ATG8s via the N-terminal LIR domain whereas let POTRA domain be in IMS, 25 amino acid length is required between the LIR motif and the POTRA domain. SAMM50 does not have such structure (Fig.6), and thus the hypothesis is not convincing at the molecular level.

4. Cooperation of p62 with SAMM50 to mediate OXPHOS-induced mitophagy
The authors suggest that p62 is involved in OXPHOS-dependent basal mitophagy. However, Fig.7 and Fig.8 do not provide sufficient data to fully support their hypothesis. The authors should show following data:
4-1, if increased amount of p62 is recovered in the mitochondrial fraction of cells grown in media containing galactose or acetoacetate (Fig.7C), the author should perform immunocytochemistry to reveal the mitochondrial localization of p62. As aforementioned, immunocytochemical data to reveal the co-localization of p62 with mitochondria (Fig. 1) were not satisfactory.
4-2, using COX8-EGFP-mCherry reporter, the authors stated that OXPHOS-dependent basal mitophagy stopped in p62 KO MEF cells (Fig. 8A). However, to conclude that p62 is involves in OXPHOS-dependent basal mitophagy, the authors should show re-emergence of red dots by exogenous p62 as complementary experiments.
4-3, in Fig. 8C, the authors stated that there is a substantial increase in ATG13 puncta (indicating autophagy induction) in WT cells upon switch to acetoacetate, while only few ATG13 puncta are observed in p62 KO cells. Moreover, several ATG13 puncta colocalize or are in close proximity to the mitochondria. However, quality of immunocytochemical data of ATG13 is not satisfactory when compared with JCB standards. The authors should improve the data quality, and should try other ATG proteins.

In conclusion, the authors' hypothesis is not well supported by experimental data, and thus their model is not convincing.

Minor comments
1. Cell types such as WT, SAMM50 KD, p62 KO, and ATG7 KO should be displayed with underlines at the top of immunoblotting panels; as WT p62 KO .
2. Fig. 8A, one picture in mCherry column is shown in yellow.
3. Fig. 8B, if 8A and 8B are derived from the same data, galactose should be acetoacetate in 8B.
4. Fig. 3A, lanes 3 and 4, SAMM50 KD - - should be + +.
5. Fig. S3H, lanes 3 and 4, SAMM50 KD - - should be + +.
6. text in page 15, line 8; "Fig.5F and 5G" should be "Fig.6F and 6G".
7. text in page 9; authors' finding that the POTRA domain of SAMM50 is dispensable for β-barrel assembly and biogenesis is not so surprising. The POTRA domain of yeast Sam50 is dispensable for cell growth and beta-barrel biogenesis and thus it does not play an essential role for precursor binding to SAM and mitochondrial protein import (Kutik et al. Cell 2008).

Reviewer #3 (Comments to the Authors (Required)):

This study investigates mechanisms underlying the basal degradation of mitochondria through selective forms of autophagy (mitophagy). Abudu et al. very thoroughly dissect the functional domains of SAM50 required for its regulation of SAM50 and MICOS complex stability, cristae organization, and mitochondrial function. Their findings also reveal that SAM50 mediates basal lysosomal degradation of SAM complexes and MICOS complexes through an LIR domain in the cytosolic facing N-terminus. The LIR domain of SAM50 interacts with specific ATG8s, which are required for SAM50-dependent lysosomal degradation. Further, SAM50-dependent lysosomal degradation is also observed in OXPHOS-inducing conditions, which requires p62. Overall, the data presented by the authors is potentially important. However, there are several technical issues that need to be addressed that preclude acceptance at this point.

Major issues

1) One problem in the Introduction is the claim of high basal mitochondrial turnover rates based on papers using the outer mitochondrial membrane probe named mitoQC. A recent paper from the Miyawaki group at Riken (https://doi.org/10.1016/j.cell.2020.04.025) using a matrix mitophagy probe argues that this prior work is incorrect. Also, two groups using a matrix mitophagy probe in Drosophila (Deficiency of parkin and PINK1 impairs age-dependent mitophagy in Drosophila. Elife 2018; Assessment of mitophagy in mt-Keima Drosophila revealed an essential role of the PINK1-Parkin pathway in mitophagy induction in vivo. FASEB J 2019; :fj201900073R) refute the prior claim of high basal mitophagy in a fly paper using the so called mitoQC probe. Please either delete this issue (high basal rate) entirely or point out the different conclusions in the literature depending on the localization of the probe. I feel the matrix probe is likely to be more accurate. However, could there be more OMM-phagy than matrix-phagy? This could relate to the current SAM50 work.
2) Sam50 staining does not look mitochondrial in Fig. 1C. What is the evidence that some or all of the Sam50 punctae is not nonspecific staining? Please compare immunostaining in WT and Sam50 KD clones #6 and #8. Also, please include these Sam50 KD controls for Fig. 4C.
3) One lane in Fig. 1B and Fid. S1B is labeled "Mitochondria". However, no indication of ER membrane or autophagosome membrane separation from mitochondria is indicated. Please probe for the ER to show the extent of contamination of the mitochondrial fraction (also in Fig. 7E). If substantial ER and other membrane contamination the claim should be that p62 binds "membranes" not mitochondria - same for Fig. 7E.
4) Figure 2 is very descriptive yielding no substantial conclusions other than pleiotropic mitochondrial problems in Sam50 KD lines. It could all be moved to a Suppl. Figure.
5) I feel the authors overinterpret the role of the N-terminus of Sam50 in protein import based simply on western blotting steady state mitochondrial proteins in Fig. 3. Sam40 KD in whole cells may have pleiotropic effects on mitochondria and the lower levels of many proteins seen in Fig. 2 may be tangential to import per se. Plus, there is still residual endogenous full length Tom50 that may mediate the needed import function of Sam50. Please substantiate these conclusions with cell free import studies or kinetic pulse chase import studies in cells or tone down this section or delete it entirely as it adds little to the main conclusions of the manuscript.
6) The data is not convincing to demonstrate whether SAMM50 complex degradation is mediated by MDV formation (see specific issues below). The Drp1 data in Fig. S4F is not compelling because Drp1 is also not required for wholesale mitophagy (Burman et al., 2017), and therefore this is not a characteristic unique to MDV degradation. At this time, there is no clear measure for detecting MDV formation other than observing vesicle budding from mitochondria by electron microscopy. With this, the specific cargo would also have to be identified within the budding vesicle, perhaps using CLEM, and shown to be degraded. This section detracts from otherwise interesting evidence (especially Fig. 4F) for selective SAM50 complex lysosomal degradation. The authors should consider removing it or providing more direct evidence for whether or not SAM50 and MICOS complexes are degraded via MDVs.
7) Fig. 5A and B should be corroborated with mtKiema fused to Mic19.
8) The reduction in ATP in galactose media (Fig. 7I) suggests that basal autophagy may be induced potentially confounding the media change interpretations. Please assess starvation mediated bulk phase autophagy using a autophagy flux probe comparing glucose and galactose.

Minor Issues

1) The terminology describing the different types of "mitophagy" are inconsistent throughout the text and confusing. Do the authors think there is a difference between "basal mitophagy", "basal piecemeal mitophagy", and "SAMM50-dependent mitophagy"? For example, the authors state

several times "SAMM50-dependent basal mitophagy". The evidence provided supports a mechanism of SAMM50-dependent lysosomal degradation of SAM50 and MICOS complexes that occurs basally, not that SAMM50 mediates basal mitophagy, which may be misconstrued as basal "wholesale" mitophagy. Please define what specific "mitophagy" is dependent on SAMM50 that is supported by the data and be consistent in referring to it throughout the text.
2) It appears the GABARAP-Q59E mutant was not discussed in the results but is mentioned in the discussion. Please include this in the results section.
3) Why is MIC19 not detected in WT cells in Fig. S3i? The western blot does not reflect the quantification in Fig. S3j.
4) There is a reference to Fig. 9C, but there is no Fig. 9C.
5) There is a clear increase of p62 detection in the mitochondrial fraction of galactose incubated WT cells in panel 7c-d. Why is the detection of p62 in the mitochondrial fraction of galactose incubated WT cells not consistent in this Fig 7e-f?
6) What is the rationale for focusing on LC3A and GABARAP as ATG8s interacting with SAMM50? Especially with the question of whether the SAMM50-dependent mitophagy is similar to "basal piecemeal mitophagy", which is mediated by LC3C?
7) What is the rationale for the GABARAP-Q59E mutant in Fig. 5h? It is not described in the text.

We are very grateful for the opportunity to submit a revised version of our manuscript and thank the expert reviewers for their constructive criticism and helpful comments that we have used to improve our paper.

In this revised manuscript, we have added new figures and revised the original figure items. Thus, the revised manuscript contains 11 new figure items; Figs. 1C, 2G, 5E, 8C, 9A, 9B, 9C, 9D, 9E, 9F and 9G. We have also revised and add new data to Figs 1B, S1B, S3I, S3J, 4D, 4E, S4E (old S4G), 6A (old 5C), 6B (old 5D), 8D (old 7E), 8E (old 7F), 8H (old 7J) and 8I (old 7K). In addition, we have replaced Fig. S1C with Fig 1C and moved. Figs. S4D to 5A, S4E to 5B, and 7I to S5D.

In the revised manuscript changed or new text is shown in red lettering.

Please find below a point-by point rebuttal for all the concerns raised by the reviewers.

Reviewer #1 (Comments to the Authors (Required)):
In this manuscript, the authors demonstrated that SAMM50, one of SAM component, directory interacts with Atg8 family proteins via LIR to mediate basal mitophagy which mainly degrades SAM and MICOS complexes. Another SAM component, MTX1, also interacts with Atg8 family to facilitate the basal mitophagy. Furthermore, the authors found that p62 interacts with SAMM50 and interaction promotes the OXPHOS-induced mitophagy. Taken together, the authors concluded that SAMM50 acts as receptor for basal mitophagy and OXPHOS-induced mitophagy and that p62 cooperates with SAMM50.
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We thank the reviewer for this important comment and agree with the reviewer that Bafilomycin A1 treatment shows that p62 is dispensable for basal mitophagy of these proteins. However, we did observe accumulation of MICOS and SAM complex proteins in p62 KO cells under basal conditions (Fig. 5A and B)(old Fig. S4D), which clearly may suggest a role of p62 in basal mitophagy. We have now edited the text on page 13 and it reads “We observed a significant increase of these mitochondrial proteins in the p62 KO cells, although there was further accumulation upon treatment with BafA1. Hence, p62 plays a role in this form of basal mitophagy, but is dispensable”.

(2) The authors explained the SAMM50 dependent basal mitophagy as piecemeal type mitophagy. Piecemeal mitophagy eliminates only a portion of mitochondria network, leaving the rest intact. Thus, morphological observation is important to distinguish piecemeal mitophagy from conventional mitophagy. In this study, most part of basal mitophagy is

observed by accumulation of SAM and MICOS proteins after bafilomycin treatment. Although their present several microscopic observations, they are not enough to distinguish piecemeal mitophagy and conventional mitophagy.

Thank you for this comment, TEM analysis has not been very informative or successful given the inability to capture such infrequent events. There is also an issue with fixed cells that will lead to loss of these fragments. However, using live cell imaging we can clearly see fragmented mitochondria that colocalizes with both p62 and hATG8 proteins (Fig. 5C, 6E and F). This is also evident in **new Fig 1C**. These fragments are degraded in the lysosome while the mitochondrial network remain intact (Fig 5C and D). Moreover, a paper from coauthor Christian Behrends group has already shown the presence of a basal piecemeal mitophagy that involves MTX1, LC3C and p62 (Le Guerroué et al 2017, PMID: **29149599**). Our live cell microscopy data align with their observation and show mitochondrial fragments, which colocalize with p62 and hATG8 puncta and are degraded in the lysosome.

(3) Although p62 is essential for the OXPHOS-induced mitophagy and p62 interacts with SAMM50 under the OXPHOS-induced mitophagy inducing condition, it is still unclear whether SAMM50 and its interaction with p62 are required for this mitophagy.

Our data show that the abundance of p62 in the mitochondrial fraction is severely reduced in SAMM50 knockdown cells (revised Fig. 8D and E). In addition, there is a clear increase in the interaction between p62 and SAMM50 under OXPHOS-induced mitophagy, but not with NIPSNAP, another interacting partner of p62 involved in damaged-induced mitophagy (Fig. 8F and G). To further test the importance of the interaction between p62 and SAMM50, we reconstituted p62 KO MEF cells with WT p62 and SAMM50 binding-deficient mutant **(New Fig. 9,E-G).** OXPHOS-induced mitophagy was significantly reduced in cells reconstituted with p62 mutant that cannot interact with SAMM50. This suggests that the interaction between p62 and SAMM50 is important for OXPHOS-induced mitophagy.

(4) Is the OXPHOS-induced mitophagy also piecemeal mitophagy?

Our new live cell imaging data show the abundance of mitochondrial fragments which colocalizes with p62, ATG13 and GABARAP during OXPHOS-induced mitophagy (**New Figs. 8C, 9A and C**). The mitochondrial network seem to be intact after 96h growth in OXPHOS media. This suggests that OXPHOS-induced mitophagy may also be a piecemeal mitophagy.

(5) Cox8-EGFP-mCherry is sensitive method to detect mitophagy (Figure 8). Is it possible to detect basal mitophagy using this system? For example, by tagging EGFP-mCherry on SAM or MICOS proteins.

Stable expression of MIC19-EGFP-mCherry seem to be deleterious to cells. We noticed excessive clumping of the mitochondria. The tandem tag is most likely too large interfering

with the functionality of MIC19. Consequently, we tagged MIC19 with Keima (**New Fig. 5E**), a pH-sensitive fluorescent probe that is predominantly excited by a short wavelength (458nm) in a neutral environment (mitochondria, green). It can also be excited by a long wavelength (561nm) in acidic environment when the mitochondria or mitochondrial fragment is delivered to the lysosome (lysosome, red). We were able to observe basal piecemeal mitophagy using this method (**New Fig. 5E**).

Reviewer #2 (Comments to the Authors (Required)):

The authors show that SAMM50 interacts directly with ATG8 family proteins and p62/SQSTM1 to act as a receptor for a basal mitophagy. Namely, they suggest SAMM50 recruits ATG8 family proteins through a canonical LIR motif and interacts with p62/SQSTM1 to mediate basal mitophagy of SAM- and MICOS components. Upon metabolic switch to oxidative phosphorylation, SAMM50 and p62 cooperate to mediate efficient mitophagy.

The authors have conducted many experiments and obtained huge amounts of data. I really respect the authors' efforts on these experiments. On the other hand, some data are unconvincing, and I think the hypothesis that SAMM50 binds to LC3 via the N-terminal LIR is unreasonable. Specific points are described below.

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1. Mitochondrial localization of p62

The authors immunoprecipitated endogenous p62 and identified various mitochondrial proteins including SAMM50 as interacting proteins under basal conditions. Given that these data are starting points of their hypothesis, p62 should localize on mitochondria. Subcellular fractionation revealed p62 is recovered in the mitochondrial fraction (Fig. 1B, the ratio of cytosol to mitochondria is almost 1:1). However, although we also examined subcellular localization of endogenous p62, we have never seen such the major distribution of p62 on mitochondria, as suggested by this fractionation experiments.

Moreover, immunocytochemical data showing co-localization of p62 with SAMM50 (Fig. 1 C) are low quality and not satisfactory when compared with JCB standards. Quality of immunocytochemical data of Fig. 1D is better than Fig. 1C, however, the data still do not support co-localization of mCherry-p62 with EGFP-MIC19 persuasively. The authors should show more convincing data for mitochondrial localization of p62.

We want to thank the reviewer for the constructive comments and suggestions. We have now performed new cellular fractionation experiments and included markers for the endoplasmic reticulum (ER), lysosomes, peroxisomes, and the Golgi (revised Fig. 1B and S1B). We have also performed live cell imaging of cells stably expressing mCherry-p62 and stained with MitoTracker (New Fig, 1C). Live cell imaging data clearly show foci of p62 that colocalize with mitochondria.

2. Lysosomal degradation of MICOS, Metaxin, and SAMM50 components.

Accumulation of SAMM50, MICOS complex proteins (MIC19 and MIC60), Metaxin

component MTX1/2, and TOM complex protein TOMM40 upon BafA1 treatment are key data. However, results vary upon experiments. Accumulation of SAMM50 in WT cells following BafA1 treatment is clearly observed in Fig.4A, Fig.4H, and Fig.S4J, whereas not obvious in Fig.4D and Fig.S4G. Similarly, accumulation of MIC19 following BafA1 treatment is clearly observed in Fig.4, whereas not obvious in Fig.5C. Accumulation of MTX1/2 following BafA1 treatment is obvious in Fig.4A and Fig.4D, whereas is not observed in Fig.7J. These variations might be derived from the secondary effects including cell toxicity. To avoid such the secondary effects, the authors may want to consider drug treatment time shorter than 24 hours.

We agree that results seem to vary upon experiments. We have now performed new experiments and re-run SAMM50 blots in Fig 4D and S4E (old S4D). We have also re-run blots for MIC19 in Fig. 6A (old 5C) and MTX1/2 in Fig 8H (old 7J).

3. Whether SAMM50 interacts with ATG8s via a LIR motif in the NTS to accelerate mitophagy.
The authors speculated that SAMM50 interacts with ATG8s for selective delivery of mitochondrial fragment to the lysosome, and found potential LIR motif in N-terminal of SAMM50. The authors show huge data including co-crystal structure of SAMM50(24-35) peptide bound to GABALAP (Fig.6), and thus I agree that N-term sequence of SAMM50 has a potential to interact with ATG8s. However, interaction between SAMM50 and ATG8s is implausible under basal physiological conditions as following reasons.
Extensive analyses have already been performed on proteins involved in beta-barrel insertion, namely SAMM50 and prokaryotic BamA. The authors described "the orientation of the N-terminal POTRA domain have been debated (Chen 2016; Habib 2007; Sommer 2011)". However, in light of the vast knowledge of SAMM50 and the structure of the BamA, it is reliable and certain that the POTRA domain localizes in the IMS (regarding this topic, the authors would agree that the POTRA domain localizes in IMS, as described in page 10). Then, is it possible that the POTRA domain localizes in IMS but the N-terminus is exposed to cytosol to bind LC3?
One possibility is that the N-terminus penetrates the center of the beta-barrel pore. Indeed, in the case of VDAC1 and FhaC, their N-termini pass through the beta-barrel pore and reach the cytosol side (see Clantin et al., "Structure of the membrane protein FhaC", Science 2007; Ujwal et. al., "The crystal structure of moue VDAC1", PNAS 2008). In the case of FhaC, the N-terminus penetrates the beta-barrel and faces the (topologically) cytosol side. In FhaC, the alpha helix H1 (29 amino acids) is almost embedded in the center of the beta barrel pore, and this length (ca. 30 amno acids) is required to penetrate the beta-barrel and expose N-terminal to cytosol. In the case of VDAC1, the N-terminal enters the beta-barrel pore, and the alpha-helix of about 30 amino acids in length is used.
Another possibility is that the N-terminal side forms an alpha-helix and penetrates the membrane in the vicinity of SAMM50 via the alpha-helix , rather than through the beta-barrel pore of SAMM50. Tom6 and Tom5 near Tom40 (beta-barrel pore) are just like this case (see Araiso et al. "Structure of the mitochondrial import gate", Nature 2019). Also in this case, Tom6 uses about 25 amino acids length to across the membrane.
In any case, to let SAMM50 interact with the cytosolic factor such as ATG8s via the N-terminal LIR domain whereas let POTRA domain be in IMS, 25 amino acid length is required

between the LIR motif and the POTRA domain. SAMM50 does not have such structure (Fig.6), and thus the hypothesis is not convincing at the molecular level.

We thank the reviewer for this knowledgeable, thorough and elaborate comment. We have deleted the sentence pointed at by the reviewer where it was said that: "the orientation of the N-terminal POTRA domain have been debated (Chen 2016; Habib 2007; Sommer 2011)". We have instead introduced a new sentence where we add two references to very recent structure work: “Very recently, cryoEM structures of Sam complexes from S. cerevisiae (Takeda et al. 2021) and the thermophilic fungus Thermothelomyces thermophilus (Diederichs et al., 2020) have been reported clearly showing that the POTRA domain is oriented towards the IMS in these fungal species.”

Our experimental results suggest that the N-terminal region of SAMM50 may be able to transverse the OMM. Myc-tagged SAMM50 is degraded upon treatment with low concentration of proteinase K (Fig. 3F) and a second band appears for SAMM50 signifying degradation similar to MTX1 which is located on the cytoplasmic side of the mitochondria. Moreover, Myc-tagged SAMM50 Δ1-40 is still degraded albeit slower than the wild type implying more membrane protection (Fig. 3H). This means that more than 40 amino acids are likely exposed and the core LIR is located at amino acids 28 to 31 of the NTS. Split fluorescence complementation experiments clearly reveal that the N-terminal of SAMM50 is exposed to the cytosol whereas the IMS-located MIC19 is not exposed (Fig. 3G). Homology modeling using the Phyre2 server suggest that the human SAMM50 POTRA domain may start at amino acid 46. However, the sequence identity between yeast Sam50 POTRA domain and human SAMM50 POTRA domain is only 23% (41% similarity when changes to chemically similar amino acids are taken into account). We have shown that a part of the region that may constitute the POTRA domain is required for interaction with MIC19. Deletion analyses reveal that amino acid sequences between aa 60-80 are required to bind to MIC19 which is located in the IMS (Fig. S4M and unpublished data). It is therefore possible that the 28 amino acid long region between the end of the core LIR (aa28-32) at amino acid 32 to amino acid 59 (which is large enough) can penetrate the membrane to expose the LIR and the remaining part of the NTS to the cytosol. If this is in the form of an alpha-helix 20-21 amino acids are required to span the 7 nm thick OMM. As the reviewer suggests this can happen through the pore of the beta-barrel or on the side of the barrel. There is also the very interesting possibility that the membrane spanning segment is actually a beta-sheet which is stabilized in the so called “lateral gate” of SAMM50 between beta-sheet 1 and beta-sheet 16 in the SAMM50 beta-barrel. For a beta-sheet an average of 10 amino acids is long enough to penetrate the OMM. For VDAC1 10 of the membrane spanning beta-sheets are exactly 10 amino acids long. This will leave enough distance even in the 16 amino acids span between the predicted start of the POTRA domain and the end of the core LIR of SAMM50 (aa 32-46). To explore this in detail will require advanced structural biology experiments that are beyond the scope of this work.

Our current data support an orientation where the most part of the POTRA domain proximal to the C-terminal β-barrel lies within the IMS where it interacts with MIC19 while the N-terminal end of this region transverses the membrane and exposes the N-terminal segment with the LIR motif. We are very confident that SAMM50 interacts with the hATG8

proteins, and that the interaction between SAMM50 and hATG8 occurs in cells and plays an important role in basal piecemeal mitophagy.

4. Cooperation of p62 with SAMM50 to mediate OXPHOS-induced mitophagy
The authors suggest that p62 is involved in OXPHOS-dependent basal mitophagy. However, Fig.7 and Fig.8 do not provide sufficient data to fully support their hypothesis. The authors should show following data:
4-1, if increased amount of p62 is recovered in the mitochondrial fraction of cells grown in media containing galactose or acetoacetate (Fig.7C), the author should perform immunocytochemistry to reveal the mitochondrial localization of p62. As aforementioned, immunocytochemical data to reveal the co-localization of p62 with mitochondria (Fig. 1) were not satisfactory.
4-2, using COX8-EGFP-mCherry reporter, the authors stated that OXPHOS-dependent basal mitophagy stopped in p62 KO MEF cells (Fig. 8A). However, to conclude that p62 is involves in OXPHOS-dependent basal mitophagy, the authors should show re-emergence of red dots by exogenous p62 as complementary experiments.
4-3, in Fig. 8C, the authors stated that there is a substantial increase in ATG13 puncta (indicating autophagy induction) in WT cells upon switch to acetoacetate, while only few ATG13 puncta are observed in p62 KO cells. Moreover, several ATG13 puncta colocalize or are in close proximity to the mitochondria. However, quality of immunocytochemical data of ATG13 is not satisfactory when compared with JCB standards. The authors should improve the data quality, and should try other ATG proteins.

In conclusion, the authors' hypothesis is not well supported by experimental data, and thus their model is not convincing.

We thank the reviewer for these valuable suggestions. We have now in the revised version improved the data quality substantially. Using live cell imaging, we have now shown that there is an increased recruitment of p62 to mitochondria under OXPHOS conditions (**New Fig. 8C**). In addition, we have reconstituted p62 KO MEF with myc-tagged WT p62 and SAMM50 binding deficient mutant of p62. We showed that OXPHOS induced mitophagy were restored when cells were reconstituted with WT p62, but the efficiency of mitophagy was significantly reduced in cells reconstituted with the SAMM50 binding deficient mutant of p62 (**New Fig. 9E-G**). Moreover, we made WT and p62 KO MEFs stably expressing mCherry-ATG13 and mCherry-GABARAP and analyzed them with live cell imaging (**New Fig. 9A-D**). Our results show an increase in both ATG13 and GABARAP puncta in WT cells in OXPHOS inducing conditions compared to p62 KO cells.

Minor comments
1. Cell types such as WT, SAMM50 KD, p62 KO, and ATG7 KO should be displayed with underlines at the top of immunoblotting panels; as WT p62 KO .
2. Fig. 8A, one picture in mCherry column is shown in yellow.
3. Fig. 8B, if 8A and 8B are derived from the same data, galactose should be acetoacetate in 8B.

4. Fig. 3A, lanes 3 and 4, SAMM50 KD - - should be + +.
5. Fig. S3H, lanes 3 and 4, SAMM50 KD - - should be + +.
6. text in page 15, line 8; "Fig.5F and 5G" should be "Fig.6F and 6G".
7. text in page 9; authors' finding that the POTRA domain of SAMM50 is dispensable for β-barrel assembly and biogenesis is not so surprising. The POTRA domain of yeast Sam50 is dispensable for cell growth and beta-barrel biogenesis and thus it does not play an essential role for precursor binding to SAM and mitochondrial protein import (Kutik et al. Cell 2008).

Thank you for pointing out these errors, we have now corrected them both in the figures and in the manuscript.

Reviewer #3 (Comments to the Authors (Required)):

This study investigates mechanisms underlying the basal degradation of mitochondria through selective forms of autophagy (mitophagy). Abudu et al. very thoroughly dissect the functional domains of SAM50 required for its regulation of SAM50 and MICOS complex stability, cristae organization, and mitochondrial function. Their findings also reveal that SAM50 mediates basal lysosomal degradation of SAM complexes and MICOS complexes through an LIR domain in the cytosolic facing N-terminus. The LIR domain of SAM50 interacts with specific ATG8s, which are required for SAM50-dependent lysosomal degradation. Further, SAM50-dependent lysosomal degradation is also observed in OXPHOS-inducing conditions, which requires p62. Overall, the data presented by the authors is potentially important. However, there are several technical issues that need to be addressed that preclude acceptance at this point.

Major issues

1) One problem in the Introduction is the claim of high basal mitochondrial turnover rates based on papers using the outer mitochondrial membrane probe named mitoQC. A recent paper from the Miyawaki group at Riken (https://doi.org/10.1016/j.cell.2020.04.025) using a matrix mitophagy probe argues that this prior work is incorrect. Also, two groups using a matrix mitophagy probe in Drosophila (Deficiency of parkin and PINK1 impairs age-dependent mitophagy in Drosophila. Elife 2018; Assessment of mitophagy in mt-Keima Drosophila revealed an essential role of the PINK1-Parkin pathway in mitophagy induction in vivo. FASEB J 2019; :fj201900073R) refute the prior claim of high basal mitophagy in a fly paper using the so called mitoQC probe. Please either delete this issue (high basal rate) entirely or point out the different conclusions in the literature depending on the localization of the probe. I feel the matrix probe is likely to be more accurate. However, could there be more OMM-phagy than matrix-phagy? This could relate to the current SAM50 work.

Our statement in the Introduction, page 4 reads “Using a transgenic mouse model expressing a pH-dependent mitochondrial tandem-tag fluorescent reporter, basal mitophagy was demonstrated in a number of tissues, particularly in tissues of high metabolic demands like heart, liver and skeletal muscles”. We do not mean to imply that this study shows that there is a high rate of basal mitophagy. The statement only implies that basal mitophagy was observed, particularly in tissues with high metabolism. We have also deleted the following

from the Introduction : « , studies on this pathway often rely extensively on overexpression of Parkin and treating cells with chemical inhibitors of mitochondrial oxidative phosphorylation to induce mitophagy. Recent work demonstrated basal mitophagy in tissues of high metabolic demand in a PINK1-knockout mouse model suggesting that the PINK1-Parkin pathway may be required under specific stress conditions in certain tissues and cell types (McWilliams et al., 2018). »
We thank the reviewer for pointing to the two papers on basal mitophagy in Drosophila which we have now also included references to. Kim et al, 2019 ; PMID: **31120803** showed using mitoKeima that basal mitophagy levels vary in Drosophila tissues. The paper reads "Similar to our previous observations in mouse tissues (17), the analysis of mt-Keima *Drosophila* larval tissues revealed varying levels of basal mitophagy.". Secondly, the paper from Wim Vandenberghe group (Cornelissen et al, 2018 ; PMID: 29809156) summarized that the mitophagy they observed in their study could be labelled as basal mitophagy. As we defined in our Introduction, basal mitophagy is a steady-state housekeeping process that continuously recycle whole or parts of the mitochondria. These two papers support the presence of basal mitophagy in several tissues.

2) Sam50 staining does not look mitochondrial in Fig. 1C. What is the evidence that some or all of the Sam50 punctae is not nonspecific staining? Please compare immunostaining in WT and Sam50 KD clones #6 and #8. Also, please include these Sam50 KD controls for Fig. 4C.

Thank you for pointing out this. Diffraction-limited deconvolution (DV) images using antibody staining produced punctate staining for SAMM50 and high level background. We have replaced Fig. 1C with live cell imaging of HeLa cells stably expressing p62 and stained with MitoTracker **(New Fig. 1C)**. In addition, we have now performed another immunostaining and confocal imaging of endogenous SAMM50 in both WT and SAMM50 KD cells (clones #6 and #8) **(New Fig. 2G)**. Fig. 4C shows colocalization between SAMM50 and LAMP2, and SAMM50 KD controls may not be relevant here because there is little to no staining of SAMM50 in the SAMM50 KD cells (**New Fig. 2G**).

3) One lane in Fig. 1B and Fid. S1B is labeled "Mitochondria". However, no indication of ER membrane or autophagosome membrane separation from mitochondria is indicated. Please probe for the ER to show the extent of contamination of the mitochondrial fraction (also in Fig. 7E). If substantial ER and other membrane contamination the claim should be that p62 binds "membranes" not mitochondria - same for Fig. 7E.

Thank you for this comment. We have now performed new cellular fractionation experiments and included markers for the endoplasmic reticulum (ER), lysosomes, peroxisomes, and the Golgi (Revised Fig. 1B and S1C). We recover p62 from mitochondrial fraction with very little contamination from other membranes.

4) Figure 2 is very descriptive yielding no substantial conclusions other than pleiotropic mitochondrial problems in Sam50 KD lines. It could all be moved to a Suppl. Figure.

In Fig. 2, we confirmed the importance of SAMM50 both in the biogenesis and assembly of β-barrel proteins and its role in maintaining cristae and stabilizing MIC19 and MIC60 of the MICOS complex. Thus, we think the figure shows data that allows the reader to appreciate the importance of SAMM50 as well as showing the defects seen in our model system with SAMM50KD that is relevant for understanding the data in the remaining main figures (We also do not have any space for these data in the supplemental figures).

5) I feel the authors overinterpret the role of the N-terminus of Sam50 in protein import based simply on western blotting steady state mitochondrial proteins in Fig. 3. Sam40 KD in whole cells may have pleiotropic effects on mitochondria and the lower levels of many proteins seen in Fig. 2 may be tangential to import per se. Plus, there is still residual endogenous full length Tom50 that may mediate the needed import function of Sam50. Please substantiate these conclusions with cell free import studies or kinetic pulse chase import studies in cells or tone down this section or delete it entirely as it adds little to the main conclusions of the manuscript.

The role of yeast Sam50 in biogenesis of mitochondrial beta-barrel OMM proteins has been very well documented. The reduction in levels of several mitochondrial proteins following human SAMM50 depletion has also been shown (Ott et al., 2012; PMID: 22252321). While the N-terminal domain of OMP85 family proteins has been shown to be important in assembly of β-barrel proteins in bacteria and chloroplasts, we here confirmed earlier reports from studies performed in yeast that this domain is dispensable in human mitochondria (Kozjak-Pavlovic et al., 2007; PMID: 17510655, Kutik et al., 2008 ; PMID: **18358813**). In addition, we also verified and confirmed the important role of SAMM50 in cristae organization ((Darshi et al., 2011; Ding et al., 2015; Ott et al., 2012 ; PMID: **22252321,** Xie et al., 2007). Yeast Sam50 has also been reported to be involved in biogenesis of Tom40 (Kozjak et al., 2003; PMID: **14570913**). We show here that the levels of human TOMM40 is significantly reduced upon SAMM50 depletion which may account for the reduction in import of several proteins.

6) The data is not convincing to demonstrate whether SAMM50 complex degradation is mediated by MDV formation (see specific issues below). The Drp1 data in Fig. S4F is not compelling because Drp1 is also not required for wholesale mitophagy (Burman et al., 2017), and therefore this is not a characteristic unique to MDV degradation. At this time, there is no clear measure for detecting MDV formation other than observing vesicle budding from mitochondria by electron microscopy. With this, the specific cargo would also have to be identified within the budding vesicle, perhaps using CLEM, and shown to be degraded. This section detracts from otherwise interesting evidence (especially Fig. 4F) for selective SAM50 complex lysosomal degradation. The authors should consider removing it or providing more direct evidence for whether or not SAM50 and MICOS complexes are degraded via MDVs.

We agree with this reviewer that there is not one clear and definite measure for detecting MDV. Electron microscopy alone did not yield any satisfactory or definite conclusions as

fragmented mitochondrial were difficult to identify under basal conditions. In the paper from the Behrends group (Le Guerroué et al 2017, PMID: **29149599**), they used DRP1 involvement and TOMM20 as substrate to compare and differentiate the involvement of MDVs in piecemeal mitophagy. Here we extended their observation and tested the involvement of MUL1 (which have been shown to induce MDV formation) (Neuspiel et al., 2008; PMID: **18207745**). We agree that no single method may be able to satisfactorily differentiate these processes and we do not rule out that MDVs may also be involved in basal mitophagy. Here we show that SAMM50-dependent basal piecemeal mitophagy is different from MUL1-mediated MDVs where TOMM20 is a substrate. In the revised text, we now write “Thus, although SAMM50-dependent basal piecemeal mitophagy shares some features with MUL1-dependent MDVs, including substrate selectivity and DRP1-independence, these two pathways are very likely distinct processes, both involved in mitochondrial quality control”.

7) Fig. 5A and B should be corroborated with mtKiema fused to Mic19.

We have now tagged MIC19 with Keima (**New Fig. 5E**) and we were able to detect lysosomal localization of MIC19-positive mitochondrial fragments under basal conditions.

8) The reduction in ATP in galactose media (Fig. 7I) suggests that basal autophagy may be induced potentially confounding the media change interpretations. Please assess starvation mediated bulk phase autophagy using a autophagy flux probe comparing glucose and galactose.

We are not sure that we understand the issue here as we think the data are clear. Metabolic switch from glucose to galactose has been shown to upregulate mitochondrial ATP production. This has been shown elsewhere (MacVicar and Lane, 2014; Melser et al.,2013; Mishra et al., 2014). In Fig. S5D (old Fig. 7I) WT and SAMM50 KD cells are compared with respect to cellular ATP production which increases somewhat upon switch to galactose in WT cells but is strongly reduced in SAMM50 KD cells. MacVicar and Lane, 2014 used both LC3 puncta assays, LC3 westerns with and without BafA1 and mCherry-GFP-LC3B tandem tag autophagy flux probe to show that autophagic flux is not altered in galactose-cultured cells.

Minor Issues

1) The terminology describing the different types of "mitophagy" are inconsistent throughout the text and confusing. Do the authors think there is a difference between "basal mitophagy", "basal piecemeal mitophagy", and "SAMM50-dependent mitophagy"? For example, the authors state several times "SAMM50-dependent basal mitophagy". The evidence provided supports a mechanism of SAMM50-dependent lysosomal degradation of SAM50 and MICOS complexes that occurs basally, not that SAMM50 mediates basal mitophagy, which may be misconstrued as basal "wholesale" mitophagy. Please define what specific "mitophagy" is dependent on SAMM50 that is supported by the data and be consistent in referring to it throughout the text.

Thank you for this comment. Basal mitophagy can be either wholesale (where the entire mitochondria and mitochondrial network are degraded) or piecemeal mitophagy (where pieces or fragments of mitochondria are degraded and the mitochondrial network is intact). Our data point to a SAMM50-dependent basal piecemeal mitophagy. Here, SAMM50 mediates lysosomal degradation of components of the SAM and MICOS complexes in a piecemeal fashion while the mitochondrial network is still intact. We have now changed SAMM50-dependent basal mitophagy to SAMM50-dependent basal piecemeal mitophagy and used this consistently in the text.

2) It appears the GABARAP-Q59E mutant was not discussed in the results but is mentioned in the discussion. Please include this in the results section.

We have now discussed GABARAP-Q59E in the result section on page 16.

3) Why is MIC19 not detected in WT cells in Fig. S3i? The western blot does not reflect the quantification in Fig. S3j.

We have now re-run new blot for MIC19 in Fig. S3I and made new quantifications for Fig. S3J.

4) There is a reference to Fig. 9C, but there is no Fig. 9C.

Here, we were referring to old Fig. 8C, this has now been corrected.

5) There is a clear increase of p62 detection in the mitochondrial fraction of galactose incubated WT cells in panel 7c-d. Why is the detection of p62 in the mitochondrial fraction of galactose incubated WT cells not consistent in this Fig 7e-f?

We have now performed new cellular fractionation experiments, which clearly show recruitment of p62 to the mitochondria (Revised Fig 8 D and E) (old 7E and F). We have also showed by live cell imaging that there is increased colocalization of p62 with mitochondria following metabolic switch to OXPHOS inducing conditions (**New Fig. 8C**).

6) What is the rationale for focusing on LC3A and GABARAP as ATG8s interacting with SAMM50? Especially with the question of whether the SAMM50-dependent mitophagy is similar to "basal piecemeal mitophagy", which is mediated by LC3C?

We noticed that LC3A (among the LC3 subfamily) and GABARAP (among the GABARAP subfamily) bound stronger to SAMM50 compared to others when immunoprecipitated from cells (Fig. 6D) (old Fig 5G). We also discovered that all the hATG8 can mediate basal piecemeal mitophagy (Fig. S5C), so it is not unique to LC3C alone.

7) What is the rationale for the GABARAP-Q59E mutant in Fig. 5h? It is not described in the text.

Q59 residue in GABARAP (which is only conserved in GABARAP subfamily and LC3C) have been shown to play a significant role in the binding of LIR containing proteins to the second hydrophobic pocket (HP2) at the LIR docking site (Wirth et al., 2019; PMID: 31053714). This further strengthened the fact that SAMM50 binds to the hATG8 through a canonical LIR motif. We have now described this in the text on page 16.

We wish to express our gratitude for the insightful and stimulating criticisms and comments made by the reviewers. This has clearly helped us to improve our manuscript significantly. We hope that the revised manuscript can be found acceptable for publication.

Yours sincerely,

Terje Johansen

Professor, Dr. scient.

April 20, 2021

RE: JCB Manuscript #202009092R

Prof. Terje Johansen
The Arctic University of Norway
Molecular Cancer Research Group, Department of Medical Biology, University of Tromsø
Tromsø 9037
Norway

Dear Prof. Johansen:

Thank you for submitting your revised manuscript entitled "SAMM50 acts with p62 in piecemeal basal- and OXPHOS-induced mitophagy of SAM- and MICOS components". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below). In your final revision, we encourage you to respond to reviewer #2's final comments.

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

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3) Figure formatting: Scale bars must be present on all microscopy images, * including inset magnifications (you may alternatively indicate the diameter of the inset)*. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

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5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

6) * Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts. *

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental display items (figures and tables). Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

14) A separate author contribution section following the Acknowledgments. All authors should be

mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

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Sincerely,

Tamotsu Yoshimori, PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The authors have addressed most comments made by reviewers. I believe that the current manuscript becomes suitable for publication.

Reviewer #2 (Comments to the Authors (Required)):

In the first round of reviewing process, I have pointed three major issues in the manuscript.

First, as a starting point of authors' hypothesis, p62 should localize on mitochondria to interact with SAMM50. However, subcellular fractionation pattern of endogenous p62 looked unnatural as almost half of p62 was recovered in the mitochondrial fraction (old Fig. 1B). Quality of immunocytochemical data showing co-localization of p62 with SAMM50 (old Fig. 1C) was low, and not satisfactory when compared with standards of JCB. Moreover, there was inconsistency in accumulation pattern of SAMM50, MIC19, and MTX1/2 following BafA1 treatment (old Figs. 4, 5, 7, and S4). These inconsistent data suggested that the immunoblotting data varied upon experiments.

Second, the authors stated that SAMM50 interacts with ATG8s via potential LIR motif in its N-terminus, for selective delivery of mitochondria fragment to the lysosomes. However, given that many papers about SAMM50 and BamA strongly suggest that the POTRA domain localizes in the IMS, it seemed impossible that the N-terminus of SAMM50 is exposed to cytosol to bind LC3 while the POTRA domain of SAMM50 localizes in IMS.

Finally, although the authors stated that p62 is involved in OXPHOS-dependent basal mitophagy, old Fig. 7 and Fig. 8 did not provide sufficient data to fully support their hypothesis. For example, the authors did not show the accelerated recruitment of p62 to mitochondria under OXPHOS conditions in immunocytochemistry. In addition, the quality of immunocytochemical data was insufficient to demonstrate a substantial increase in ATG13 puncta (indicative of autophagy induction) upon acetoacetate conditions in WT cells.

Now, in this revised manuscript, the authors have added new data to solve these concerns. Regarding the first issue, quality of immunocytochemical data for co-localization of p62 with SAMM50 (new Fig. 1C) improved much during revision, and distribution ratio of endogenous p62 in mitochondrial and cytosolic fraction looks reasonable in new Fig. 1B. Accumulation pattern of SAMM50, MIC19, and MTX1/2 became almost consistent among new Figs. 4, 6, 8, and S4.

Regarding the second issue, there was a remarkable progress in structural analysis of SAMM50. During revision term, cryoEM structures of SAMM50 from yeast and the thermophilic fungus have been reported. As I mentioned in the previous Reviewer Comments, these two papers clearly showed that the POTRA domain is oriented towards the IMS (Extended Fig. 7 in Takeda et al., Nature 2021; Fig. 3 in Diederichs et al., Nature Commun. 2020). However, the authors gathered further data to surmise that the most part of the POTRA domain proximal to the C-terminal β-barrel lies within the IMS while the N-terminal end of this region transverses the membrane and exposes the N-terminal segment with the LIR motif into cytosol. Unfortunately, we do not have structural information of mammalian SAMM50 yet to judge whether the aforementioned hypothesis is valid.

Regarding the last issue, the authors have shown an increased recruitment of p62 to mitochondria under OXPHOS conditions in immunocytochemistry (new Fig. 8C). Moreover, the authors showed

that OXPHOS-induced mitophagy were restored when p62 KO MEFs were reconstituted with WT p62, while the efficiency of mitophagy was significantly reduced in p62 KO MEFs reconstituted with the SAMM50 binding deficient mutant of p62 (new Fig. 9). These new results -especially new Fig 9- convincingly support their hypothesis.

Considering the structure of fungi SAMM50 proteins, I still feel reluctant to accept the authors' hypothesis that the N-terminal side of SAMM50 is exposed to the cytoplasm that interacts with LC3 for mitophagy. However, it is difficult to deny the authors' hypothesis thoughtlessly without knowledge on the structure of mammalian SAMM50. In addition, the authors addressed my other comments appropriately. I thus think the current manuscript is now suitable for publication in JCB.

Reviewer #3 (Comments to the Authors (Required)):

The authors adequately addressed all my concerns in the revised manuscript.