Mitophagy via Drp1-mediated outer membrane severing and inner membrane ubiquitination

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Review Timeline:

| Event                      | Date       |
|----------------------------|------------|
| Submission Date            | 2020-06-09 |
| Editorial Decision         | 2020-07-15 |
| Revision Received          | 2021-02-02 |
| Editorial Decision         | 2021-03-05 |
| Revision Received          | 2021-03-13 |

Monitoring Editor: Thomas Langer
Scientific Editor: Melina Casadio

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

DOI: https://doi.org/10.1083/jcb.202006043
July 15, 2020

Re: JCB manuscript #202006043

Dr. Mariusz Karbowski
University of Maryland School of Medicine
111 S. Penn Street Suite 104
Baltimore, MD 21201

Dear Dr. Karbowski,

Thank you for submitting your manuscript entitled "Drp1-dependent outer mitochondrial membrane severing and inner membrane ubiquitination mediate mitochondrial proteotoxicity-induced mitophagy in the absence of Parkin". We appreciate your patience with the peer review process of the paper. The manuscript has been evaluated by expert reviewers, whose reports are appended below. Unfortunately, after an assessment of the reviewer feedback, our editorial decision is against publication in JCB.

You will see that all reviewers appreciated the interesting nature of your observations but commented on the lack of mechanistic insight and raised points of criticism that undermine the central findings of the manuscript. It will require substantial experimentation to address some of these concerns, such as the molecular basis and relevance of cytochrome c mosaicism. We feel that the points raised by the reviewers are more substantial than can be addressed in a typical revision period and preclude further consideration at JCB. If you wish to expedite publication of the current data, it may be best to pursue publication at another journal.

Given interest in the topic, if you are interested in substantially revising the manuscript to address the reviewers' concerns, we would be willing to discuss a resubmission to JCB. To ensure that you do not embark on time- and resource-consuming revisions that may not be sufficient for re-review at JCB, we'd be happy to discuss resubmission to the journal through an appeal detailing your strategy to address the reviewers' points. In our view, for publication in JCB, additional evidence is required (1) in support of mitophagy (rather than alternative possibilities outlined by Rev #1) using established methods in the field (Rev#1 and #3), (2) to support OMM rupture (for instance by EM, Rev#1), and (3) to show the relevance of cytochrome c mosaicism for outer membrane severing and apoptosis (Revs#1 and #2). The manuscript would also benefit from additional experimental support for the mechanism of induction of this pathway (protein misfolding and/or OXPHOS disassembly) (Revs#2 and #3). Technical issues such as data quantification (Rev #1) and the statistical evaluation of the data (Rev#2) would also need to be addressed rigorously. Please note that our policy for appealed manuscripts is that priority and novelty would be reassessed at resubmission.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss the reviewer comments further once you've had a chance to consider the points raised in this letter. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.
Reviewer #1 (Comments to the Authors (Required)):

The current manuscript describes the cellular response to reduce mitochondrial translation fidelity in actinonin-treated human cancer cell lines (HCT116 and HeLa) that express low levels or no Parkin, respectively. The authors observe an interesting redistribution of cytochrome c resulting in a mosaic accumulation within mitochondria with reduced membrane potential in a Drp1-dependent manner. In addition, using fluorescence microscopy, the authors discover mitochondria that display circular ubiquitination signals but disrupted signal for the outer mitochondrial protein TOM20, which the authors interpret as a rupture of the outer mitochondrial membrane and inner membrane protein ubiquitination. These structures emerge in a Drp1-dependent manner and increase in number upon autophagy inhibition, suggesting that they are targeted and removed by mitophagy. Consistent with this model, the authors detect p62 and OPTN, two autophagy receptor proteins at these ubiquitinated mitochondria.

While the manuscript describes interesting phenomena, namely cytochrome c redistribution and OMM rupture and IMM protein ubiquitination-induced mitophagy, the data are not convincing yet. There are three major weaknesses in the manuscript:

(1) Direct evidence for induction and turnover of Drp1 and "high" cyto. c mitochondria by mitophagy is missing.

Although the authors describe a detectable reduction in Drp1 protein levels upon ABT-737 and STS treatment in figure 1A, data in figure 1B show that these apparent differences are not significant/reproducible. Thus, all data shown in figure 1 need to be normalized for loading and quantified to assess whether apparent effects are real.

In addition, steady-state protein levels, inherently being a product of both translation and degradation, are insufficient to demonstrate a change in protein degradation. To test for a change in protein stability of Drp1, the authors would need to perform pulse-chase experiments. Steady-state measurements of mitochondrial proteins do not support a general mitophagic turnover of the organelle; protein levels of ATP5A1, SDHB, COX2, TOM20, TOM40 are unchanged and NDUFB8 shows the opposite behavior than Drp1 in Figures 1A-E. How do the authors reconcile these data with their proposed induction of mitophagy?

Accumulation of OMM-ruptured, IMM ubiquitinated mitochondria in ATG5-/- cells is consistent with mitophagic turnover but not proof. For example, ATG5-/- mitochondria may be affect by Atg12-Atg3 conjugates independent of ATG5's function in autophagy (Radoshevich et al. Cell, 2010). The authors need to perform standard assays in the field to assess mitophagy: mtKeima FACS, mitochondrial GFP-RFP signal analysis (yellow vs red (autolysosomes)), time-lapse analysis of defective mitochondria being engulfed by LC3-marked autophagosomes.

The changes in protein crosslinks shown in figure 7 are not convincing and need to be quantified.
Moreover, what is the nature of these crosslinks and on what basis do the authors consider them to be a readout for an accumulation of abnormal OXPHOS complexes? Why are non-crosslinked protein levels not changed? This again does not support mitophagy mediated turnover/quality control. The authors should perform BN-PAGE of supercomplexes.

(2) OMM ruptured and IMM protein ubiquitination are not convincingly shown yet

The data is consistent with OMM rupture. However, EM analysis is required to directly demonstrate it (compare to Yoshii et al. and Wei et al. see below). An alternative explanation might be that, instead of OMM rupture, TOM20 is degraded in a localized manner resulting the impression of absence of the OMM at some areas of the mitochondria. The OMM ubiquitination leads to OMM protein turnover by the proteasome upon membrane dissipation. Could the lack of TOM20 signal be caused by proteasome turnover of TOM20 on mitochondria with low membrane potential?

The data are consistent with IMM protein ubiquitination, but are indirect. The authors need to directly show ubiquitination of IMM proteins by Western blot or proteomics analyses in dependence of Acn, Acn+CP treatment, Drp1 and autophagy.

An alternative explanation might be that, instead of OMM rupture, TOM20 is degraded in a localized manner resulting the impression of absence of the OMM at some areas of the mitochondria. The OMM ubiquitination leads to OMM protein turnover by the proteasome upon membrane dissipation. Could the lack of TOM20 signal be caused by proteasome turnover of TOM20 on mitochondria with low membrane potential?

In this context, the authors neither cite nor discuss critical literature:

Yoshii et al., JBC 2011 show rupture of OMM during Parkin-mediated mitophagy
Wei et al., Cell 2017 identify Phb2 as receptor for mitophagy upon OMM rupture.
Both papers show that OMM rupture depends on UPS; does epoxomicin treatment prevent OMM rupture and IMM ubiquitination?

Abeliovich et al. Nature Comm. 2013: shows involvement of mitochondrial dynamics in the segregation of mitochondrial matrix proteins during mitophagy in yeast. Similar mechanisms might apply to cyto c.

(3) What is the functional connection of cyto c accumulation to OMM rupture, IMM ubiquitination, or mitophagy? While cyto c mosaicism is an interesting phenomenon, its functional relevance is unclear.

Is cyto c mosaicism caused by cyto c redistribution or due to protein translation and preferred import into mitochondria with low membrane potential?
Does cyto c accumulation/mosaicism depend on Parkin/Pink1?
Parkin is expressed at low levels in HCT116 cells. Is Parkin/Pink1 required for cyto c mosaicism, OMM rupture, IMM ubiquitination, and mitophagy?
The authors should characterize parkin-/- HCT116 cells.
Loss of membrane potential precedes or coincides with induction of cyto c accumulation; is loss of membrane potential sufficient to explain induction of mitophagy (s. discussion of Yoshii et al and Wei et al.)
Are p62 and/or OPTN required for degradation of these structures?
Does OMM rupture lead to a loss of mitochondrial cyto c?
Minor points:

I don't think the phrase "the process is conserved" is appropriate when comparing two human cancer cell lines (Page 8).

Figure legends should include more experimental information including duration of treatments.

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

The authors of this study observed that treatment of HCT116 cells with actinonin (Acn), which reduces translation fidelity of mitochondrial ribosomes, decreases Drp1 protein abundance and increases autophagy. In actinonin-treated cells they found a "mosaic" pattern of cytochrome c (mitochondria with higher or lower fluorescence intensity of cytochrome c) that was dependent on Drp1 but not on Bax/Bak expression. Live-cell time lapse imaging indicated that "high" cytochrome c mitochondria were characterized by a reduction of the mitochondrial membrane potential. Acn induced circular ubiquitin-positive structures at mitochondria that increased in abundance upon autophagy inhibition by BafA1 but were not observed in Drp1-deficient cells. These circular ubiquitin-positive structures colocalized with Tim23 and SDHA and were therefore attributed to inner mitochondrial membrane (IMM) ubiquitination. The autophagy adaptors p62 and optineurin were also found at sites of IMM ubiquitination. When overexpressing Parkin-mCherry together with cytochrome c-GFP in HeLa cells, the amount of "high" cytochrome c mitochondria per cell was reduced, suggesting that Parkin primes "high" cytochrome c mitochondria for degradation by mitophagy. Immunofluorescence analysis indicated colocalization of Parkin-mCherry and ubiquitin with Tom20. Therefore the authors concluded that in the presence of Parkin outer mitochondrial membrane (OMM) ubiquitination and canonical mitophagy occurs.

This study describes the interesting phenomenon of IMM ubiquitination mediated by Drp1 through OMM sewering in Parkin-deficient or low Parkin expressing cells induced by Acn. In contrast, Parkin overexpression in Acn-treated cells causes OMM ubiquitination and canonical PINK1/Parkin-induced mitophagy that is independent of Drp1 (as described earlier by Burman et al., 2017). Most of the finding are based on sophisticated imaging approaches. However, the physiological relevance of the observations remains unclear and the "red thread" is missing. In addition, several mechanistic aspects have not adequately been addressed. First, the phenomenon described is attributed to mitochondrial proteotoxicity based on using the drug Acn, an inhibitor of mitochondrial peptide deformylase (PDF) that blocks the growth of rapidly proliferating cells, but does not seem to affect normal cell growth. To claim mitochondrial proteotoxicity as the trigger for the effects observed in this study, other conditions causing mitochondrial proteostasis dysregulation need to be tested. Second, the relationship between "high" cytochrome c mitochondria, OMM sewering and apoptosis has not been addressed. Formation of "high" cytochrome c mitochondria has been shown to be independent of Bax/Bak, but OMM sewering mediated by Drp1 could allow cytochrome c to translocate to the cytoplasm and induce apoptosis. Is IMM ubiquitination associated with "high" or "low" cytochrome c? Does inhibition of autophagy influence apoptosis in Acn-treated cells? Third, the comparison of HeLa cells +/- Parkin overexpression is not an adequate model to test for a possible role of endogenous Parkin in this paradigm of mitochondrial stress. Notably, Parkin expression is transcriptionally upregulated in various stress conditions through the integrated stress response. Therefore, even in cells expressing Parkin at low abundance under steady state, Parkin effects may be relevant under stress.
Figure 1:
In general, the effects of Acn on Drp1 abundance are rather minor and the rescue by BafA1 (Fig. 1C) or ATG5-/- (Fig. 1D) is difficult to evaluate by the blots shown. Quantification of Fig. 1A is obviously based on scanning X-Ray films and performing image analysis. The dynamic range of this method is low compared to CCD-based detection of luminescence signals or laser detection. Furthermore, it is not clear how normalization of the Western blot signals has been done.

Figure 2:
The quantification of the mosaic cytochrome c pattern shown in Figure 2F looks like an "all or nothing" effect. What is the ratio of "high" to "low" cytochrome c mitochondria?

Figure 7:
The blots show high molecular weight complexes of OXPHOS components after Acn treatment and cross-linking that are degraded by mitophagy. The effects are rather minor and require quantification.

Statistics:
The authors used two-tailed Student's t-test throughout their study. This statistical test is only applicable for the comparison of two parametric datasets. Gaussian distribution can be tested by the Kolmogorov-Smirnov test. In Figure 1B, the authors performed a Student's t-test with n=3 for each group. An n number of 3 does not allow to test for Gaussian distribution, which is a prerequisite for a t-test. Therefore, the non-parametric U-test is required. When multiple comparisons are done within a dataset (as in Figure S2E and S2F), a one-way ANOVA (parametric) or a Kruskal-Wallis test and post-hoc test needs to be performed to correct for type I error inflation. To perform statistical analysis on time-lapse experiments (as performed in Figure 3C-E), a multifactorial ANOVA is recommended or the endpoint analysis should be labelled accordingly.

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

Previous studies have shown that the antibacterial agent, actinonin, causes a reduction in the fidelity of translation on mitochondrial ribosomes and affects mitochondrial functions, such as mitochondrial protein synthesis, membrane potential, and mitophagy. In the present manuscript, Oshima et al. reported that actinonin induces ubiquitination of the mitochondrial inner membrane and accumulation of cytochrome c in a small population of mitochondria that show the loss of the membrane potential. This ubiquitination was independent of PINK1 and parkin and required Drp1. Drp1 was proposed to sever the outer membrane. The authors suggest that assembly (but not amounts) of OXPHOS subunits are affected when cells are treated with actinonin. Regions of mitochondria that are ubiquitinated are also associated with autophagy adaptor proteins, such as p62 and optineurin. The authors propose that aberrant OXPHOS assembly induces mitophagy through Drp1-mediated outer membrane rupture, which exposes the inner membrane to ubiquitination. This study found several very interesting observations and provides a new model for Parkin-independent mitophagy. However, some of the data are preliminary and current data do not strongly support this interesting hypothesis. Addressing several points could substantiate the work.

Major points

1. Because actinonin induces the recruitment of ubiquitin and autophagy adaptors to mitochondria
in WT but not Drp1 KO cells, the authors propose that actinonin promotes mitophagy in a Drp1-dependent manner. However, since the levels of most mitochondrial proteins did not decrease (Tom20, Tom40 and OXPHOS subunits), it is not convincing that this process is really related to mitophagy. The authors also suggest that this mitophagy mechanism causes degradation of Drp1. They should directly examine mitophagy in WT and Drp1 KO cells in the presence of actinonin.

2. The authors show that outer membrane severing requires Drp1. Is this activity incomplete mitochondrial division which depends on its receptor proteins such as MFF and Mid49/51 or is it mediated by the membrane remodeling activity of Drp1 through interactions with mitochondrial lipids, such as cardiolipin, independently of the receptor proteins? Distinguishing these two modes of action would reveal the mechanistic role of Drp1.

3. The authors found that cytochrome c accumulates in a small population of mitochondria when cells are treated with actinonin. This accumulation was lost when Drp1 was absent and mitochondria were highly connected (Fig. 2). This finding is a very interesting, but the authors did not address whether or how this accumulation is involved in the proposed mitophagy pathway. Are mitochondria with high levels of cytochrome c the ones that are ubiquitinated in a parkin-independent manner? Is this accumulation of cytochrome c necessary for ubiquitination? Is the accumulation specific to cytochrome c, or are other OXPHOS subunits also accumulated?

4. The authors examined OXPHOS assembly using chemical cross linkers and antibody cocktails (which recognize multiple OXPHOS subunits). These data are preliminary. It is unclear which crosslinked bands correspond to which subunits or which complexes. The assembly status of the OXPHOS complexes need to be analyzed using BN-PAGE.

5. Another major weakness of the current study is that only actinonin is used to alter OXPHOS assembly or mitochondrial translational fidelity. Additional approaches that change OXPHOS assembly, such as the use of heteroplasmic cybrid cells carrying mtDNA mutations or cells lacking complex I assembly factors, would strengthen the model.

Minor points

6. In Fig. 1C and D, the authors should quantify band intensity of Drp1 since its changes are modest, and a cytosolic loading control, such as GAPDH, should be included.

7. Fig. 4I is not described in the text.
Point-by-point response to the reviewers' critiques:

We thank the reviewers for their critical reading and interest in our work. Their constructive critiques and suggestions were carefully considered and addressed by new experiments. The manuscript's significant changes include adding new data and clarification of some issues in the text. We believe that the inclusion of several new evidence pieces, in many cases orthogonally supporting conclusions of the original version of this work, and adding further depth to work, resulted in a strikingly improved manuscript. We hope that the reviewers agree with this assessment.

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

The current manuscript describes the cellular response to reduce mitochondrial translation fidelity in actinonin-treated human cancer cell lines (HCT116 and HeLa) that express low levels or no Parkin, respectively. The authors observe an interesting redistribution of cytochrome c resulting in a mosaic accumulation within mitochondria with reduced membrane potential in a Drp1-dependent manner. In addition, using fluorescence microscopy, the authors discover mitochondria that display circular ubiquitination signals but disrupted signal for the outer mitochondrial protein TOM20, which the authors interpret as a rupture of the outer mitochondrial membrane and inner membrane protein ubiquitination. These structures emerge in a Drp1-dependent manner and increase in number upon autophagy inhibition, suggesting that they are targeted and removed by mitophagy. Consistent with this model, the authors detect p62 and OPTN, two autophagy receptor proteins at these ubiquitinated mitochondria.

While the manuscript describes interesting phenomena, namely cytochrome c redistribution and OMM rupture and IMM protein ubiquitination-induced mitophagy, the data are not convincing yet. There are three major weaknesses in the manuscript:

(1) Direct evidence for induction and turnover of Drp1 and "high" cyto. c mitochondria by mitophagy is missing.

Although the authors describe a detectable reduction in Drp1 protein levels upon ABT-737 and STS treatment in figure 1A, data in figure 1B show that these apparent differences are not significant/reproducible. Thus, all data shown in figure 1 need to be normalized for loading and quantified to assess whether apparent effects are real.
We further focused the manuscript on the effects of reduced mitochondrial translation fidelity and decided to remove the STS and ABT-737 data, originally shown in Figure 1, from the current version. We believe that those data were not strongly linked to the main topic and message of this work, and therefore, their removal does not affect the quality and scientific depth of our manuscript.

In addition, steady-state protein levels, inherently being a product of both translation and degradation, are insufficient to demonstrate a change in protein degradation. To test for a change in protein stability of Drp1, the authors would need to perform pulse-chase experiments. Steady-state measurements of mitochondrial proteins do not support a general mitophagic turnover of the organelle; protein levels of ATP5A1, SDHB, COX2, TOM20, TOM40 are unchanged and NDUFB8 shows the opposite behavior than Drp1 in Figures 1A-E. How do the authors reconcile these data with their proposed induction of mitophagy?

In contrast to the mitophagy models in which prolonged treatments with uncouplers or OXPHOS inhibitors lead to eliminating the mitochondria from the cells, the model of reduced MTF that we describe here induces much less pronounced mitophagy. The data show that only a few mitochondria per cell are affected at the given time, and the changes in mitochondrial abundance per cell are not detectable. Therefore, the protein changes effected by this selective mitophagy are likely challenging to detect (e.g., ATP5A, SDHB, etc.). On the other hand, the increase in NDUFB8 or Cox2 reflects the direct mechanism by which Actinonin affects mitochondrial proteome (see new Figure 2 and discussion of the model on pages 9-10 of the revised manuscript). Furthermore, other than Drp1, substrates eliminated by this selective mitophagy mode (especially OXPHOS and IMM proteins) need to be identified. Our subsequent research will focus on addressing this gap.

Importantly, the induction of mitophagy is now supported with additional data, including accumulation of LC3 on the mitochondria with severed OMM and ubiquitinated IMM (seen new Figure 7F-J) and accumulation of K63 Ub chains in light mitochondria from Acm-treated cells. Importantly, LC3 localization at autophagosomes is one of the most commonly used and well-established methods in detecting autophagy. Therefore, we believe that mechanisms other than mitophagy are unlikely.

Accumulation of OMM-ruptured, IMM ubiquitinated mitochondria in ATG5-/− cells is consistent with mitophagic turnover but not proof. For example, ATG5-/− mitochondria may be affect by Atg12-Atg3 conjugates independent of ATG5's function in autophagy (Radoshevich et al. Cell, 2010).

Thank you for suggesting this interesting possibility. Our new data indicate that Atg12-Atg5 conjugates are critical for autophagy in cells with reduced MTF. First, we found that autophagy adaptors
p62 and optineurin localized to ubiquitinated IMM in Acn-treated or Acn plus bafilomycin A1 co-treated WT cells and Acn-treated ATG5-/- cells. In contrast, LC3 accumulates on ubiquitinated IMM in Acn-treated or Acn plus bafilomycin A1 co-treated WT cells, but not in similarly treated ATG5-/- HCT116 cells (see new Figure 7F-J). Furthermore, consistent with the critical role for ATG5 in the model of mitophagy we describe here, no LC3 processing was detected in ATG5-/- cells but was evident in WT cells (data now shown). Thus, since Atg5 acts downstream of autophagy adaptors and upstream of autophagosome assembly, these data indicate that Atg12-Atg5 conjugates are implicated in the process.

The manuscript by Radoshevich et al. found the role of Atg12-Atg3 conjugates in mitochondrial homeostasis and cell death. It is possible that these noncanonical Atg12 conjugates also influence changes described by us. However, the data we show, especially the relationship between LC3 accumulation/processing and expression status of ATG5, clearly points to the mechanism implicating ATG5-dependent autophagy in mitochondrial response to reduced MTF.

The authors need to perform standard assays in the field to assess mitophagy: mtKeima FACS, mitochondrial GFP-RFP signal analysis (yellow vs red (autolysosomes)), time-lapse analysis of defective mitochondria being engulfed by LC3-marked autophagosomes.

We propose that the reduced MTF-induced mitophagy occurs in a very limited capacity, e.g., only a small number of the mitochondria within each cell are affected. This mitophagy mode could resemble the local elimination of faulty mitochondria/misfolded proteins, as reported by Burman et al. (JCB;2017). Specifically, it affects only a small subset of mitochondria and does not lead to extensive degradation/elimination of mitochondria from the cell. Thus, the scope is different from the well-studied mitophagy induced by treatments with uncoupling agents and ectopic expression of Parkin that leads to massive degradation of mitochondria in the cell; therefore, can be detected by FACS and other high throughput "standard" methods. However, new data included in the revised manuscript (Figure 7F-J) clearly show that autophagy is indeed activated. First, the mitochondria with severed/ruptured OMM and ubiquitinated IMM accumulate in ATG5-/- cells and WT cells co-treated with autophagy inhibitor (see Figure 4I in the revised manuscript). Second, autophagy adaptors and upstream autophagy machinery (e.g., LC3) are activated and colocalize with Ub on the IMM with severed OMM. Third, ectopically expressed Parkin is activated, although it switches the mechanism of autophagy in cells with reduced MTF. Fourth, mitochondrial fission protein Drp1, which regulates this process, is also degraded in an autophagy (ATG5)-dependent manner.

The changes in protein crosslinks shown in figure 7 are not convincing and need to be quantified.
Moreover, what is the nature of these crosslinks and on what basis do the authors consider them to be a readout for an accumulation of abnormal OXPHOS complexes? Why are non-crosslinked protein levels not changed? This again does not support mitophagy mediated turnover/quality control. The authors should perform BN-PAGE of supercomplexes.

We agree with the reviewer. However, we performed the NATIVE gel analyses of OXPHOS proteins, and a substantial majority of these proteins localized almost exclusively to high molecular weight “supercomplexes.” We were not able to gain any new insights into the process. Considering the rather sketchy nature of our original cross-linking data, these data were removed from the revised work. They will be used as the basis for our further studies. We believe that other data added to the revised manuscript more than compensate for removing these studies, making the new manuscript more focused.

(2) OMM ruptured and IMM protein ubiquitination are not convincingly shown yet

The data is consistent with OMM rupture. However, EM analysis is required to directly demonstrate it (compare to Yoshii et al. and Wei et al. see below). An alternative explanation might be that, instead of OMM rupture, TOM20 is degraded in a localized manner resulting the impression of absence of the OMM at some areas of the mitochondria. The OMM ubiquitination leads to OMM protein turnover by the proteasome upon membrane dissipation. Could the lack of TOM20 signal be caused by proteasome turnover of TOM20 on mitochondria with low membrane potential?

We appreciate this suggestion. We conducted several new experiments to test the nature of mitochondria with a discontinuous Tom20 signal. We investigated other than Tom20 OMM proteins, including Tom40 and Fis1 (new Figure S8A, B). They also show discontinuous signals and colocalize with Tom20. Second, all analyzed mitochondria with discontinuous Tom20 and IMM ubiquitination are invariably deficient in cytochrome c signal (new Figure 4J). Independent analyses of p62-positive mitochondria with intermittent Tom20 signal also show that 100% of mitochondria are deficient in cytochrome c (new Figure 7K). Since all of the mitochondria with discontinuous Tom20 are permeable for cytochrome c, we conclude that the discontinuous Tom20 (Fis1 and Tom40) signal represents severed OMM. This conclusion was further supported by the cell fractionation studies (new Figures 4K, 5A, and 6D). Finally, the mass spectroscopy analyses of protein ubiquitinated in mitochondria from Acn-treated cells identified a total of five mitochondrial proteins (see new Figure 6A and Supplemental spreadsheet). One of these proteins was p62, two CPOX and TRAP1 localize to the IMM side facing IMS. Biochemical
analyses also verified that these proteins are ubiquitinated (new Figure 6B-D). These data support the notion that through the severed OMM Ub ligase(s) could gain access to and ubiquitinate and/or interact with CPOX and TRAP1. We believe that other scenarios are much less likely.

**The role of UPS-mediated severing of the OMM in our model of mitochondrial proteotoxicity.** The evidence indicates that proteasomal degradation of the OMM proteins is not required for OMM severing. First, co-treatment of cells with Acn and MG132 did not inhibit severing of the OMM. Second, while Acn induced proteasome-dependent degradation of some OMM proteins, such as Mfn1 and Mfn2, levels of Tom20, Tom40, Fis1 (used by us to detect severed OMM) were not detectably affected. Furthermore, the OMM severing and IMM ubiquitination were also detected in HeLa cells that naturally do not express Parkin. Thus, the mechanism proposed by Yoshii et al. in which Parkin-mediated ubiquitination of the OMM proteins facilitates their proteasomal degradation may not be valid in the model we describe. Because the manuscript is already long and exceeds the JCB size limits, these data are not shown in the revised manuscript but can be included if the reviewer thinks it is necessary and editors agree.

The data are consistent with IMM protein ubiquitination, but are indirect. The authors need to directly show ubiquitination of IMM proteins by Western blot or proteomics analyses in dependence of Acn, Acn+CP treatment, Drp1 and autophagy.

We agree with the reviewer that the data shown in the original version of our manuscript was not direct and did not wholly support IMM ubiquitination. We performed mass spectroscopy of mitochondria from control (DMSO-treated) and Acn-treated WT and ATG5−/− HCT116 cells. These analyses identified five mitochondrial proteins with a significant increase of potential ubiquitination in Acn-treated versus DMSO-treated samples (see new Figure 6A and Supplemental spreadsheet). Notably, one of these proteins was p62 (which we independently identified to localize to ubiquitinated IMM; see Figure 7). The other two proteins, CPOX and TRAP1, were earlier reported to reside in the IMS, with significant subset binding IMM (see revised manuscript for more details; page 15). Ubiquitination of these proteins, especially in Acn-treated ATG5−/− cells, was also verified by Ub pull-down assay (see new Figure 6B, D). The fact that ubiquitinated CPOX and TRAP1 accumulate in light mitochondria from ATG5−/− cells is also consistent with imaging data included in the original version of the work. Specifically, Acn-induces fragmentation of mitochondria and shifts some mitochondria into light membrane fractions (see new Figures 4K and 5A). The mitochondria showing OMM severing are small in size and likely copurify with the light membranes.
Identification of CPOX and TRAP1 is a proof of principle, and other IMM proteins may also be ubiquitinated. Nevertheless, we believe that data in the revised manuscript strongly and more directly support the notion that in mitochondria with severed OMM the IMM proteins are ubiquitinated.

Notably, these data are also crucial for another reason. We found that like cytochrome c, CPOX also shows the mosaic distribution in Acn-treated cells. Moreover, increased levels of cytochrome c were detected in the same mitochondria showing high CPOX levels (new Figure 6E-G). Thus, it is plausible to assume that the formation of mosaic mitochondria could be linked to IMM protein ubiquitination and subsequent mitophagy. This idea will be further tested in planned studies.

To test whether cytochrome c mosaicism, OMM rupture, and IMM ubiquitination occur upon delayed Parkin-mediated mitophagy, the authors should examine ATG5-/− HCT116 cells after FCCP, OA, Rot treatment for cyto c distribution, OMM rupture and IMM ubiquitination. In the presence of efficient Parkin-mediated mitophagy upon dissipation of MM potential, cyto c mosaicism, OMM rupture, and IMM protein ubiquitination might not be visible due to rapid turnover of mitochondria.

We tested the degree to which FCCP induces the mitochondrial mosaicism, OMM rupture, and IMM ubiquitination in untransfected or Parkin-YFP-expressing WT and ATG5-/− cells. We have never seen the mosaic cytochrome c distribution in FCCP-treated cells (either Parkin-YFP positive or negative). We also found that FCCP inhibits the Acn-induced formation of mosaic mitochondria in Parkin-deficient cells. Therefore, the data support the notion that in Acn-treated cells, OMM rupture and IMM ubiquitination do not occur in cells ectopically expressing Parkin and that FCCP and dissipation of mitochondrial membrane potential is not mechanistically linked to mitochondrial mosaicism. However, the possibility that membrane potential changes are important downstream of other Acn-induced mitochondrial changes (e.g., changes in mitochondrial proteome) cannot be excluded.

In this context, the authors neither cite nor discuss critical literature:

Yoshii et al., JBC 2011 show rupture of OMM during Parkin-mediated mitophagy
Wei et al., Cell 2017 identify Phb2 as receptor for mitophagy upon OMM rupture.
Both papers show that OMM rupture depends on UPS; does epoxomicin treatment prevent OMM rupture and IMM ubiquitination?

We found that inhibition of proteasome does not affect OMM severing/rupture and IMM ubiquitination. Specifically, OMM severing and IMM ubiquitination were detected in cells co-treated with
the proteasome inhibitor MG132 and Acn. Because the manuscript is already long and exceeds the JCB limits, these data are not shown in the revised manuscript but can be included if the reviewer thinks it is necessary and editors agree.

Abeliovich et al. Nature Comm. 2013: shows involvement of mitochondrial dynamics in the segregation of mitochondrial matrix proteins during mitophagy in yeast. Similar mechanisms might apply to cyto c.

We apologize for these omissions. Now, these citations were incorporated and cited in the current version of the manuscript.

(3) What is the functional connection of cyto c accumulation to OMM rupture, IMM ubiquitination, or mitophagy? While cyto c mosiacism is an interesting phenomenon, its functional relevance is unclear.

Our new data provide some answers to these critical questions. First, the only protein we found to clearly show the mosaic distribution and similar staining pattern as cytochrome c is an IMS/IMM protein CPOX. High levels of CPOX and cytochrome c were detected in the same mitochondria (new Figure 6E-G). Another IMS/IMM protein, TRAP1, showed some mosaicism but its association with high cytochrome c mitochondria was much less pronounced than CPOX’s (new Figure S9A). However, we tested many other mitochondrial proteins, including different subunits of OXPHOS complexes, mitochondrial ribosomal proteins, etc., and they showed uniform distribution in control and Acn-treated cells. Thus, while other, currently unidentified proteins may show a similar pattern like cytochrome c and CPOX, the mosaic distribution of cytochrome c, CPOX, and TRAP1 is likely to have functional significance. While at this time we are not able to answer the reviewer’s question of whether the “high cytochrome c mitochondria are necessary for ubiquitination,” we believe that the fact that CPOX and TRAP1 were identified by mass spectrometry and verified by ubiquitin pull down to be the ubiquitinated in Acn-treated cells (new Figure 6A-D) suggest that high cytochrome c/CPOX mitochondria are subsequently displaying OMM severing and IMM ubiquitination. Other studies will be necessary to eliminate the speculative nature of this conclusion. However, the fact that high cytochrome c mitochondria are preferentially targeted by ectopically expressed Parkin supports the possibility that they contain clues targeting them for elimination in Parkin-deficient cells.

Is cyto c mosaicism caused by cyto c redistribution or due to protein translation and preferred import into mitochondria with low membrane potential?

Since direct dissipation of mitochondrial membrane potential with FCCP does not change the distribution and signal intensity of cytochrome c, the mechanism proposed by the reviewer is unlikely.
Moreover, FCCP treatment inhibits Acn-induced mitochondrial mosaicism, further pointing to another scenario.

Does cyto c accumulation/mosaicism depend on Parkin/Pink1?

The data show that mitochondrial mosaicism does not depend on Parkin. HeLa cells do not express this protein, but the formation of mosaic cytochrome c mitochondria (and CPOX, which was identified in studies addressing the reviewer’s concerns) occurs (new Figures 1G and S1C,D).

Parkin is expressed at low levels in HCT116 cells. Is Parkin/Pink1 required for cyto c mosaicism, OMM rupture, IMM ubiquitination, and mitophagy?

The data (new Figure S8E) and published literature indicate that HeLa cells do not express Parkin. HeLa clone used in our study was the same as described by Burman et al. (Burman et al.; JCB 2017). They were extensively analyzed with different methods and found to be deficient in Parkin expression (Burman et al.; JCB 2017).

The authors should characterize parkin-/- HCT116 cells.

We tested the WT and Parkin-/- M17 neuroblastoma cells but were not able to detect mitochondrial ubiquitination regardless of the trigger used (e.g., FCCP or Acn) in neither WT nor Parkin-/- cells. This is a very surprising observation. However, we believe that investigating the underlying reason may be outside of the scope of this manuscript. Regardless, since Parkin deficient HeLa cells show mitochondrial mosaicism (new Figures 1G and S1C, D), the OMM rupture and IMM ubiquitination (new Figure S8C, D), these processes appear to be Parkin-independent.

Loss of membrane potential precedes or coincides with induction of cyto c accumulation; is loss of membrane potential sufficient to explain induction of mitophagy (s. discussion of Yoshii et al and Wei et al.)

Direct pharmacological dissipation of mitochondrial membrane potential with FCCP does not change the distribution and signal intensity of cytochrome c. Furthermore, FCCP treatment inhibits Acn-induced mitochondrial mosaicism and IMM ubiquitination. Thus, the data indicate that while mitochondrial membrane decline is associated with Acn-induced mitochondrial changes, it is unlikely that it is the primary trigger of mitophagy in this model.
Are p62 and/or OPTN required for degradation of these structures?

We do not know. However, since autophagosomes (LC3; see new Figure 7F-I) localize to mitochondria with severed OMM and ubiquitinated IMM, this possibility is likely. We appreciate this question. However, in this manuscript, we describe a new pathway. The nature of this, and similar discoveries, is that they induce many questions. These questions, including the role of p62 and OPTN in the degradation of mitochondria with severed OMM and ubiquitinated IMM, will be addressed in the future.

Does OMM rupture lead to a loss of mitochondrial cyto c?

This is an important point. Indeed, using Airyscan superresolution imaging and cell fractionation (new Figures 4JK, 5A, and 7K), we found that mitochondria with severed OMM are deficient in cytochrome c. In our view, these data strongly support the notion that the discontinuous Tom20 signal reflects the OMM severing, but not redistribution or local degradation of the OMM proteins.

Minor points:

I don't think the phrase "the process is conserved" is appropriate when comparing two human cancer cell lines (Page 8).

We agree with the reviewer. Although in the revised manuscript, we added more cell types (e.g., M17 neuroblastoma and MEFs), additional studies are required to verify the degree of conservation. We removed this statement from the revised manuscript.

Figure legends should include more experimental information including duration of treatments.

We corrected this issue and added durations of treatments and other details to the figure legends and figures.

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

The authors of this study observed that treatment of HCT116 cells with actinonin (Acn), which reduces translation fidelity of mitochondrial ribosomes, decreases Drp1 protein abundance and increases
autophagy. In actinonin-treated cells they found a "mosaic" pattern of cytochrome c (mitochondria with higher or lower fluorescence intensity of cytochrome c) that was dependent on Drp1 but not on Bax/Bak expression. Live-cell time lapse imaging indicated that "high" cytochrome c mitochondria were characterized by a reduction of the mitochondrial membrane potential. Acn induced circular ubiquitin-positive structures at mitochondria that increased in abundance upon autophagy inhibition by BafA1 but were not observed in Drp1-deficient cells. These circular ubiquitin-positive structures colocalized with Tim23 and SDHA and were therefore attributed to inner mitochondrial membrane (IMM) ubiquitination. The autophagy adaptors p62 and optineurin were also found at sites of IMM ubiquitination. When overexpressing Parkin-mCherry together with cytochrome c-GFP in HeLa cells, the amount of "high" cytochrome c mitochondria per cell was reduced, suggesting that Parkin primes "high" cytochrome c mitochondria for degradation by mitophagy. Immunofluorescence analysis indicated colocalization of Parkin-mCherry and ubiquitin with Tom20. Therefore the authors concluded that in the presence of Parkin outer mitochondrial membrane (OMM) ubiquitination and canonical mitophagy occurs.

This study describes the interesting phenomenon of IMM ubiquitination mediated by Drp1 through OMM sewing in Parkin-deficient or low Parkin expressing cells induced by Acn. In contrast, Parkin overexpression in Acn-treated cells causes OMM ubiquitination and canonical PINK1/Parkin-induced mitophagy that is independent of Drp1 (as described earlier by Burman et al., 2017). Most of the finding are based on sophisticated imaging approaches.

However, the physiological relevance of the observations remains unclear and the "red thread" is missing. In addition, several mechanistic aspects have not adequately been addressed. First, the phenomenon described is attributed to mitochondrial proteotoxicity based on using the drug Acn, an inhibitor of mitochondrial peptide deformylase (PDF) that blocks the growth of rapidly proliferating cells, but does not seem to affect normal cell growth. To claim mitochondrial proteotoxicity as the trigger for the effects observed in this study, other conditions causing mitochondrial proteostasis dysregulation need to be tested.

In the first version of the manuscript, we applied a pharmacological modification of mitochondrial translation, including reducing mitochondrial translation fidelity with Acn and inhibition of this process with chloramphenicol (CP). Based on separate and combined treatments with these compounds and available literature, we concluded that abnormal mitochondrial translation causes mitochondrial phenotypes we described. New data included in the revised manuscript support these earlier thoughts. Also, they indicate that abnormal protein load on the IMM could be the specific trigger of mitochondrial mosaicism and subsequent mitophagy. First, by generating mtDNA-deficient cells, we removed mitochondrial translation from the system. No mosaic mitochondria, OMM severing, and IMM
ubiquitination were detected in Acn-treated mtDNA-deficient cells (new Figure 2C-E), supporting the role for active mitochondrial translation in mitochondrial alterations we describe.

Moreover, although shown in the original version of the manuscript, but not discussed, Acn-induced accumulation of some mtDNA encoded proteins (new Figure 2B, F). This accumulation was inhibited by CP or mtDNA removal (new Figure 2B, F), suggesting that abnormal IMM proteins could be implicated in mitochondrial changes described by us. Consistent with this, we found that ectopic expression of IMM localized complex I protein (NDUFA12) leads to Drp1-dependent mosaic distribution of cytochrome c without any additional treatment (new Figure 2G-J). We believe that these new data markedly increase understanding of the mechanism leading to mitochondrial mosaicism and subsequent mitophagy. We hope that the reviewer agrees with our assessment.

Second, the relationship between "high" cytochrome c mitochondria, OMM sewer and apoptosis has not been addressed. Formation of "high" cytochrome c mitochondria has been shown to be independent of Bax/Bak, but OMM sewer mediated by Drp1 could allow cytochrome c to translocate to the cytoplasm and induce apoptosis. Is IMM ubiquitination associated with "high" or "low" cytochrome c? Does inhibition of autophagy influence apoptosis in Acn-treated cells?

Thank you for pointing these critical issues. We carefully tested apoptosis activation in WT and autophagy-deficient ATG5/−/ HCT116 cells. Our data indicate that in Acn-treated cells, markers of apoptosis activation, caspase 9, and PARP processing can be detected not earlier than at 48hr of treatment. In comparison, treatment with small-molecule BH3 mimetic ABT-737 led to robust apoptosis activation at ~12hr of treatment. While apoptosis activation in ABT-737-treated cells was more pronounced in ATG5/−/ cells, there was no difference in Acn-treated cells (new Figure S4F). Thus, one can conclude that apoptosis activation in Acn-treated cells is not directly related to the OMM severing that occurs much earlier (~6hr of Acn treatment) and is not affected by the autophagy activation. Given that only a few mitochondria per cell show severing of the OMM, and thereby cytochrome c release, the extent of OMM permeabilization may not be sufficient to trigger activation of programmed cell death. However, we believe that the high glucose (4.5mM) in the media may also be responsible for dampened cell death response. Specifically, the lack of the effect of reduced mitochondrial translation fidelity and subsequent mitochondrial defects on cell viability could be due to the Crabtree effect. This comment stimulated us. We are now setting up experiments to test the degree to which altered growth conditions (e.g., glucose availability) affect reduced mitochondrial translation fidelity-induced cell death and the role of autophagy in the process. However, we believe that these studies are out of the current manuscript's scope and will be pursued in the near future.
Third, the comparison of HeLa cells +/- Parkin overexpression is not an adequate model to test for a possible role of endogenous Parkin in this paradigm of mitochondrial stress. Notably, Parkin expression is transcriptionally upregulated in various stress conditions through the integrated stress response. Therefore, even in cells expressing Parkin at low abundance under steady state, Parkin effects may be relevant under stress.

In principle, we agree with the reviewer that HeLa cells +/- Parkin overexpression is not the best model. However, many highly influential studies, some published in top-tier journals such as the Journal of Cell Biology, apply this model. Since other approaches have often validated data from HeLa +/- Parkin model, we believe this approach can be highly revealing. Nevertheless, we took the following actions to address this point. First, we toned down some statements in the revised manuscript (e.g., instead of saying “Parkin deficient,” we now say “low level of Parkin expressing” when we discuss HCT116 cells). Moreover, HeLa cells do not express Parkin, as shown by us using the most sensitive Western blot detection (see Figure S8E) and reported by Burman et al. (JCB, 2017) using 5’ RACE. Using the same clone of HeLa cells as Burman et al., we found that Acn-treatment leads to OMM severing and IMM ubiquitination, supporting that these mitochondrial changes do not require Parkin activity (new Figure S8C, D). Additional evidence, such as the fact that ubiquitinated IMM in Acn-treated HCT116 cells is deficient in phosphorylated Ub, in contrast to the cells ectopically expressing Parkin-YFP (Figure S10), further support this notion. As pointed by reviewer #1, Yoshi et al. (JBC, 2011) showed that in CCCP-treated cells, Parkin-mediated ubiquitination of the OMM proteins leads to the proteasome-dependent degradation of these proteins including Tom20 and Tom40 and the OMM rupture followed by subsequent degradation of remaining mitochondrial components by autophagy. The elegant studies by Yoshi et al. were performed using Parkin overexpressing and Parkin-overexpression deficient MEFs. Although MEFs express Parkin endogenously, proteasomal degradation of the OMM proteins was not apparent in Parkin-overexpression deficient MEFs. Regardless, inhibition of proteasomal activity does not affect OMM severing in described here model of reduced mitochondrial translation fidelity, supporting the notion that OMM severing and IMM ubiquitination does not require Parkin to occur.

Major points:
Figure 1:
In general, the effects of Acn on Drp1 abundance are rather minor and the rescue by BafA1 (Fig. 1C) or ATG5 +/- (Fig. 1D) is difficult to evaluate by the blots shown. Quantification of Fig. 1A is obviously based on scanning X-Ray films and performing image analysis. The dynamic range of this method is low.
compared to CCD-based detection of luminescence signals or laser detection. Furthermore, it is not clear how normalization of the Western blot signals has been done.

We used the CCD camera detection. In most of the experiments, we used a stable OMM protein Tom20 as a reference. In the revised manuscript, all critical Western blot experiments were done several times and quantified. Actin was also used as a loading control in some cell fractionation experiments.

Figure 2:
The quantification of the mosaic cytochrome c pattern shown in Figure 2F looks like an "all or nothing" effect. What is the ratio of "high" to "low" cytochrome c mitochondria?

Yes, the data show that at 4hr and 6hr of treatment, ~100% of cells display mosaic distribution of cytochrome c. To address the reviewer's inquiry, we performed the time course analyses of mosaic cytochrome c in Acn-treated WT HCT116 cells (new Figure 1 C). We also quantified the abundance of high cytochrome c mitochondria at a different time of treatment with Acn. We found that at 4hr and 6hr of treatment, ~15% of mitochondria within each cell have increased cytochrome c signal. These data are included as new Figure 1D. Similar quantifications in Acn-treated cells ectopically expressing Parkin revealed that more high cytochrome c mitochondria are detected in ATG5-/- than in WT HCT116 cells (new Figure 8G), supporting that high cytochrome c mitochondria are indeed targeted by mitophagy.

Figure 7:
The blots show high molecular weight complexes of OXPHOS components after Acn treatment and cross-linking that are degraded by mitophagy. The effects are rather minor and require quantification.

Considering a rather sketchy nature of our original cross-linking data, also pointed by reviewers#1 and #3, these data were removed. They will be used as the basis for further studies. We believe that other data added to the revised work more than compensate for removing these studies, making the current manuscript more focused.

Statistics:
The authors used two-tailed Student's t-test throughout their study. This statistical test is only applicable for the comparison of two parametric datasets. Gaussian distribution can be tested by the Kolmogorov-Smirnov test. In Figure 1B, the authors performed a Student's t-test with n=3 for each group. An n number of 3 does not allow to test for Gaussian distribution, which is a prerequisite for a t-test. Therefore, the non-parametric U-test is required. When multiple comparisons are done within a dataset (as in Figure
S2E and S2F), a one-way ANOVA (parametric) or a Kruskal-Wallis test and post-hoc test needs to be performed to correct for type I error inflation. To perform statistical analysis on time-lapse experiments (as performed in Figure 3C-E), a multifactorial ANOVA is recommended or the endpoint analysis should be labelled accordingly.

We appreciate the reviewer’s advice. We performed the statistical analyses as suggested.

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

Previous studies have shown that the antibacterial agent, actinonin, causes a reduction in the fidelity of translation on mitochondrial ribosomes and affects mitochondrial functions, such as mitochondrial protein synthesis, membrane potential, and mitophagy. In the present manuscript, Oshima et al. reported that actinonin induces ubiquitination of the mitochondrial inner membrane and accumulation of cytochrome c in a small population of mitochondria that show the loss of the membrane potential. This ubiquitination was independent of PINK1 and parkin and required Drp1. Drp1 was proposed to sever the outer membrane. The authors suggest that assembly (but not amounts) of OXPHOS subunits are affected when cells are treated with actinonin. Regions of mitochondria that are ubiquitinated are also associated with autophagy adaptor proteins, such as p62 and optineurin. The authors propose that aberrant OXPHOS assembly induces mitophagy through Drp1-mediated outer membrane rupture, which exposes the inner membrane to ubiquitination. This study found several very interesting observations and provides a new model for Parkin-independent mitophagy. However, some of the data are preliminary and current data do not strongly support this interesting hypothesis. Addressing several points could substantiate the work.

**Major points**

1. Because actinonin induces the recruitment of ubiquitin and autophagy adaptors to mitochondria in WT but not Drp1 KO cells, the authors propose that actinonin promotes mitophagy in a Drp1-dependent manner. However, since the levels of most mitochondrial proteins did not decrease (Tom20, Tom40 and OXPHOS subunits), it is not convincing that this process is really related to mitophagy. The authors also suggest that this mitophagy mechanism causes degradation of Drp1. They should directly examine mitophagy in WT and Drp1 KO cells in the presence of actinonin.

We propose that the reduced mitochondrial translation fidelity induces mitophagy that occurs in a very limited capacity, e.g., only a small number of the mitochondria within each cell is affected at the
given time. In our view, this mitophagy mode resembles the local elimination of faulty mitochondria/misfolded proteins from the mitochondrial network, as reported by Burman et al. (JCB;2017). It affects only a small subset of mitochondria and does not lead to the robust degradation/elimination of mitochondria. Thus, the scope is different from the well-studied mitophagy induced by treatments with uncoupling agents and ectopic expression of Parkin that leads to massive degradation of mitochondria in the cell; therefore, it can be detected by FACS and other high throughput "standard" methods. However, new data included in the revised manuscript (Figure 7F-J) clearly show that autophagy is indeed activated. First, the mitochondria with severed OMM and ubiquitinated IMM accumulate in autophagy-deficient ATG5/-/- cells and WT cells co-treated with Acn plus autophagy inhibitor bafilomycin A1 (new Figure 4I). Second, autophagy adaptors and upstream autophagy machinery (e.g., LC3) are activated and colocalize with Ub on the mitochondria with severed OMM. Third, ectopically expressed Parkin is activated, although it switches the mechanism of autophagy in cells with reduced mitochondrial translation fidelity. Fourth, mitochondrial fission protein Drp1, which regulates this process, is also degraded in an autophagy (ATG5)-dependent manner. We believe that the evidence, including new data, strongly supports the point that reduced mitochondrial translation fidelity activates mitophagy and hope that the reviewer agrees.

2. The authors show that outer membrane severing requires Drp1. Is this activity incomplete mitochondrial division which depends on its receptor proteins such as MFF and MiD49/51 or is it mediated by the membrane remodeling activity of Drp1 through interactions with mitochondrial lipids, such as cardiolipin, independently of the receptor proteins? Distinguishing these two modes of action would reveal the mechanistic role of Drp1.

Thank you for this suggestion. We found that mosaic cytochrome c distribution was apparent in WT mouse embryonic fibroblasts (MEFs) and almost completely inhibited in Mff/-/-, MiD51/-/- and Drp1/-/- MEFs. The effect of MiD49 knockout was less pronounced but significant (new Figures 1G and S2). The role of Mff in mosaic cytochrome c distribution was confirmed in time-lapse experiments and cell fractionation Western blot studies. Similar to Drp1/-/- HeLa cells mosaic cyt. c-GFP formation was much less pronounced in Mff/-/- HeLa cells than in WT HeLa cells (new Figure 3F and Movie S3). Supporting a direct role for Drp1 in the process, and regulation by Mff, cell fractionation also revealed that Acn-induced accumulation of Drp1 in the mitochondrial fractions from WT, but not Mff/-/- cells (new Figure S5E). Thus, we conclude that in cells with reduced mitochondrial translation fidelity, mitochondrial receptors of Drp1, especially Mff and MiD51, are vital for mitochondrial mosaicism.
3. The authors found that cytochrome c accumulates in a small population of mitochondria when cells are treated with actinonin. This accumulation was lost when Drp1 was absent and mitochondria were highly connected (Fig. 2). This finding is a very interesting, but the authors did not address whether or how this accumulation is involved in the proposed mitophagy pathway. Are mitochondria with high levels of cytochrome c the ones that are ubiquitinated in a parkin-independent manner? Is this accumulation of cytochrome c necessary for ubiquitination? Is the accumulation specific to cytochrome c, or are other OXPHOS subunits also accumulated?

Our new data provide some answers to these questions. First, the only protein we found to clearly show the mosaic distribution and similar staining pattern as cytochrome c is an IMS/IMM protein CPOX. High levels of CPOX and cytochrome c were detected in the same mitochondria (new Figure 6E-G). Another IMS/IMM protein, TRAP1, showed some mosaicism but its association with high cytochrome c mitochondria was much less pronounced than CPOX’s (new Figure S9A). However, we tested many other mitochondrial proteins, including different subunits of OXPHOS complexes, mitochondrial ribosomal proteins, etc., and they showed uniform distribution in control and Acn-treated cells. Thus, while other, currently unidentified, proteins may show a similar pattern like cytochrome c and CPOX, the mosaic distribution of cytochrome c, CPOX, and TRAP1 is likely to have functional significance. While at this time we are not able to answer the reviewer’s question of whether the “high cytochrome c mitochondria are necessary for ubiquitination,” we believe that the fact that CPOX and TRAP1 were identified by mass spectrometry (total five mitochondrial proteins were identified) and verified by ubiquitin pull down to be the ubiquitinated in Acn-treated cells (new Figure 6A-D) suggest that high cytochrome c/CPOX mitochondria are subsequently displaying OMM severing and IMM ubiquitination. Other studies will be necessary to reduce the speculative nature of this conclusion. However, the fact that high cytochrome c mitochondria are preferentially targeted by ectopically expressed Parkin further supports the possibility that they contain clues targeting them for elimination also in Parkin-deficient cells.

4. The authors examined OXPHOS assembly using chemical cross linkers and antibody cocktails (which recognize multiple OXPHOS subunits). These data are preliminary. It is unclear which crosslinked bands correspond to which subunits or which complexes. The assembly status of the OXPHOS complexes need to be analyzed using BN-PAGE.

We agree with the reviewer. However, we performed the NATIVE gel analyses of OXPHOS proteins, and a substantial majority of these proteins localized almost exclusively to high molecular weight “supercomplexes.” We were not able to gain any new insights into the process. Considering a rather sketchy nature of our original cross-linking data, these data were removed and used as the basis
for further studies. We believe that other data added to the revised version of the manuscript more than compensate for removing these studies, making the revised manuscript more focused.

5. Another major weakness of the current study is that only actinonin is used to alter OXPHOS assembly or mitochondrial translational fidelity. Additional approaches that change OXPHOS assembly, such as the use of heteroplasmic cybrid cells carrying mtDNA mutations or cells lacking complex I assembly factors, would strengthen the model.

In the first version of the manuscript, we applied a pharmacological modification of mitochondrial translation, including reducing mitochondrial translation fidelity with Acn and inhibition of this process with chloramphenicol (CP). Based on separate and combined treatments with these compounds and available literature, we concluded that abnormal mitochondrial translation causes mitochondrial phenotypes we described. The new data included in the revised manuscript support these earlier thoughts. Also, they indicate that abnormal protein load on the IMM could be the specific trigger of mitochondrial mosaicism and subsequent mitophagy. First, by generating mtDNA-deficient cells, we removed mitochondrial translation from the system. No mosaic mitochondria, OMM severing, and IMM ubiquitination were detected in Acn-treated mtDNA-deficient cells (new Figure 2C-E), supporting the role for active mitochondrial translation in mitochondrial alterations we describe.

Moreover, although shown in the original version of the manuscript, but not discussed, Acn-induced accumulation of some mtDNA encoded proteins (new Figure 2B, F). This accumulation was inhibited by CP or mtDNA removal (new Figure 2B, F), suggesting that abnormal IMM proteins could be implicated in mitochondrial changes described by us. Consistent with this, we found that ectopic expression of IMM localized complex I protein (NDUFA12) leads to Drp1-dependent mosaic distribution of cytochrome c without any additional treatment (new Figure 2G-J). We believe that these new data markedly increase understanding of the mechanism leading to mitochondrial mosaicism and subsequent mitophagy. We hope that the reviewer agrees with our assessment.

Minor points
6. In Fig. 1C and D, the authors should quantify band intensity of Drp1 since its changes are modest, and a cytosolic loading control, such as GAPDH, should be included.

Done.

7. Fig. 4I is not described in the text.
We apologize for this problem. We carefully checked the current version of the manuscript for similar issues.
Dear Dr. Mariusz Karbowski,

Thank you for submitting your revised manuscript entitled "Drp1-dependent outer mitochondrial membrane severing and inner membrane ubiquitination mediate mitochondrial proteotoxicity-induced mitophagy in the absence of Parkin". You’ll see that the reviewers find that the changes strengthened the study and they are now supportive of publication. We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below) and pending revisions to address Rev#2’s remaining comments concerning the statistical evaluation of some data. Please include a response to these comments in a cover letter with your final files.

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

1) Titles, eTOC: Please consider the following revision suggestions aimed at increasing the accessibility of the work for a broad audience and non-experts.

- Title suggestions to make the advance clearer, more concise, and more accessible to a broad audience:

Parkin-independent mitophagy via Drp1-mediated outer membrane severing and inner membrane ubiquitination

Parkin-independent, Drp1-mediated piecemeal turnover of mitochondria upon proteotoxicity

- Running title (50 characters max, including spaces, but we can accommodate a small extension as such): Mechanism of Parkin-independent Drp1-mediated mitophagy

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Please include a summary statement on the title page of the resubmission. It should start with "First author name(s) et al..." to match our preferred style.

**please revise to make sure the statement is written in the 3rd person**

2) JCB Articles can have up to 10 main and 5 supplemental figures. While we could have one or two
more supplemental figures if needed, could you please try to combine some of the supplemental
data and/or move some to the main figures? Each figure can span up to 1 entire page as long as all
panels fit on the page. Thank you in advance for your efforts.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset
magnifications. Please add scale bars to S1 (all, including magnifications), S2 all (including mags), S7
all, S10 all, 7KFGH

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly
described in the figure legend. The number of independent data points (n) represented in a graph
must be indicated in the legend. Statistical methods should be explained in full in the materials and
methods. For figures presenting pooled data the statistical measure should be defined in the figure
legends.
- Please double check all figure legends for pooled data to include definitions of n

5) 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.
- For all cell lines, vectors, constructs/cDNAs, etc. - all genetic material: please include database /
    vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic
    features *even if described in other published work or gifted to you by other investigators*
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- Sequences should be provided for all oligos: primers, si/shRNA, gRNAs, etc.
- 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 reconstructions, surface or volume
    rendering, gamma adjustments, etc.).

6) A summary paragraph of all supplemental material should appear at the end of the Materials and
methods section.
- please include one brief descriptive sentence per item.

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Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Thomas Langer, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

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

The authors provide a number of new experiments that provide interesting insight into this newly discovered pathway. In sum, the authors addressed my concerns.

Reviewer #2 (Comments to the Authors (Required)):
In their revised version, the authors addressed most of the concerns. I am still not fully satisfied with their answer to the question whether there is a relationship between "high" cytochrome c mitochondria and OMM sewering/IMM ubiquitination. The authors stated in their rebuttal letter that they "performed the statistical analyses as suggested". This applies for data shown in Fig. 1D, but not for data shown in Fig. 1C. In Fig. 1C the experiment has been performed three times with 150 cell/condition each. For using ANOVA a Gaussian distribution of the data is a prerequisite; this cannot be addressed with 3 biological replicates only. Also, it is not possible to mix up technical (150 cell/condition) with biological replicates (experiment performed three times). Here, a Kruskal-Wallis plus post-hoc test would be the appropriate statistical test. This also applies to Fig. 2K-P and all other experiments with biological replicates N=3-4.

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

The authors have addressed most of my original concerns and greatly substantiated their work during the revision. I now strongly support publishing this very interesting paper, which provides many novel findings, in JCB.