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Parkin inhibits BAK and BAX apoptotic function by distinct mechanisms during mitophagy

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Transaction Report:

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

1st Editorial Decision 13 July 2018

Thank you for submitting your manuscript on Parkin-mediated BAK ubiquitination following mitophagic stress to The EMBO Journal. We have now received three referee reports on your study, which are enclosed below for your information.

As you can see, the referees concurred with us on the overall interest of your findings. However, they also raise several critical points that need to be addressed before they can support publication at The EMBO Journal. In particular, referee #2 and #3 are concerned that the study fails to address the physiological relevance of Parkin-mediated BAK ubiquitination. Also, referee #3 requests you to test the effects of disease-associated parkin mutants on BAK ubiquitination and the type of ubiquitin linkage on BAK. In addition, referee #1 and #3 ask you to discuss in deep the conflicting literature on BAK proteasomal degradation. Finally, all the reviewers stress the lack of appropriate quantification for all the experiments.

Addressing these issues through decisive additional data as suggested by the referees would be essential to warrant publication in The EMBO Journal. Given the overall interest of your study, I would thus like to invite you to revise the manuscript in response to the referee reports. Please note that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the

conceptual advance presented by your study. However, please contact me as soon as possible upon publication of any related work in order to discuss how to proceed.

Thank you again for the opportunity to consider this work for publication, and please feel free to contact me with any questions about submission of the revised manuscript to The EMBO Journal. I look forward to your revision.

REFEREE REPORTS

Referee #1:

Parkin is a cytoprotective molecule that functions to prevent spillage of mitochondrial contents upon mitochondrial damage/depolarisation, by initiating the orderly demise of the damaged mitochondrial section via mitophagy. In highly damaged mitochondria, or in cells receiving other forms of cell-death cues, pores in the outer mitochondrial membrane release cytochrome c to activate apoptosis. This process is mediated by members of the Bcl family of proteins, and eventually executed by pore-forming proteins BAX and BAK. Multiple mechanisms of how Parkin interacts with BAX/BAK mediated have been proposed. The exciting manuscript by Bernadini et al illuminates new mechanisms of how Parkin limits BAX/BAK mediated apoptosis.

The manuscript is very convincing and molecularly sound, and while the authors are limited by the difficulty to study endogenous processes and basal mitophagy for the lack of tools, there is little doubt that the identified mechanism would be physiologically relevant. The next step, a KI mouse mutating the relevant BAK residue, could be interesting but is outside the scope of this manuscript.

From the ubiquitin angle, some of the experiments are spectacular. It is rare that single ubiquitination sites are found functionally relevant, and then rigorously tested in vitro and ex vivo to show a mechanistic effect.

My comments are mostly minor, relating to figure/experiments and to the text. I support publication once these changes have been made.

Experimental comments
Fig 1A is hard to follow, and I am not sure what I am looking at. The contrast is unclear and while there seem to be some effects with Parkin overexpression, it is not clear what the authors study. I suggest that the data is in some form quantified and better explained.

In Fig 1B, the GAPDH blot should not be cut like this, and the upper band explained.

In Fig 1C, full membrane should be shown - Ub smears are often most obvious at high molecular weight. Another experiment could be to incubate with USP2 to see if any BAX is recovered in the TUBE pull down to rule out that the Ab fails to detect ubiquitinated BAX. How many Lys does BAX have?

What I am missing in Fig 1 is an explanatory Fig that encapsulates the question that this manuscript addresses, as a guide to the reader.

The Ubiquitin Fig. 2 is really very nice and clear. With only 2 Lys residues in BAX, it was maybe unfortunate that only one was mutated (see comment on structure figs below).

Many studies revealed Parkin substrates and sites after A/O, in HeLa cells, e.g. Saraf 2012, Ordureau 2018. Has K113 been identified in these studies? Why has it not, is there an obvious explanation ? (Eg peptide gets too long after modification, low abundance etc). This should be commented on.

The structure Fig in 2D is underwhelming. The groove is not clear, nor is it clear where / how BAK dimerises. The two colours have too little contrast. The relation fo the membrane and where the second site is located should be shown. This needs to be clarified with further Figures/panels.

Fig. 4C - the axis is labelled with liposome release - what is released? Rephrase for clarity.

Fig 5 - How many Cys residues does BAK have and were mutated? Should be mentioned in the text. Could be illustrated in an alignment / structure fig in EV.

What happens when you add Ub G76C to purified mitochondria with and without CuPhe? A Ub blot should be shown. Clearly it works and serves the mechanistic purpose, so no concerns, but it would be interesting to see the background. If this experiment went through rounds of optimisation, this could be interesting to include a bit more in detail in the methods. As such, the experiment is a really nice way to study site-specific ubiquitination, and will be useful for ubiquitin community.

Comments on text

Page numbers! Line numbers!

Introduction
P2 : two recent reviews by Harper (NRMCB) and Youle (Curr Biol) should be cited with Padman.
P3 : refs need checking.
Ia) for Ser65 phosphorylation include Wauer, EMBOJ 2015
Ib) that binding of pUb leads to release of the REP is not correct. Rephrase.
II) the sentence : "binding of phosphoUb is required for parkin activation ..." cites 2 papers before phosphoUb was discovered (Chaugule & Trempe) rephrase/expand or delete.
III) the fact that pUb binding enables Parkin phosphorylation was shown in structural work by several groups (Kazlauskaite (EMBO Rep), Sauve (EMBO J), Kumar (EMBO J), Wauer (Nature)) and not already in 2012. Rephrase.
IV) we have just recently learned how pParkin is activated (Gladkova in press, also bioRXiv) - could be cited.
V) adaptor recruitment : cite Heo, Ordureau Mol Cell, and OPTN papers from Dikic and Holzbaur.
VI) not sure about the comment on 'slower kinetics' - there is just less Parkin present in these cells. Kinetics seems not the right word. It was also interesting in that papers that some sites seem to change between systems. See comment above on further discussion of these data.

P4 bottom, sentence starting "Among..." needs references.
P5 paragraph on PD seems out of place.

Results
P6: this part is kept very vague as a clear mechanism is not presented. The idea that BAX recruitment may be blocked through VDAC-ubiquitination is attractive. The conclusion that this is indirect is fine.

One issue relates to the mechanism of VDAC loss - to what extend is what Ordureau see (2018) proteasomal loss (little/none according to their experiments) and what is loss by mitophagy? The last sentences in this section should take this into account.

P7/8 top - same as previous comment - loss of these proteins is and should be by mitophagy not proteasomal degradation.

P8: explain TUBEs at first mention further above
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P13 -uibiquitinated

P16 the paper is mostly about BAK yet the proposed clinical angle is on BAX. Consider modulating BAK ubiquitination as a mechanism of protection, eg by limiting its DUB.

Here, USP30 could finally be mentioned. The group of Urbe/Clague have shown that knockdown of USP30 improved the cytotoxic effect of Abt737 (Liang, EMBO Rep 2015). This seems very relevant here and should be mentioned/cited.

Referee #2:

Parkin is a neuroprotective E3 ubiquitin ligase associated with autosomal recessive Parkinsonism. Protective functions of Parkin include removal of damaged mitochondria by mitophagy and prevention of apoptotic cell death by several mechanisms. In their study, Bernardini and coworkers reported another anti-apoptotic function of Parkin. They observed that Parkin can directly ubiquitinate BAK at a conserved lysine residue (K113) on the periphery of its hydrophobic groove. This ubiquitination event interferes with the interaction of activating BH3-only proteins and with BAK homo-dimerization that ultimately is required for mitochondrial outer membrane permeabilization. The authors speculate that inhibition of BAK activation by Parkin prevents errant apoptosis and facilitates mitophagy.

Overall, the study addresses an interesting aspect of apoptosis regulation. Whereas the data on BAK ubiquitination and its impact on activation, oligomerization and membrane pore formation are convincing, the data on Parkin are weak and mostly indirect. Unfortunately, the study lacks evidence to support a role of endogenous Parkin in the ubiquitination of BAK. Experiments were performed with overexpressed HA-Parkin in HeLa cells combined with anisomycin A and oligomycin treatment that might have Parkin-independent effects. If BAK is ubiquitinated only after Parkin overexpression plus anisomycin A/oligomycin treatment (as suggested by Figure 5E), I am concerned about the relevance of this effect.

Major points:
1. Throughout the manuscript there is no quantification of Western blot data. Although most experiments were obviously performed in triplicate, no statistics are presented that would allow an evaluation of reproducibility and/or variability of the data presented.

2. Figure 1A:
These images are not convincing. Provide quantification or perform other state-of-the-art apoptosis assays.

3. Figure 1B:
Quantification of these effects from at least three independent experiments is missing.

4. Figure 1C:
Compare to cells not overexpressing Parkin to test for Parkin-independent effects of anisomycin A/oligomycin.

5. Figure 2:
Quantifications are missing.

6. Figure 3A:
Do you observe PINK1 stabilization under these conditions? Show PINK1 expression levels. Demonstrate an effect of endogenous Parkin by using cell lines silenced for Parkin expression or primary cells from Parkin knockout mice. Comparing HeLa cells +/- Parkin overexpression is not an adequate model to prove a role for endogenous Parkin.

7. Figure 3B:
There seems to be no difference in the levels of ubiquitinated BAK between WT and WT-HA-Parkin cells (blot 1 and 2, lanes 10 and 12), whereas there is a difference in the ubiquitination of Mfn2. What is the rationale and evidence that Parkin is a relevant ligase for the ubiquitination of BAK? Is Parkin interacting with BAK under apoptotic conditions? Are there any supportive data from mass spectrometry?

8. Figure 4 D:
Since the effects seem to be quite variable, quantifications of independent experiments are required.

9. Figure 5C:
The statistics display a student's t-test with n = 3. An n of 3 does not allow to test for Gaussian distribution that is a prerequisite for a t-test.

10. Figure 5E:
It would be interesting here to include BAX immunoblotting for comparison.

Referee #3:

This is an interesting report by Bernadini and colleagues who demonstrated that the apoptotic function of BAK and BAX in cells treated with antimycin and oligomycin (AO) is suppressed in the presence of Parkin. The authors showed that following AO-induced mitochondrial depolarization, Parkin ubiquitinates BAK at Lysine 113 that impairs its activation and oligomerization, and thereby its apoptotic function. They further showed that ubiquitinated BAK Impairs its ability to permeabilize membranes, thus potentially limiting mitochondrial membrane permeabilization. Taken together, these findings suggest a mechanism whereby cell death is restricted in the presence of Parkin during Parkin/PINK1-mediated mitophagy. Although an attractive proposition, I have a number of comments below that need to be clarified/addressed by the authors:

1. Fig. 1A: It is difficult to interpret the data. Are these live or detached cells? If it is the latter, Parkin expression seems to promote cell death in the absence of AO treatment. If it is the former, Parkin expression seems to promote cell death in the presence of AO treatment. Quantification of data is clearly necessary here.

2. Fig. 1B: It would be informative to show alongside this the scenario in cells not expressing HA-parkin.

3. Ubiquitinated BAK is apparently stable over the time course that the authors have examined. It would be informative to determine if these are K63-linked (or other) ubiquitin chains, which Parkin is known to mediate. Also informative to examine whether disease-associated Parkin mutants compromise BAK ubiquitination.

4. Is BAK a target of Parkin-mediated ubiquitination in vitro?

5. Related to the above, the authors stated that "modified BAK was actually reduced upon MG132 treatment". I assumed that they are referring to the results on Fig. 2E. Curiously, the reduction was not seen in EV1B.

6. Fig. 4D: The dimerization of BAK-Ub appears to be delayed rather than impaired. Curiously, in the presence of 25 mM TCEP, the reduction of BAK-Ub dimers and oligomers remains evident.

7. In the article by Chan NC et al. (2011), BAK is seen clearly degraded with time after CCCP treatment. In addition, Holloway A, et al. (2014) reported that Lysine 113 of BAK is critical for its proteasomal degradation. These reports seem to contradict the current findings by the authors.

8. AO treatment is known to trigger a plethora of events other than mitophagy. The authors may need to be cautious in interpreting that Parkin-mediated effects on BAK/BAX is associated with mitophagy.

1st Revision - authors' response 8 October 2018

We are pleased that the reviewers found our manuscript interesting and thank them for their positive feedback and suggestions. We have addressed each point either experimentally or otherwise as detailed in the point-by-point rebuttal below. We feel that these suggestions contribute to a significantly strengthened revised manuscript.

Referee #1:

Parkin is a cytoprotective molecule that functions to prevent spillage of mitochondrial contents upon mitochondrial damage/depolarisation, by initiating the orderly demise of the damaged mitochondrial section via mitophagy. In highly damaged mitochondria, or in cells receiving other forms of cell-death cues, pores in the outer mitochondrial membrane release cytochrome c to activate apoptosis. This process is mediated by members of the Bcl family of proteins, and eventually executed by pore-forming proteins BAX and BAK. Multiple mechanisms of how Parkin interacts with BAX/BAK mediated have been proposed. The exciting manuscript by Bernadini et al illuminates new mechanisms of how Parkin limits BAX/BAK mediated apoptosis.

The manuscript is very convincing and molecularly sound, and while the authors are limited by the difficulty to study endogenous processes and basal mitophagy for the lack of tools, there is little doubt that the identified mechanism would be physiologically relevant. The next step, a KI mouse mutating the relevant BAK residue, could be interesting but is outside the scope of this manuscript.

From the ubiquitin angle, some of the experiments are spectacular. It is rare that single ubiquitination sites are found functionally relevant, and then rigorously tested in vitro and ex vivo to show a mechanistic effect.

My comments are mostly minor, relating to figure/experiments and to the text. I support publication once these changes have been made.

Experimental comments
Figure 1A is hard to follow, and I am not sure what I am looking at. The contrast is unclear and while there seem to be some effects with Parkin overexpression, it is not clear what the authors study. I suggest that the data is in some form quantified and better explained.
We apologise that the images did not clearly show the protective effect of activating Parkin in BAX-mediated apoptosis. We now instead show new data to quantify the cell death by LDH assay confirming that Parkin inhibits BAX-mediated cell death in response to BH3 mimetics (new Figure 1B).

In Figure 1B, the GAPDH blot should not be cut like this, and the upper band explained. The GAPDH blot was a reprobe of the TIMM44 blot (the upper band being TIMM44 solely in the membrane fractions). We have now amended the blot image to show both bands as suggested (Figure 1C in revised manuscript).

In Figure 1C, full membrane should be shown - Ub smears are often most obvious at high molecular weight. Another experiment could be to incubate with USP2 to see if any BAX is recovered in the TUBE pull down to rule out that the Ab fails to detect ubiquitinated BAX. How many Lys does BAX have? Human BAX has 8 lysines. We now show the full blot up to >150 kDa and still see no evidence of poly-ubiquitinated BAX. We failed to detect ubiquitinated BAX with a monoclonal antibody 3C10 (Iyer et al, Nature Comms 2016, PMID:27217060) that recognises an epitope within amino acids 33-55 that does not contain a lysine (DRAGRMGGEAPELALDPVPQDAS). We also failed to detect ubiquitinated BAX with a monoclonal antibody (49F9, Czabotar et al Cell 2013, PMID:23374347) that recognizes a different epitope (amino acids 11-25), although this epitope incorporates a lysine (K21). In addition, we failed to detect significant loss of non-ubiquitinated BAX upon activation of Parkin that might suggest that a major population of BAX was becoming ubiquitinated. Although we cannot exclude that a minor population of BAX is ubiquitinated that is detectable by mass spectrometry (Sarraf et al Nature), the marked effect on BAX-mediated cell death suggests an indirect mechanism.

What I am missing in Figure 1 is an explanatory Figure that encapsulates the question that this manuscript addresses, as a guide to the reader. Thank you for this suggestion. We have inserted a schematic as suggested (new Figure 1A).

The Ubiquitin Figure 2 is really very nice and clear. With only 2 Lys residues in BAX, it was maybe unfortunate that only one was mutated (see comment on structure figs below).

Many studies revealed Parkin substrates and sites after A/O, in HeLa cells, e.g. Saraf 2012, Ordureau 2018. Has K113 been identified in these studies? Why has it not, is there an obvious explanation ? (Eg peptide gets too long after modification, low abundance etc). This should be commented on. This is an interesting question as we clearly see significant ubiquitination of BAK in different cell types including with endogenous Parkin (new Figure 3C). We believe that the major reason is, as suggested by the reviewer, the length of the ubiquitinated BAK peptide. Ubiquitination of BAK ablates K113 as a tryptic site, thus resulting in a large 39+2 amino acid tryptic peptide (Y89-R127+ GG) that would complicate identification using standard LC-MS/MS workflows. Additionally, proteasome inhibitors used in some di-Gly proteomics analyses (inc. Sarraf et al) reduce Ub-BAK in HeLa cells rather than stabilise it (Figure 2B and 2D), and may also contribute to the inability to detect Ub-BAK. We have now included discussion of this in the manuscript (Discussion text, p18).

The structure Figure in 2D is underwhelming. The groove is not clear, nor is it clear where / how BAK dimerises. The two colours have too little contrast. The relation fo the membrane and where the second site is located should be shown. This needs to be clarified with further Figures/panels. We have now amended this figure to improve the contrast to show the binding groove and how ubiquitin modification at the surface could impact on BAK activation by BH3-only proteins and BAK homodimer formation. We also show the structure of the modelled C-terminal TM anchor to highlight the K210 localisation in the IMS. Please note that although evidence indicates that the BAK TM domain spans the MOM (Iyer et al CDD, 2014), the orientation of the soluble portion of BAK with respect to the MOM is hypothetical.

Figure 4C - the axis is labelled with liposome release - what is released? Rephrase for clarity. Corrected.

Figure 5 - How many Cys residues does BAK have and were mutated? Should be mentioned in the text. Could be illustrated in an alignment / structure Figure in EV. We apologise for this oversight. BAK has two endogenous cysteines (C14 and C166). We have previously shown that mutation of both Cys does not significantly affect BAK apoptotic activity (Dewson et al Mol Cell 2008, PMID:18471982; Westphal et al PNAS, PMID:25228770).

What happens when you add Ub G76C to purified mitochondria with and without CuPhe? A Ub blot should be shown. Clearly it works and serves the mechanistic purpose, so no concerns, but it would be interesting to see the background. If this experiment went through rounds of optimisation, this could be interesting to include a bit more in detail in the methods. As such, the experiment is a really nice way to study site-specific ubiquitination, and will be useful for ubiquitin community. We thank the reviewer for this recommendation. We now show the accompanying ubiquitin blot in Figure 6B. We also now show a control experiment that the addition of Ub-G76C alone to mitochondria (neither with or without CuPhe) causes cytochrome *c* release (new Figure EV5A).

Comments on text

Page numbers! Line numbers! Now added.

Introduction
P2 : two recent reviews by Harper (NRMCB) and Youle (Curr Biol) should be cited with Padman. Reviews now added as recommended.
P3 : refs need checking. Checked and corrected where required.
Ia) for Ser65 phosphorylation include Wauer, EMBOJ 2015. Added.
Ib) that binding of pUb leads to release of the REP is not correct. Rephrase. Corrected.
II) the sentence : "binding of phosphoUb is required for parkin activation ..." cites 2 papers before

phosphoUb was discovered (Chaugule & Trempe) rephrase/expand or delete. Corrected.
III) the fact that pUb binding enables Parkin phosphorylation was shown in structural work by several groups (Kazlauskaite (EMBO Rep), Sauve (EMBO J), Kumar (EMBO J), Wauer (Nature)) and not already in 2012. Rephrase. Corrected.
IV) we have just recently learned how pParkin is activated (Gladkova in press, also bioRXiv) - could be cited. Gladkova paper is now cited.
V) adaptor recruitment : cite Heo, Ordureau Mol Cell, and OPTN papers from Dikic and Holzbaur. Papers now cited.
VI) not sure about the comment on 'slower kinetics' - there is just less Parkin present in these cells. Kinetics seems not the right word. It was also interesting in that papers that some sites seem to change between systems. See comment above on further discussion of these data. We have now amended this text to, "…a recent study shows that endogenous Parkin ubiquitinates similar substrates in neurons, although some site and temporal differences were observed (Ordureau et al 2018)."

P4 bottom, sentence starting "Among..." needs references. Sarraf et al, Nature has now been added here. This paper shows proteomics analysis to identify that various BCL-2 proteins are ubiquitinated following induction of mitophagy. We discuss specific BCL-2 family members (and cite the relevant studies) in the subsequent sentences.
P5 paragraph on PD seems out of place. We have moved the discussion of the importance of Parkin function in PD to the beginning of the Introduction to place our study in context.

Results
P6: this part is kept very vague as a clear mechanism is not presented. The idea that BAX recruitment may be blocked through VDAC-ubiquitination is attractive. The conclusion that this is indirect is fine. One issue relates to the mechanism of VDAC loss - to what extend is what Ordureau see (2018) proteasomal loss (little/none according to their experiments) and what is loss by mitophagy? The last sentences in this section should take this into account. We agree that the recent study by the Harper lab suggests that Parkin does not significantly promote proteasomal degradation of MOM proteins such as VDAC2. We have now modified the wording of our conclusions to indicate that VDAC2 ubiquitination may preclude interaction of BAX with VDAC2 (Results text, p8).

P7/8 top - same as previous comment - loss of these proteins is and should be by mitophagy not proteasomal degradation. Amended.

P8: explain TUBEs at first mention further above. The data (Figure EV2 in the revised manuscript) was actually from cell lysates rather than a TUBE pull down. We apologise for this error that we have now corrected.
P8: substrate of in SHSY5Y cells - delete of. Corrected.
P8: "BAK ubiquitination was stable over time" - BAK protein or BAK Ub or both? Rephrase. Amended.

P9: it is speculation that Lys210 cannot be targeted. I could imagine that this eg prevents BAK insertion into membrane? Our data show that mutation of K113R alone is sufficient to prevent detectable BAK ubiquitination suggesting that if K210 is ubiquitinated it is only a very minor population that we barely detect by immunoblotting. That ubiquitination of K210 could impair mitochondrial targeting is an interesting suggestion. However, we note that our experiments involving UBA enrichment were performed on total cell lysates, not just mitochondrial fractions, so we would capture both the mitochondrial and mis-localised cytosolic populations. In addition, we have previously shown that BAK is constitutively anchored in the mitochondrial outer membrane via its C-terminal transmembrane domain with its extreme C-terminus in the intermembrane space (Iyer et al, CDD 2015, PMID:25744027), and therefore inaccessible to Parkin-mediated ubiquitination. We cannot exclude that following mitochondrial outer membrane permeabilisation during apoptosis that this C-terminal lysine can be ubiquitinated, and that this may account for some of the modified BAK dimers (Parkin-independent) seen in Figure 4B, and we have now discussed this more thoroughly in the text (p12). Nevertheless, our data clearly show that K113 is the predominant substrate lysine following activation of Parkin.

P9: HeLa cells were engineered - be more explicit - engineered these days means crispr'd... Corrected.

P10: conformation change -> conformational change. Corrected.

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P11: Bid experiments should be more clearly explained. The BID experiments are now more clearly described.
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P13 -uibiquitinated Corrected.

P16 the paper is mostly about BAK yet the proposed clinical angle is on BAX. Consider modulating BAK ubiquitination as a mechanism of protection, eg by limiting its DUB. We have now mentioned the potential to inhibit DUBs to inhibit BAK activity (Discussion, p20).

Here, USP30 could finally be mentioned. The group of Urbe/Clague have shown that knockdown of USP30 improved the cytotoxic effect of Abt737 (Liang, EMBO Rep 2015). This seems very relevant here and should be mentioned/cited. We agree that the work of the Urbe lab on USP30 is highly relevant to our study, and we have now cited it (Discussion text, p17).

Referee #2:

Parkin is a neuroprotective E3 ubiquitin ligase associated with autosomal recessive Parkinsonism. Protective functions of Parkin include removal of damaged mitochondria by mitophagy and prevention of apoptotic cell death by several mechanisms. In their study, Bernardini and coworkers reported another anti-apoptotic function of Parkin. They observed that Parkin can directly ubiquitinate BAK at a conserved lysine residue (K113) on the periphery of its hydrophobic groove. This ubiquitination event interferes with the interaction of activating BH3-only proteins and with BAK homo-dimerization that ultimately is required for mitochondrial outer membrane permeabilization. The authors speculate that inhibition of BAK activation by Parkin prevents errant apoptosis and facilitates mitophagy.

Overall, the study addresses an interesting aspect of apoptosis regulation. Whereas the data on BAK ubiquitination and its impact on activation, oligomerization and membrane pore formation are convincing, the data on Parkin are weak and mostly indirect. Unfortunately, the study lacks evidence to support a role of endogenous Parkin in the ubiquitination of BAK. Experiments were performed with overexpressed HA-Parkin in HeLa cells combined with anisomycin A and oligomycin treatment that might have Parkin-independent effects. If BAK is ubiquitinated only after Parkin overexpression plus anisomycin A/oligomycin treatment (as suggested by Figure 5E), I am concerned about the relevance of this effect.

Major points:
1. Throughout the manuscript there is no quantification of Western blot data. Although most experiments were obviously performed in triplicate, no statistics are presented that would allow an evaluation of reproducibility and/or variability of the data presented. As suggested, we have now included quantitation of immunoblots from independent experiments to support reproducibility (new Figures 1C, 1D, 2B, 3A, 3D, 5D, 6D and 6E).

2. Figure 1A:
These images are not convincing. Provide quantification or perform other state-of-the-art apoptosis assays. We apologise for the lack of clarity in the presented images. We have now replaced this data with quantitation of cell death using LDH assay (new Figure 1B), to confirm that AO-treatment of Parkin expressing cells limits apoptosis induced by BH3-mimetics, consistent with the blockade in

cytochrome c release.

3. Figure 1B:
Quantification of these effects from at least three independent experiments is missing. Quantitation of non-ubiquitinated proteins and cytochrome c release is now included (new Figure 1C and 1D) to show that in repeated experiments BAX levels at mitochondria and subsequent cytochrome c release are reduced following AO-treatment of Parkin-expressing cells.

4. Figure 1C:
Compare to cells not overexpressing Parkin to test for Parkin-independent effects of anisomycin A/oligomycin. We now show new data with Parkin-null cells confirming that the inhibition of cytochrome c release following AO treatment is Parkin-dependent (new Figure 1D). AO-treatment did reduce mitochondrial BAX levels, although not to the extent as in Parkin-expressing cells.

5. Figure 2:
Quantifications are missing. Quantifications of the blots in Figure 2B have now been added to show that AO induced BAK ubiquitination is reduced upon MG132 treatment. Figure 2A, 2C and 2D present descriptive data showing clear changes in BAK ubiquitination and so we feel that quantitation of these data would not be informative.

6. Figure 3A:
Do you observe PINK1 stabilization under these conditions? Show PINK1 expression levels. Demonstrate an effect of endogenous Parkin by using cell lines silenced for Parkin expression or primary cells from Parkin knockout mice. Comparing HeLa cells +/- Parkin overexpression is not an adequate model to prove a role for endogenous Parkin. We thank the reviewer for this interesting question. Our data indicate that BAK-mediated mitochondrial damage during BH3 mimetic-induced apoptosis promotes Parkin activity. Although, we did not observe PINK1 stabilisation on mitochondria following BH3-mimetic induced apoptosis (new Figure EV4A), the induced Parkin activity was clearly PINK1 dependent as ubiquitination of Mfn2 was not detected in HeLa cells engineered to lack PINK1 using CRISPR/Cas9 gene editing (new Figure 4C and 4D, new Figure EV4B). To our knowledge, this is the first evidence that mitochondrial damage during apoptosis can promote PINK1-dependent Parkin activity.

We also now show that BAK ubiquitination, albeit limited, is detectable in SH-SY5Y neuroblastoma cells that express endogenous Parkin (new Figure 3B and C). CRISPR-mediated deletion of Parkin confirmed that BAK ubiquitination in these cells in response to AO is Parkin-dependent. HeLa cells overexpressing Parkin is the standard model for Parkin-mediated mitophagy and, as noted by reviewer #1, studying the endogenous process of Parkin-mediated mitophagy is challenging. Few Parkin substrates have been confirmed with endogenous Parkin and detecting marked ubiquitination of even a canonical substrates such as VDAC1 (termed “tier1” substrates by Ordureau et al, Mol Cell 2018, PMID:29656925) with endogenous Parkin is difficult. That we detect BAK ubiquitination mediated by endogenous Parkin strongly supports its physiological relevance as an important substrate.

7. Figure 3B:
There seems to be no difference in the levels of ubiquitinated BAK between WT and WT-HA-Parkin cells (blot 1 and 2, lanes 10 and 12), whereas there is a difference in the ubiquitination of Mfn2. What is the rationale and evidence that Parkin is a relevant ligase for the ubiquitination of BAK? Is Parkin interacting with BAK under apoptotic conditions? Are there any supportive data from mass spectrometry? This is a very relevant point. Our data in Figure 3 (Figure 4 in the revised manuscript) indicate that after BAK dimers have been triggered following induction of apoptosis (see Figure 4A, lanes 2 and 4), the subsequent mitochondrial outer membrane permeabilisation can promote PINK1-dependent Parkin activity leading to Mfn2 ubiquitination (new Figure 4B-D). Importantly, once BAK is dimerised following BH3 mimetic treatment, even though Parkin is activated, BAK ubiquitination by Parkin is impaired (Figure 4A, compare lanes 3 and 4), suggesting that K113 in the BAK dimerisation site is inaccessible. Hence, we propose that the low levels of ubiquitinated BAK (Figure 4A and 4B in revised manuscript) after BH3 mimetic treatment are Parkin-independent. This may also involve ubiquitination of K210 in the extreme C-terminus of BAK, which, due to its location in the mitochondrial intermembrane space, only becomes accessible following mitochondrial outer membrane permeabilisation. We have now expanded the text and also

added a schematic (new Figure 4E) to explain this data more thoroughly.

8. Figure 4 D:
Since the effects seem to be quite variable, quantifications of independent experiments are required. Quantification of the data in Figure 4D (Figure 5D in the revised manuscript) from independent experiments is now shown.

9. Figure 5C:
The statistics display a student's t-test with n = 3. An n of 3 does not allow to test for Gaussian distribution that is a prerequisite for a t-test. We thank the reviewer for this comment. We used the Student's t-test under the assumption of normal distribution, but we agree that 3 independent experiments are insufficient to test this and for the calculation of a robust P value by Student's t-test (or any statistical test; Halsey et al, Nature Methods 2015, PMID:25719825). In accordance with EMBO J guidelines for data analyses of biological experiments where the number of independent repeats is limited, we have removed this statistical analysis in favour of showing the individual data points from independent experiments (with SD), whilst supporting our conclusions using orthogonal assays.

10. Figure 5E:
It would be interesting here to include BAX immunoblotting for comparison. These experiments involve mitochondria isolated from wild-type HeLa cells in which endogenous BAX is predominantly localised to the cytosol with little/no mitochondrial BAX (Arnoult et al PNAS 2004, PMID:15148411; Tsuruta et al JBC 2002, PMID:11842081; Zhou and Chang JCS 2008, PMID:18544634), hence supporting the rationale why BAK is the mitochondrial target of Parkin to limit apoptosis following mitochondrial damage.

Referee #3:

This is an interesting report by Bernadini and colleagues who demonstrated that the apoptotic function of BAK and BAX in cells treated with antimycin and oligomycin (AO) is suppressed in the presence of Parkin. The authors showed that following AO-induced mitochondrial depolarization, Parkin ubiquitinates BAK at Lysine 113 that impairs its activation and oligomerization, and thereby its apoptotic function. They further showed that ubiquitinated BAK Impairs its ability to permeabilize membranes, thus potentially limiting mitochondrial membrane permeabilization. Taken together, these findings suggest a mechanism whereby cell death is restricted in the presence of Parkin during Parkin/PINK1-mediated mitophagy. Although an attractive proposition, I have a number of comments below that need to be clarified/addressed by the authors:

1. Figure 1A: It is difficult to interpret the data. Are these live or detached cells? If it is the latter, Parkin expression seems to promote cell death in the absence of AO treatment. If it is the former, Parkin expression seems to promote cell death in the presence of AO treatment. Quantification of data is clearly necessary here. Please also see responses above. We apologise that the images were not sufficiently clear. We now include a quantitative measure of cell death using LDH assay (new Figure 1B) to show that AO-treatment inhibits BAX-mediated cell death specifically in Parkin-expressing cells.

2. Figure 1B: It would be informative to show alongside this the scenario in cells not expressing HA-parkin. Please also response to reviewer #2. Consistent with the death assay data by LDH assay (new Figure 1B), we now show evidence with quantified immunoblots from 3 independent experiments that AO-treatment of HeLa cells not expressing Parkin does not impair cytochrome c release induced by BH3-mimetics (new Figure 1D).

3. Ubiquitinated BAK is apparently stable over the time course that the authors have examined. It would be informative to determine if these are K63-linked (or other) ubiquitin chains, which Parkin is known to mediate. Also informative to examine whether disease-associated Parkin mutants compromise BAK ubiquitination. We note that the predominant species following mitochondrial damage is mono-ubiquitinated BAK, consistent with its stability over time rather than leading to proteasomal degradation by K48-linked poly-ubiquitination. Our data that BAK is not targeted for degradation by the proteasome by K48-linkage is supported by a recent study indicating that Parkin

(including endogenous) does not promote significant proteasomal degradation of mitochondrial substrates. (Ordureau et al, Mol Cell 2018, PMID:29656925). In addition, using deubiquitinase assay (Hospenthal et al, Nature Protocols 2015), we now show that K11 is the predominant linkage on poly-ubiquitinated BAK (new Figure EV2D), consistent with its stability over time and the ubiquitination profile catalyzed by Parkin during mitophagy (Cunningham et al 2015, Nature Cell Biol, PMID:25621951).

As suggested by the reviewer we have now tested a number of PD-associated Parkin mutants. As expected, those mutants that had reduced capacity to ubiquitinate Mfn2 and VDAC1 also had reduced capacity to ubiquitinate BAK (new Figure 3D).

4. Is BAK a target of Parkin-mediated ubiquitination in vitro? We agree that these in vitro experiments would be of interest, however these experiments are extremely challenging. Firstly, producing active recombinant Parkin is not straight-forward. Parkin is auto-inhibited and not only requires phosphorylation (that can be mimicked by a phosphomimetic mutation at Ser65), but also needs to bind S65-phosphorylated ubiquitin. Moreover, full-length, wild-type recombinant BAK cannot be expressed due to its instability (Leshchiner et al PNAS 2013, PMID:23404709). Hence, any such experiments would have to be done on a mutated form of BAK. For these reasons, we have employed orthogonal cell-based assays showing that ubiquitination of full-length wild-type BAK occurs in various cell types (including endogenous) is triggered only in Parkin-expressing cells (either over-expressed or endogenous), and that PD-associated mutations in Parkin impair BAK ubiquitination to strongly support that BAK is a novel Parkin substrate.

5. Related to the above, the authors stated that "modified BAK was actually reduced upon MG132 treatment". I assumed that they are referring to the results on Figure 2E. Curiously, the reduction was not seen in EV1B. The effect of MG132 was less pronounced in EV1B (EV2B in the revised manuscript) as this is in MEFs rather than HeLa cells. However, the di-ubiquitinated form of BAK (highlighted by * in Figure EV2B) was still reduced in MEF. This is now indicated in the text (p9).

6. Figure 4D: The dimerization of BAK-Ub appears to be delayed rather than impaired. Curiously, in the presence of 25 mM TCEP, the reduction of BAK-Ub dimers and oligomers remains evident. The reviewer is correct that in our model membrane system, the conjugation of a single ubiquitin molecule to BAK, delayed, but did not completely block, its oligomerisation, and in light of this we have described the effect of ubiquitination as “impairing” activation/oligomerisation. Nevertheless, this attenuation of oligomerisation was sufficient to significantly reduce BAK apoptotic function on model membranes (Figure 5C in revised manuscript) and also on mitochondria (and Figure 6B and 6D in revised manuscript). In addition, conjugation of >1 ubiquitin molecule to BAK as is detected in cells would likely enhance this inhibitory effect on BAK oligomerisation.

TCEP treatment in general did reduce BAK activity on liposomes and we believe that this is due to interfering with the targeting of BAK-His to the nickel-NTA phospholipids. Importantly, however, while there was a clear defect in BAK-mediated liposome permeabilisation and oligomerisation when ubiquitin was conjugated to BAK in the absence of TCEP, there was minimal difference in the presence of TCEP.

7. In the article by Chan NC et al. (2011), BAK is seen clearly degraded with time after CCCP treatment. In addition, Holloway A, et al. (2014) reported that Lysine 113 of BAK is critical for its proteasomal degradation. These reports seem to contradict the current findings by the authors. Holloway et al reported BAK degradation in response to viral infection which likely involves a different E3 ubiquitin ligase and so a different ubiquitin linkage (presumably K48). Our data show that ubiquitinated BAK, i) is predominantly mono-ubiquitinated, ii) is relatively stable over time, and iii) is not stabilised upon proteasomal inhibition, support non-degradative linkage. In addition, we now show new data with a deubiquitinase assay that BAK is predominantly K11-linked by Parkin (new Figure EV2D). This non-degradative signal is also more consistent with the physiological role of Parkin in mitophagy (Ordureau et al, Mol Cell 2018, PMID:29656925) where proteasome-mediated degradation of mitochondrial substrates was limited in favour of Lys11 and Lys63 non-degradative ubiquitination (Cunningham et al 2015, Nature Cell Biol, PMID:25621951).

8. AO treatment is known to trigger a plethora of events other than mitophagy. The authors may need to be cautious in interpreting that Parkin-mediated effects on BAK/BAX is associated with mitophagy. We thank the reviewer for this suggestion. We now show that Parkin-mediated

ubiquitination of BAK also occurs following treatment with the mitochondrial uncoupling agent, CCCP, or a mtHSP90 inhibitor and Parkin activator, GTPP (Munch and Harper Nature, 2016 PMID:27350246; Fiesel et al Oncotarget 2017, PMID:29290944) (new Figure 3A), confirming that BAK is ubiquitinated in response to a variety of mitophagy stimulators. Of note, AO treatment in the absence of Parkin or upon expression of Parkin mutants does not affect cell death (new Figure 1B) or cytochrome c release (Figure 6E in revised manuscript).

2nd Editorial Decision 6 November 2018

Thank you for submitting a revised version of your manuscript. It has now been seen by the original referees whose comments are shown below.

As you will see they find that all criticisms have been sufficiently addressed and recommend the manuscript for publication. However, before we can officially accept your manuscript, there are a few editorial issues concerning text and figures that I would kindly ask you to address in a final revised version.

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

REFEREE REPORTS

Referee #1:

The authors have addressed my comments entirely, and added additional data, most notably showing endogenous BAK ubiquitination in a newly engineered cell system, in which without Parkin, BAK ubiquitination is strongly reduced. This adds BAK in my mind to the list of strongly validated Parkin substrates, and the explanation why it is poorly detected in the wealth of MS studies makes sense.

The new schematic figures are very helpful, figures are really very good. A recent paper from Gehring in NSMB should be cited alongside the Gladkova Nature paper.

I fully support publication of this important work.

Referee #2:

Most points raised by the reviewers were adequately addressed.

Referee #3:

This is a revised version of the original manuscript that also represents a significantly improved version of the report. Overall, the authors have addressed the majority of my comments to my satisfaction. The significance of the findings remains, i.e. the elucidation of a mechanism whereby cell death is restricted in the presence of Parkin during Parkin/PINK1-mediated mitophagy.

2nd Revision - authors' response 8 November 2018

Authors made requested editorial changes.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

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The data shown in figures should satisfy the following conditions:

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Each figure caption should contain the following information, for each panel where they are relevant:

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- an explicit mention of the biological and chemical entity(ies) that are being measured.
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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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5. For every figure, are statistical tests justified as appropriate?	Statistical tests were not appropriate for 3-4 independent biological replicates.
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7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All cell lines were routinely tested to confirm Mycoplasma negativity.

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