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# LUBAC assembles a ubiquitin signaling platform at mitochondria for signal amplification and transport of NF-κB to the nucleus

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Dear Konstanze,

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by two referees and their comments are provided below.

As you can see from the comments, the referees find the analysis interesting and insightful. The referees bring up constructive comments that would improve the study. I would therefore like to invite you submit a revised version should you be able to address the raised concerns. It would be helpful to discuss the revision plan and I am available to do so via email or a video call.

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: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Thank you for the opportunity to consider your work for publication. I look forward to discussing your revisions further with you.

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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I have attached a PDF with helpful tips on how to prepare the revised version.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (18th Oct 2022).

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, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

If you require more time to complete the revisions let me know as as I can grant an extension.

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Referee #1:

The authors report the identification of a signaling platform on mitochondria that contributes to TNF-induced NFkB activation. They observe recruitment of the LUBAC subunits HOIP and HOIL-1L to and the deposition of M1-linked ub chains at mitochondria in a manner partially dependent on PINK1 catalytic activity. The authors further report recruitment of the NFkB-controlling IKK complex to mitochondria, where it causes localized activation of NFkB. Subsequent translocation of NFkB into the nucleus appears partially dependent on nucleus-mitochondria contact sites.

The manuscript contains with no doubt interesting observations, in particular the identification on mitochondria of a LUBAC-dependent signaling platform for the enhancement of TNF signaling. However, the authors fail to relate their manuscript to previous publications reporting conceptually similar signaling platforms on cytosol-invading bacteria. Signaling platforms on bacteria also rely on LUBAC recruitment (Noad, Nat Immunol 2017) and are also antagonized by Otulin (van Wijk, Nat Immunol 2017). The authors similarly fail to cite the literature on the NFkB inducing activity of Parkin (for example Sha, Hum Mol Gen 2010).

Overall, the results presented here support the authors' conclusions. However, several issues need to be addressed to strengthen the manuscript.

1. Large parts of the manuscript rely on the isolation of mitochondria, for example the demonstration of LUBAC recruitment to and M1-deposition at mitochondria. However, the purity of the mitochondria preps is not carefully addressed and the possibility of other organelles contaminating the material needs to be ruled out. For example Fig1A, B, C, E. And also Fig5: It seems remarkable that p65/RelA itself would accumulate on mitochondria, as it normally is known to shuttle from the cytosol into the nucleus as a soluble protein.
2. I thought initially that the white / black bands in Western blots would correspond to the detection method, i.e. ECL vs fluorescence. However, in Fig1E even corresponding samples differ (e.g. HOIP, Sharpin, TIM23, TNFR1, GAPDH but not HOIL-1L). The issue is more than cosmetic - for example the absence of TNFR1 and GAPDH in the mitochondrial fraction, rather than confirming the absence of membrane and cytosole contamination, could well be a technical problem since the positive control (lysate) was clearly handled differently. In general terms, it is regrettable that blots in Fig1 are mosaic with mitochondria and lysates not on the same gel.
3. Fig1A: Why are there two HOIP bands in the mitochondria fraction? Since the two blots are separated, which of the two bands corresponds to the band in whole cell lysates?
4. Why is the Otulin band in the mitochondria fraction not sensitive to siRNA depletion?
5. Fig1F: I wasn't aware that mitochondria are constitutively and strongly positive for K48 ub chains. Again, how clean is the mitochondrial fraction?
6. Fig2A: The anti-PINK blot has many bands, two are claimed to represent PINK. It would be helpful if the authors would demonstrate the specificity of their anti-PINK antibody.
7. Fig2C: A positive control (for example CCCP treatment) for the mKeima experimental setup should be provided.
8. Fig2D: The authors demonstrate a ubiquitin smear in PINK IPs (2D). Is this smear M1 linked ubiquitin as claimed in the manuscript? Disappearance of the smear upon Otulin expression is consistent with, but does not prove the identify of, the M1 linkage type as the Otulin effect could be indirect.
9. Fig2F: TNF treatment does not cause enhanced M1 linked ubiquitylation of endogenous PINK, as would be expected from earlier(overexpression) experiments.
10. Page9: The authors wish to test '... whether PINK1 increases TNF-induced M1-ubiquitination at mitochondria by phosphorylating ubiquitin.' This question remains unresolved - the authors demonstrate an effect of PINK1 on M1-ub and on p-S65-Ub but they haven't shown the occurrence of pS65 in M1-linked ub chains.
11. Fig4E: A positive control for the activity of the dominant negative IkB is needed.
12. Fig5C: The authors report an effect of PINK1 on NFkB translocation. Confirmation by reporter gene activation is advisable. Similarly in Fig6B for the effect of Miro1/2 on NFkB translocation.
13. Is the TNF-induced relocalization of mitochondria towards the nucleus dependent on PINK1?
14. The authors propose that PINK1-dependent phosphorylation of ubiquitin stabilizes M1-linked chains. However, since the authors also report physical interaction between PINK1 and HOIP (FigS2), it seems equally possible that the PINK1-dependent enhancement of M1-linked ubiquitylation is due to its interaction with LUBAC and stimulation of its E3 activity. Assessing the role of PINK1 in Otulin ko cells could clarify the situation.

Referee #2:

Wu et al. investigate the role of mitochondria in the amplification of innate immune responses. They focus on NF-kB, whose persistent activation causes inflammation but also allows for the survival and functioning of neurons. A ey factor in this process is TNF, which acts as a neuroprotective cytokine and activates NF-kB. This requires the ubiquitination of NEMO by the linear ubiquitin chain assembly complex (LUBAC), whose catalytic subunit is HOIP. NEMO activates IKK, which then cause dissociation and degradation of the NF-kB inhibitor IkB. Parkin supports this pathway, while OTULIN acts as a deubiquitinase. Given this link, the authors propose a role of mitochondria in NF-kB activation.
In their MS-based data, the authors demonstrate that TNF increases mitochondrial ubiquitination and stabilizes Pink1 without inducing mitophagy. mitochondrial localization of OTULIN whose activity is inhibited by Pink1.
The authors present a convincing story that is currently mostly weakened by a lack of controls in multiple figures. A large portion of the paper relies on biochemical assays, which is ok for the most part. However, the spatial location of nucleus-mitochondria contact sites in Figure 6 has not been determined. This is important new insight that could generate important cell biological knowledge.

Specific points
1. The fractionation in Figure 1A does not provide any information about the relative localization of OTULIN or HOIP. A quantitative fractionation protocol demonstrating this should be performed to assess the relative mitochondrial protein amounts.
2. A positive control of the mt-Keima probe should accompany the shown negative results, since the absence of mitophagy is considered a key characteristic of TNF treatment. Also, there are no timepoints between 30 min and 16h, which is a very long time during which technically mitochondria could have undergone autophagic elimination.
3. Controls are also missing on Figure 4A, where total Bax is not measured. While I trust the authors, this should be added. Moreover, quantification of the Bax increase should be measured versus total Bax rather than GAPDH. Similarly, total

cytochrome c is not shown. The switch to SH-SY5Y cells for the caspase-3 assay is not optimal.
4. Why is mitochondrial HOIP not shown in figure 4F? The signal of this protein changes over the figures, it should remain the same after TNF treatment (Figure 1), but here it increases in total lysates. The authors should address this variability.
5. Figures 4F and 4G should show lysate controls for the proteins involved consistently.
6. In Figure 5B, it is mentioned that there is already p65 on mitochondria before the treatment, but this is not actually measured with phospho-p65. Similarly, the amounts of phospho-p65 are not given for the input in Figure 5D.
7. The spatial consequences of nuclear SPLICS signals are unclear. In Figure 6E-I, are the signals for the contact sensor seen on the interior corresponding to mitochondria invading the nucleus? Or are these signals due to different selection of the plane. This warrants further investigation of the nuclear volume. Is it containing mitochondria? This could be investigated by EM analysis or improved video analysis.

Minor points:
1. On page 11, there is a grammar mistake: "we triggering the mitochondrial retrograde response..."

**EMBOJ-2022-112006**

**Rebuttal Letter**

We would like to thank the referees for their helpful suggestions and constructive criticism. In the following, we addressed their concerns point by point.

The revised version includes substantial new data and information:

**1.** An *in situ* proximity ligation assay to verify the presence of the activated NF-κB subunit p65 at mitochondria (Fig. 5B, C).

**2.** More experimental evidence that mitochondrial M1-ubiquitination induced by TNF or OTULIN silencing does neither stimulate mitophagy nor affect CCCP-induced mitophagy:

- mt-mKeima time course experiment in cells treated with FCCP, TNF or OTULIN siRNAs (Fig. EV2C);
- quantification of FCCP-induced Parkin translocation +/- TNF treatment (Fig. EV2B);
- quantification of CCCP-induced Parkin translocation +/- OTULIN overexpression (Fig. EV2D);
- quantification of CCCP-induced clearance of mitochondria +/- OTULIN overexpression (Fig. EV2E).

**3.** Stabilization of mature PINK1 in cells silenced for OTULIN expression (Fig. 2A).

**4.** Phospho-S65-ubiquitin and phospho-S65-Parkin levels at mitochondria in response to 15 min TNF compared to 90 min CCCP treatment (Fig. 2B).

**5.** Sensitivity of the TNF-induced phospho-S65-ubiquitin-specific signal to OTULIN (Fig. 2F).

**6.** NF-κB luciferase reporter assays to show that PINK1 or Miro1/2 silencing decreases NF-κB transcriptional activity (EV4D and E).

**7.** TNF-induced M1-linked ubiquitin, OTULIN and HOIP in whole cell lysates and mitochondrial fractions on a single blot (Fig. 1B).

**8.** Schematic workflow for the preparation and purification of mitochondrial fractions and immunoblots to test the purity of mitochondrial fractions (Fig. EV1A and B).

**9.** Validation of the PINK1-specific antibody in cells silenced for PINK1 expression (Fig. EV1C and D).

**10.** An NF-κB luciferase reporter assay and a p65 nuclear translocation assay to demonstrate that IκB-2S prevents NF-κB activation (Appendix Fig. 1SA and B).

**11.** We indicated for each experiment, which M1-ubiquitin antibody (1E3 or 1F11/3F5/Y102L) was used to explain differences in the signal intensities. Please note that the original commercial 1E3 antibody has not been available for more than a year so that we had to switch to the 1F11/3F5/Y102L antibody provided by Genentech through a Material Transfer Agreement.

**Point-by-Point response**

**Referee #1**

The authors report the identification of a signaling platform on mitochondria that contributes to TNF-induced NFkB activation. They observe recruitment of the LUBAC subunits HOIP and HOIL-1L to and the deposition of M1-linked ub chains at mitochondria in a manner partially dependent on PINK1 catalytic activity. The authors further report recruitment of the NFkB-controlling IKK complex to mitochondria, where it causes localized activation of NFkB. Subsequent translocation of NFkB into the nucleus appears partially dependent on nucleus-mitochondria contact sites.

The manuscript contains with no doubt interesting observations, in particular the identification on mitochondria of a LUBAC-dependent signaling platform for the enhancement of TNF signaling. However, the authors fail to relate their manuscript to previous publications reporting conceptually similar signaling platforms on cytosol-invading bacteria. Signaling platforms on bacteria also rely on LUBAC recruitment (Noad, Nat Immunol 2017) and are also antagonized by Otulin (van Wijk, Nat Immunol 2017). The authors similarly fail to cite the literature on the NFkB inducing activity of Parkin (for example Sha, Hum Mol Gen 2010).

In the revised manuscript, we discussed in detail similarities and differences between the bacterial and mitochondrial signaling platform generated by LUBAC. We also elaborate on the previous literature on Parkin/PINK1-induced NF-kB activation, including (Akundi et al., 2011, Henn et al., 2007, Ingles-Prieto et al., 2021, Lee & Chung, 2012, Lee et al., 2012, Meka et al., 2015, Meschede et al., 2020, Muller-Rischart et al., 2013, Sha et al., 2010, Wang et al., 2018).

Overall, the results presented here support the authors' conclusions. However, several issues need to be addressed to strengthen the manuscript.

1. Large parts of the manuscript rely on the isolation of mitochondria, for example the demonstration of LUBAC recruitment to and M1-deposition at mitochondria. However, the purity of the mitochondria preps is not carefully addressed and the possibility of other organelles contaminating the material needs to be ruled out. For example Fig1A, B, C, E. And also Fig5: It seems remarkable that p65/RelA itself would accumulate on mitochondria, as it normally is known to shuttle from the cytosol into the nucleus as a soluble protein.

By combining differential centrifugation with isopycnic density gradient centrifugation for purification of mitochondria, we were able to considerably increase the purity of the mitochondrial fraction. Of course, it is not possible to get a "pure" mitochondrial fraction, since mitochondria physically interact with other cellular organelles, particularly the ER.

To address the reviewer's concerns, we added several additional information and approaches. In the **new Fig. EV1A**, we illustrated the workflow for the preparation and purification of mitochondria from mammalian cells. We also included representative Western blots of the whole cell lysates and the mitochondrial fractions probed for ER and plasma membrane markers (**new Fig. EV1B**). Most importantly, we demonstrated that the mitochondrial fraction does not contain TNF receptors to exclude the possibility that the mitochondrial signals for M1 ubiquitin and NF-kB signaling components derive from TNFR1 signaling complexes assembled at the plasma membrane. In support of a mitochondrial signaling platform, we showed an increase in mitochondrial M1 ubiquitin also by immunocytochemistry and fluorescence microscopy (Fig. 1D). Moreover, we performed proximity ligation *in situ* assays supporting the presence of phospho-S536-p65 in close vicinity to TOM20 at the mitochondrial outer membrane (**new Fig. 5B, C**).

Regarding the transport of p65 to the nucleus, a regulated transport of p65 may have advantages particularly in polarized cells, such as neurons, where p65 needs to travel long distances from the periphery to the nucleus. In support of this notion, it has been shown previously that p65/p50 NF-κB heterodimers are selectively localized at synapses (Meffert et al., 2003) and that retrograde transport of p65 in hippocampal neurons in response to synaptic stimulation requires the dynein/dynactin motor complex (Mikenberg et al., 2007, Shrum et al., 2009).

2. I thought initially that the white / black bands in Western blots would correspond to the detection method, i.e. ECL vs fluorescence. However, in Fig1E even corresponding samples differ (e.g. HOIP, Sharpin, TIM23, TNFR1, GAPDH but not HOIL-1L). The issue is more than cosmetic - for example the absence of TNFR1 and GAPDH in the mitochondrial fraction, rather than confirming the absence of membrane and cytosole contamination, could well be a technical problem since the positive control (lysate) was clearly handled differently. In general terms, it is regrettable that blots in Fig1 are mosaic with mitochondria and lysates not on the same gel.

We agree with the reviewer that it would have been better to show all samples on the same blot. In the **new Fig. 1B**, the abundance of M1-ubiquitin, OTULIN and HOIP in purified

mitochondrial fractions and whole cell lysate are shown on the same blot to confirm the robustness of our findings. Please note that for the blot shown in Fig. 1B we had to use the 1F11/3F5/Y102L M1-ubiquitin antibody instead of the 1E3 antibody (Fig. 1A), since the 1E3 antibody is no longer available (i.e. it can be purchased but does not work as well as the original clone). This is why we had to use the 1F11/3F5/Y102L M1-ubiquitin antibody provided by Genentech for the more recent experiments.
Notably, there are some variations in the abundance of proteins recruited to mitochondria in response to TNF treatment, which can be explained by the fact that these signaling proteins are non-covalently and in some cases indirectly bound to the outer mitochondrial membrane, increasing the risk of losing these proteins during the elaborate and time-consuming purification of mitochondria. In addition, there are variations in the intensity of the TNF response based on variabilities in the biological activity of the recombinant protein and on differences in the expression levels of cellular TNF receptors.

3. Fig1A: Why are there two HOIP bands in the mitochondria fraction? Since the two blots are separated, which of the two bands corresponds to the band in whole cell lysates?

The lower band in the mitochondrial fraction corresponds to the HOIP-specific band in whole cell lysates. Interestingly, in most cell lines we see a second, higher molecular weight band for HOIP in mitochondrial fractions and partly in whole cell lysates. This band disappears in cells silenced for HOIP expression by RNA interference, therefore we assume that the second band represents a posttranslationally modified HOIP species. We are currently following up on this phenomenon to find out which kind of HOIP modification occurs at mitochondria.

4. Why is the Otulin band in the mitochondria fraction not sensitive to siRNA depletion?

We repeatedly observed that the mitochondrial fraction of OTULIN is less sensitive to the siRNA. Our interpretation would be that the mitochondrial localization of OTULIN decreases its turnover and thus increases its half-life, so that it is more difficult to deplete mitochondrial OTULIN.

5. Fig1F: I wasn't aware that mitochondria are constitutively and strongly positive for K48 ub chains. Again, how clean is the mitochondrial fraction?

There is constitutive ubiquitination of mitochondrial outer membrane proteins, mostly involving K48-linked ubiquitin chains. Actually, our data are consistent with what has been

observed in other studies. Ordureau et al. have shown that K48-linked ubiquitin chains are present at a relatively high abundance under steady state by Western blotting (Fig. 1E) and mass spectrometry (Fig. S1H) (Ordureau et al., 2014).
The amount of K48-linked chains may appear high the experiment shown in the previous Fig. 1F (now Fig. 1G), since M1-linked ubiquitination is a low abundant type of ubiquitin linkage. As a consequence, we need considerable amounts of proteins for detection, which gives the impression that K48-linked ubiquitin, which is the most abundant ubiquitin linkage, is extremely strong.

6. Fig2A: The anti-PINK blot has many bands, two are claimed to represent PINK. It would be helpful if the authors would demonstrate the specificity of their anti-PINK antibody.

We validated the PINK1-specific antibody in cells silenced for PINK1 expression by RNA interference (**new Fig. EV1C and D**).

7. Fig2C: A positive control (for example CCCP treatment) for the mKeima experimental setup should be provided.

The positive control for the mt-mKeima ratiometric analysis was antimycin A/oligomycin treatment (10 µM each) for 1 h. We included the data in the revised version. In addition, we performed a time course experiment over 8 h, in which we compared mt-mKeima fluorescence intensities in cells treated with FCCP, TNF, or OTULIN-specific siRNA (**new Fig. EV2C**).

8. Fig2D: The authors demonstrate a ubiquitin smear in PINK IPs (2D). Is this smear M1 linked ubiquitin as claimed in the manuscript? Disappearance of the smear upon Otulin expression is consistent with, but does not prove the identify of, the M1 linkage type as the Otulin effect could be indirect.

The fact that the ubiquitin signal was dependent on catalytically active HOIP (Fig. 2D, lane 3) and disappeared upon increased expression of OTULIN (Fig. 2D, lane 6) strongly argues in favor of M1-linked ubiquitin. When catalytically inactive C885A was expressed, the signal intensity was strongly reduced (Fig. 2D, lane 4). The weak signal remaining can be explained by the fact that overexpressed HOIL-1L increased endogenous HOIP activity. We used a ubiquitin antibody instead of the M1-specific antibody for this experiment, because the M1-ubiquitin antibody 1E3 was not available at that time and we did not yet have the permission from Genentech to use the 1F11/3F5/Y102L antibody. Thus, we had to circumvent this

problem by genetically modifying the expression levels of HOIP or OTULIN. Since these are the only proteins that exclusively assemble or disassemble M1-linked ubiquitin chains, linear ubiquitination can be studied with unique specificity.

Moreover, for the analysis of endogenous PINK1, which we consider more relevant, we were able to use the M1-specific 1F11/3F5/Y102L antibody from Genentech (Fig. 2F). Here, we controlled for M1 specificity by adding recombinant OTULIN to the cell lysates to avoid artefacts by overexpression (see workflow below). This approach confirmed the initial observation with overexpressed proteins and showed that TNF treatment increases ubiquitination of endogenous PINK1 which is sensitive to OTULIN.

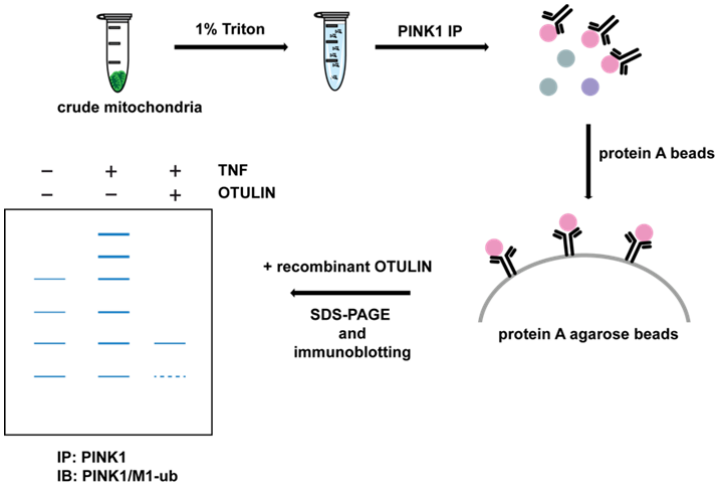


**Fig. 1: Workflow for endogenous PINK1 immunoprecipitation shown in Fig. 2D.** Crude mitochondria were extracted from cells, lysed in 1% Triton X-100 lysis buffer on ice for 5 min, then centrifuged at 20k x g for 20 min at 4˚C. The lysates were incubated with rabbit anti-PINK1 antibody or IgG as a control overnight at 4˚C. The samples were then incubated with pre-washed protein A beads for 3 h, then washed three times with lysis buffer and eluted with 2 x LSB. To validate the M1-ubiquitin-posiitve signals in the TNF-treated samples, the beads were washed three times with 1% Triton X-100 lysis buffer, and twice with the low salt buffer and then incubated with recombinant OTULIN (2 nM) for 1 h at 25˚C to hydrolyze M1-linked ubiquitin chains. The reactions were terminated by elution with 2 x LSB and the samples were analyzed by immunoblotting.

9. Fig2F: TNF treatment does not cause enhanced M1 linked ubiquitylation of endogenous PINK, as would be expected from earlier(overexpression) experiments.

Both the PINK1 and the M1-ubiquitin blot show an increase in endogenous PINK1 ubiquitination in response to TNF treatment (compare lane 1 and 2 of Fig. 2F). We added

another experiment to the Source Data confirming increased M1-ubquitination of endogenous PINK1 upon TNF stimulation, see below.

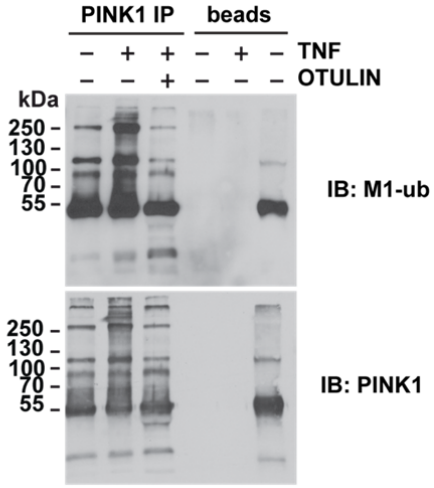


**Fig. 2: Endogenous PINK1 is modified with M1-ubiquitin after TNF treatment.** HEK293T cells were treated with TNF (25 ng/ml, 15 min), then endogenous PINK1 was immunoprecipitated from mitochondrial fractions. To validate the specificity of the M1-ubiquitin signals, the TNF-treated sample was incubated with recombinant OTULIN to hydrolyze M1-ubiquitin chains. The samples were analyzed by immunoblotting (IB) using antibodies against M1-ubiquitin and PINK1.

10. Page9: The authors wish to test '... whether PINK1 increases TNF-induced M1-ubiquitination at mitochondria by phosphorylating ubiquitin.' This question remains unresolved - the authors demonstrate an effect of PINK1 on M1-ub and on p-S65-Ub but they haven't shown the occurrence of pS65 in M1-linked ub chains.

Several approaches let to the conclusion that PINK1 phosphorylates M1-linked ubiquitin chains, although it is also conceivable that K63-linked polyubiquitin is phosphorylated by PINK1. First, PINK1 phosphorylated M1-linked ubiquitin *in vitro* (Fig. EV6B). Second, catalytically active PINK1 increased pS65-ubiquitination of NEMO (Fig. 5D), which is mostly ubiquitinated by M1- and K63-linked chains. Finally, we see a TNF-induced increase in the pS65-ubiquitin signal when endogenous PINK1 is immunoprecipitated; this signal is sensitive to recombinant OTULIN added to cell lysates, indicating that the phosphorylated ubiquitin chains were linked via M1 (**new Fig. 2F**).

11. Fig4E: A positive control for the activity of the dominant negative IkB is needed.

We included a **new Appendix Fig. S2** to show that the dominant-negative IκBα-2S mutant prevents the TNF-induced nuclear translocation of p65 (**Fig. S2A**) and NF-κB transcriptional activity (**Fig. S2B**).
The luciferase assay shown in Fig. S2B corresponds to the apoptosis assay shown in Fig. 4E and confirms that the experimental paradigm we chose to test an NF-κB-independent function of TNF in preventing apoptosis is suitable.

12. Fig5C: The authors report an effect of PINK1 on NFkB translocation. Confirmation by reporter gene activation is advisable. Similarly in Fig6B for the effect of Miro1/2 on NFkB translocation.

We performed luciferase reporter assays in cells silenced for PINK1 or Miro1/2 expression. TNF-induced NF-κB transcriptional activity was significantly reduced in both PINK1- and Miro1/2-silenced cells (**new Fig. EV4D and E**).
The PINK1-dependent effect on NF-κB transcriptional activity is consistent with studies performed by Sha et al., who observed a decrease in NF-κB activity in response to TNF, PMA, or rotenone treatment in cells silenced for PINK1 expression (Sha et al., 2010)(Fig. 9). Since Sha et al. quantified luciferase activity after 3 h treatment plus 8 h recovery, we chose an earlier time point (3 h) to test whether there are already differences at this stage.

13. Is the TNF-induced relocalization of mitochondria towards the nucleus dependent on PINK1?

We tested this, but surprisingly, cells silenced for PINK1 expression showed an increase in mitochondria-nucleus contact sites already under basal conditions. However, we observed that acute PINK1 silencing induces mitochondrial fragmentation and obviously causes mitochondrial damage, consistent with our previous work (Exner et al., 2007, Ingles-Prieto et al., 2021, Klein et al., 2014, Lutz et al., 2009). Thus, the loss of PINK1 expression obviously triggers a mitochondrial stress response, which results in increased proximity between mitochondria and the nucleus.

14. The authors propose that PINK1-dependent phosphorylation of ubiquitin stabilizes M1-linked chains. However, since the authors also report physical interaction between PINK1 and HOIP (FigS2), it seems equally possible that the PINK1-dependent enhancement of M1-linked ubiquitylation is due to its interaction with LUBAC and stimulation of its E3 activity. Assessing the role of PINK1 in Otulin ko cells could clarify the situation.

We fully agree. PINK1 is strongly stabilized in cells overexpressing LUBAC components and complex formation seems to contribute to this phenomenon (Fig. 2D). In cells silenced for OTULIN expression, PINK1 is clearly stabilized (**new Fig. 2A**). However, in OTULIN knockdown cells, mitochondrial HOIP is increased (Fig. 1A, B), thus we cannot discriminate whether PINK1 stabilization is based on PINK1/HOIP complex formation or HOIP-mediated ubiquitination of PINK1. Based on the fact that PINK1 stabilization is markedly reduced when catalytically inactive C885A HOIP is expressed (Fig. 2D), although PINK1 interacts with C885A HOIP, we concluded that HOIP-induced ubiquitination of PINK1 at least contributes to its stabilization.

**Referee #2**

Wu et al. investigate the role of mitochondria in the amplification of innate immune responses. They focus on NF-kB, whose persistent activation causes inflammation but also allows for the survival and functioning of neurons. A ey factor in this process is TNF, which acts as a neuroprotective cytokine and activates NF-kB. This requires the ubiquitination of NEMO by the linear ubiquitin chain assembly complex (LUBAC), whose catalytic subunit is HOIP. NEMO activates IKK, which then cause dissociation and degradation of the NF-kB inhibitor IkB. Parkin supports this pathway, while OTULIN acts as a deubiquitinase. Given this link, the authors propose a role of mitochondria in NF-kB activation.

In their MS-based data, the authors demonstrate that TNF increases mitochondrial ubiquitination and stabilizes Pink1 without inducing mitophagy. mitochondrial localization of OTULIN whose activity is inhibited by Pink1.

The authors present a convincing story that is currently mostly weakened by a lack of controls in multiple figures. A large portion of the paper relies on biochemical assays, which is ok for the most part. However, the spatial location of nucleus-mitochondria contact sites in Figure 6 has not been determined. This is important new insight that could generate important cell biological knowledge.

Specific points

1. The fractionation in Figure 1A does not provide any information about the relative localization of OTULIN or HOIP. A quantitative fractionation protocol demonstrating this should be performed to assess the relative mitochondrial protein amounts.

In the revised version, we included new data allowing the assessment of the relative localization of OTULIN and HOIP at mitochondria, see **new Fig. 1B**.

2. A positive control of the mt-Keima probe should accompany the shown negative results, since the absence of mitophagy is considered a key characteristic of TNF treatment. Also, there are no timepoints between 30 min and 16h, which is a very long time during which technically mitochondria could have undergone autophagic elimination.

The positive control for the mt-mKeima ratiometric analysis (antimycin A/oligomycin, 10 µM for 1 h) is now included in the **new Fig. 2C**. In addition, we performed a time course experiment over 8 h, in which we compared mt-mKeima fluorescence intensities in cells treated with FCCP, TNF, or OTULIN-specific siRNA (**new Fig. EV2C**).
To further strengthen our data on TNF/M1-linked ubiquitin and mitophagy, we performed additional experiments. We observed that TNF pretreatment has no effect on FCCP-induced Parkin translocation to mitochondria (**new Fig. EV2B**). Moreover, OTULIN overexpression did neither affect CCCP-induced Parkin translocation to mitochondria (**new Fig. EV2D**) nor the CCCP-induced clearance of mitochondria (**new Fig. EV2E**). In conclusion, we did not find any evidence that M1-ubiquitination affects PINK1/Parkin-dependent mitophagy. This is consistent with previous publications from Wade Harper's lab, showing that M1 ubiquitin chains are not implicated in mitophagy regulation (Ordureau et al., 2018, Ordureau et al., 2014).

3. Controls are also missing on Figure 4A, where total Bax is not measured. While I trust the authors, this should be added. Moreover, quantification of the Bax increase should be measured versus total Bax rather than GAPDH. Similarly, total cytochrome c is not shown. The switch to SH-SY5Y cells for the caspase-3 assay is not optimal.

We fully agree with the reviewer that total Bax levels should have been used for quantification. Unfortunately, we do not have total Bax levels for all replicates. However, we quantified Bax recruitment for one example, calculating the ratio between normalized total Bax (to GAPDH) and normalized mitochondrial Bax (to TIM23). This confirmed our results shown in Fig. 4A, see Fig. 3 below.

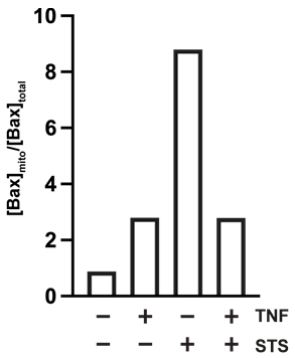

**Fig. 3. Quantification of Bax recruitment to mitochondria.** HeLa cells were treated with STS (1 µM, 1 h) with or without a 15 min pretreatment with TNF (25 ng/ml) and then harvested. Purified mitochondrial fractions were analyzed by immunoblotting using antibodies against Bax and M1-ubiquitin. Mitochondrial Bax signals were normalized to TIM23, and total Bax signals were normalized to GAPDH. The ratio between normalized mitochondrial and normalized total Bax is shown for one representative experiment.

We used SH-SY5Y cells for the caspase-3 assays, since we wanted to show that the anti-apoptotic effect is seen in different cell lines with different approaches (ultrastructural analysis of cristae morphology, mitochondrial Bax recruitment, cytochrome c release, caspase-3 activation). We also quantified the decrease in mitochondrial cytochrome c by immunocytochemistry and fluorescence microscopy using CellProfiler, which confirmed the results from other approaches, see Fig. 4 below. As the fast, transcription-independent anti-apoptotic effect of TNF is only a minor aspect of our manuscript, we hope that the consistent effects shown by these different approaches is convincing enough.

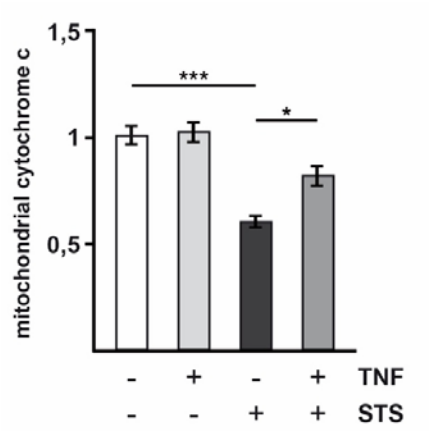


**Fig. 4: TNF reduces STS-induced mitochondrial cytochrome c release.** HeLa cells were treated with STS (1 µM) for 1 h with or without a 15 min TNF pretreatment (25 ng/ml). Then the cells were fixed and analyzed by immunocytochemistry and fluorescence microscopy using cytochrome c and Hsp60 antibodies. Images were analyzed using CellProfiler for overlapping cytochrome c and Hsp60 signals. Data represent the means +/- standard error (n=21; Kruskal-Wallis test); *p ≤ 0.05, ***p ≤ 0.001.

4. Why is mitochondrial HOIP not shown in figure 4F? The signal of this protein changes over the figures, it should remain the same after TNF treatment (Figure 1), but here it increases in total lysates. The authors should address this variability.

The idea of the experiments shown in Fig. 4F and 4G was to compare the STS-induced cytochrome c release into the cytoplasm. To show mitochondrial HOIP, a higher amount of cells would have been required for mitochondrial purification.
We added another replicate of the experiment shown in Fig. 4F to the Source Data to confirm that the decrease in cytochrome c release after TNF pretreatment is consistent (see Fig. 5 below).

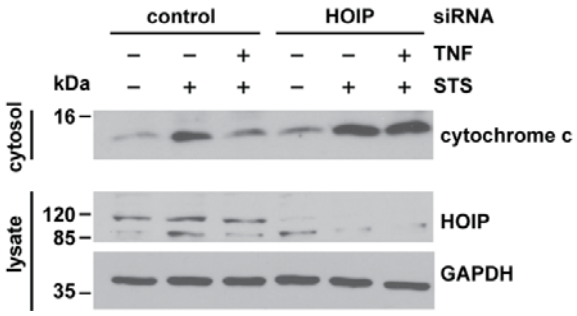


**Fig. 5. The protective effect of TNF is dependent on HOIP expression.** HeLa cells were transiently transfected with control or HOIP siRNA. 48 h after transfection, cells were treated with STS (1 µM, 1 h) with or without a 15 min TNF pretreatment (25 ng/ml) and then harvested. The cytosolic fractions were analyzed by immunoblotting using cytochrome c antibodies. HOIP silencing efficiency was analyzed in whole cell lysates using antibodies against HOIP. GAPDH was immunoblotted as input control.

Concerning the amount of HOIP and other signaling components detected in mitochondrial fractions, there are some variabilities. This is due to the fact that these signaling proteins are non-covalently and in some cases indirectly bound to the outer mitochondrial membrane, increasing the risk of losing these proteins during the elaborate and time-consuming purification of mitochondria. In addition, there are variations in the intensity of the TNF response based on variabilities in the biological activity of the recombinant protein and on differences in the expression level of cellular TNF receptors.

5. Figures 4F and 4G should show lysate controls for the proteins involved consistently.

For Figure 4F we labeled the lysate controls.

6. In Figure 5B, it is mentioned that there is already p65 on mitochondria before the treatment, but this is not actually measured with phospho-p65. Similarly, the amounts of phospho-p65 are not given for the input in Figure 5D.

To confirm our data by an independent approach and to avoid variations caused by the mitochondrial purification procedure, we performed a proximity ligation assay (PLA). *In situ* PLA using anti-TOM20 and anti-phospho-S536-p65 (p-p65) antibodies revealed the presence of p-p65 at mitochondria and an increased abundance upon TNF treatment (**new Fig. 5B, C**).
The input in Fig. 5D (now Fig. 6B) is shown in the revised version.

7. The spatial consequences of nuclear SPLICS signals are unclear. In Figure 6E-I, are the signals for the contact sensor seen on the interior corresponding to mitochondria invading the nucleus? Or are these signals due to different selection of the plane. This warrants further investigation of the nuclear volume. Is it containing mitochondria? This could be investigated by EM analysis or improved video analysis.

The images shown in Fig. 6E-I are indeed Z-projections derived from different confocal stacks over several planes acquired every 0.3 µm. This is specifically performed to avoid variability in the number of contacts over single confocal planes, thus the whole number of contacts is considered in our analysis. The signal present in the central part of the nucleus is indeed plane-dependent and represent the contact sites occurring in the top and bottom part of the nucleus. It is also a clear indication of the effect in terms of increase in mitochondria-nucleus contacts as well as of the specificity of the newly generated SPLICS reporter.

Minor points:
1. On page 11, there is a grammar mistake: "we triggering the mitochondrial retrograde response..."

Typo fixed, thanks for mentioning.

**References**

Akundi RS, Huang Z, Eason J, Pandya JD, Zhi L, Cass WA, Sullivan PG, Bueler H (2011) Increased mitochondrial calcium sensitivity and abnormal expression of innate immunity genes precede dopaminergic defects in Pink1-deficient mice. PLoS ONE 6: e16038

Exner N, Treske B, Paquet D, Holmstrom K, Schiesling C, Gispert S, Carballo-Carbajal I, Berg D, Hoepken HH, Gasser T, Kruger R, Winklhofer KF, Vogel F, Reichert AS, Auburger G, Kahle PJ, Schmid B, Haass C (2007) Loss-of-function of human PINK1 results in mitochondrial pathology and can be rescued by parkin. J Neurosci 27: 12413-8

Henn IH, Bouman L, Schlehe JS, Schlierf A, Schramm JE, Wegener E, Nakaso K, Culmsee C, Berninger B, Krappmann D, Tatzelt J, Winklhofer KF (2007) Parkin mediates

neuroprotection through activation of IkappaB kinase/nuclear factor-kappaB signaling. J Neurosci 27: 1868-78

Ingles-Prieto A, Furthmann N, Crossman SH, Tichy AM, Hoyer N, Petersen M, Zheden V, Biebl J, Reichhart E, Gyoergy A, Siekhaus DE, Soba P, Winklhofer KF, Janovjak H (2021) Optogenetic delivery of trophic signals in a genetic model of Parkinson's disease. PLoS Genet 17: e1009479

Klein P, Muller-Rischart AK, Motori E, Schonbauer C, Schnorrer F, Winklhofer KF, Klein R (2014) Ret rescues mitochondrial morphology and muscle degeneration of Drosophila Pink1 mutants. EMBO J 33: 341-55

Lee HJ, Chung KC (2012) PINK1 positively regulates IL-1beta-mediated signaling through Tollip and IRAK1 modulation. J Neuroinflammation 9: 271

Lee HJ, Jang SH, Kim H, Yoon JH, Chung KC (2012) PINK1 stimulates interleukin-1beta-mediated inflammatory signaling via the positive regulation of TRAF6 and TAK1. Cell Mol Life Sci 69: 3301-15

Lutz AK, Exner N, Fett ME, Schlehe JS, Kloos K, Lammermann K, Brunner B, Kurz-Drexler A, Vogel F, Reichert AS, Bouman L, Vogt-Weisenhorn D, Wurst W, Tatzelt J, Haass C, Winklhofer KF (2009) Loss of Parkin or PINK1 Function Increases Drp1-dependent Mitochondrial Fragmentation. J Biol Chem 284: 22938-51

Meffert MK, Chang JM, Wiltgen BJ, Fanselow MS, Baltimore D (2003) NF-kappa B functions in synaptic signaling and behavior. Nat Neurosci 6: 1072-8

Meka DP, Muller-Rischart AK, Nidadavolu P, Mohammadi B, Motori E, Ponna SK, Aboutalebi H, Bassal M, Annamneedi A, Finckh B, Miesbauer M, Rotermund N, Lohr C, Tatzelt J, Winklhofer KF, Kramer ER (2015) Parkin cooperates with GDNF/RET signaling to prevent dopaminergic neuron degeneration. J Clin Invest 125: 1873-85

Meschede J, Sadic M, Furthmann N, Miedema T, Sehr DA, Muller-Rischart AK, Bader V, Berlemann LA, Pilsl A, Schlierf A, Barkovits K, Kachholz B, Rittinger K, Ikeda F, Marcus K, Schaefer L, Tatzelt J, Winklhofer KF (2020) The parkin-coregulated gene product PACRG promotes TNF signaling by stabilizing LUBAC. Sci Signal 13

Mikenberg I, Widera D, Kaus A, Kaltschmidt B, Kaltschmidt C (2007) Transcription factor NF-kappaB is transported to the nucleus via cytoplasmic dynein/dynactin motor complex in hippocampal neurons. PLoS ONE 2: e589

Muller-Rischart AK, Pilsl A, Beaudette P, Patra M, Hadian K, Funke M, Peis R, Deinlein A, Schweimer C, Kuhn PH, Lichtenthaler SF, Motori E, Hrelia S, Wurst W, Trumbach D, Langer

T, Krappmann D, Dittmar G, Tatzelt J, Winklhofer KF (2013) The E3 ligase parkin maintains mitochondrial integrity by increasing linear ubiquitination of NEMO. Mol Cell 49: 908-21

Ordureau A, Paulo JA, Zhang W, Ahfeldt T, Zhang J, Cohn EF, Hou Z, Heo JM, Rubin LL, Sidhu SS, Gygi SP, Harper JW (2018) Dynamics of PARKIN-Dependent Mitochondrial Ubiquitylation in Induced Neurons and Model Systems Revealed by Digital Snapshot Proteomics. Mol Cell 70: 211-227 e8

Ordureau A, Sarraf SA, Duda DM, Heo JM, Jedrychowski MP, Sviderskiy VO, Olszewski JL, Koerber JT, Xie T, Beausoleil SA, Wells JA, Gygi SP, Schulman BA, Harper JW (2014) Quantitative proteomics reveal a feedforward mechanism for mitochondrial PARKIN translocation and ubiquitin chain synthesis. Mol Cell 56: 360-75

Sha D, Chin LS, Li L (2010) Phosphorylation of parkin by Parkinson disease-linked kinase PINK1 activates parkin E3 ligase function and NF-kappaB signaling. Hum Mol Genet 19: 352-63

Shrum CK, Defrancisco D, Meffert MK (2009) Stimulated nuclear translocation of NF-kappaB and shuttling differentially depend on dynein and the dynactin complex. Proc Natl Acad Sci U S A 106: 2647-52

Wang Y, Shan B, Liang Y, Wei H, Yuan J (2018) Parkin regulates NF-kappaB by mediating site-specific ubiquitination of RIPK1. Cell Death Dis 9: 732

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