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Structural basis for feedforward control in the PINK1/parkin pathway

Véronique Sauvé, George Sung, Emma MacDougall, Guennadi Kozlov, Anshu Saran, Rayan Fakih, Edward Fon, and Kalle Gehring
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Corresponding author(s): Kalle Gehring (kalle.gehring@mcgill.ca)

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(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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Thank you for submitting your manuscript on phospho-ubiquitin activation of Parkin to The EMBO Journal, and apologies for the delay in getting back to you with evaluations from referees in this case. We have now received the below-copied reports from four reviewers with expertise in structural biology of ubiquitination (refs 2 and 4) and in the PINK1/Parkin mitophagy pathway (refs 1 and 3). As you will see, the referees acknowledge the importance of the topic and the potential interest of your findings, but especially referees 2 and 4 raise concerns about the wider significance of the present results, a concern also echoed in referee 3's comments on recent physiological studies. In addition, the structural referees also consider parts of the presented in vitro results to require further follow-up.

As these issues would appear to affect the eventual suitability of this work for The EMBO Journal and it is not clear whether they could be easily addressed during revision, I would in this case appreciate hearing from you how you would envision responding to the referees' points should you be given the opportunity to revise this work for The EMBO Journal. Therefore, please carefully consider the attached reports and send back a brief point-by-point response outlining how the referees' comments might be addressed/clarified, should you be given the opportunity to revise this work for The EMBO Journal. With your proposal in hand, we could further discuss via email or schedule a Zoom call to directly discuss what could and what could not be done here, before taking a definitive decision on this manuscript. It would be great if you could get back to me with such a response over the course of this week or by early next week.

Referee #1 (Report for Author)

Previous studies have shown that Parkin either lacking its PINK1 phosphorylation site (Ser65) or lacking its entire Ubl domain still retained mitophagic activity, albeit at a reduced rate. However, the mechanism behind how Parkin is activated in this context remained unknown. In this manuscript, Sauve et al discover that pUb can bind the RING0 domain within Parkin (where pUbl binds) and that this enables exposure of the catalytic cysteine in RING2 and therefore activation of Parkin. Parkin can therefore bind pUb at two separate sites which explains why S65A mutants of Parkin can still be activated. By having both pUbl and pUb activation modalities via RING0, the authors propose increased robustness of the pathway. Overall, the data in the manuscript are predominantly clear and convincing, and the discoveries help to close the loop on our understanding of Parkin activation mechanisms. The work will be of interest to researchers in multiple fields including Parkin biology, mitophagy and ubiquitin ligases.

1. Figure 1C: Despite not being active in mitophagy, can the authors show whether the mutants, alone or in combination can translocate to mitochondria? How do the mutant protein expression levels compare to WT?

2. Figure 1C: It would be beneficial to show whether the T240R mutant can efficiently expose its active site cysteine in RING2 using the Ub-vinyl sulfone experiments?

3. Given that the trans activity mechanism maybe slow/less efficient with two mutant proteins, can the authors also confirm that following a longer CCCP treatment has evidence of mitophagy e.g. 8h and 12h?

4. Figure 2: In the text the authors refer to pUb or pUbl concentrations required for Parkin activation e.g. "Addition of pUbΔG76 to wild-type R0RBR led to detectable crosslinking at 20 μM and 50% at 200 μM." Can the authors clarify how many times these experiments were repeated or were the conclusions drawn from single experiments? It would be more convincing to provide some quantitative data and n=3 of the Ub-VS crosslinking experiments to strongly support the data in this figure.

5. Figure 3A: It would be interesting to determine whether the Ub-Parkin chimera can drive mitophagy in cells. This would demonstrate that it is functional and active within a cellular context.

6. Figure 4 and the statement "Polyubiquitin chains can be phosphorylated by PINK1 in vitro and polyphosphorylated ubiquitin chains have been detected in cells following mitochondrial depolarization (Ordureau et al, 2014; Wauer et al, 2015b).": It should be noted that typically only the terminal ubiquitin is phosphorylated in cells (Swatek et al (2019) Nature). Therefore, it may be unlikely that Parkin encounters a poly-phosphorylated ubiquitin chain. This does not invalidate the in vitro experiments since they are still highly informative. However, it is unclear whether the pUb chain type and therefore its length will be an important factor in cells. What I think the in vitro data indicates is that Parkin may become more strongly activated on substrates that have two surface lysines at the right distance apart to bridge the two binding sites on Parkin. But, by having its UBL domain Parkin can circumvent this and add more robustness to its activation and expand its activation/recruitment substrate repertoire. It would be worthwhile to discuss these possibilities (at the author's discretion), however, the finding that terminal Ub molecules are typically phosphorylated must be referred to in the context of the pUb chain data.

Minor:

1. The following sentence should be modified to include the discovery of Parkin auto-inhibition by Chaugule et al (2011) EMBO J: "X-ray structures of parkin have revealed that it adopts an autoinhibited conformation in basal cell conditions (Riley et al, 2013; Trempe et al, 2013; Wauer & Komander, 2013)"

2. Although only a discussion piece, given that it was also raised in the abstract, are there species along the evolutionary path which lack the Parkin UBL domain?

Referee #2 (Report for Author)

Parkin is an RBR-type E3 ligase that plays a central role in mitochondrial quality control. The mechanism of Parkin activation has been an area of intense research due to the observation that autosomal mutations in the PARK2 gene which codes for Parkin are associated with early-onset Parkinson's disease. Parkin is cytosolic and autoinhibited under basal cell conditions. Upon sensing of mitochondrial damage, the kinase PINK1 phosphorylates ubiquitin molecules on the mitochondrial surface, which induces accumulation of Parkin on mitochondria and its subsequent activation. This is a multi-step process that involves binding of phosphorylated ubiquitin and phosphorylation by PINK1, which in turn induce structural rearrangements within Parkin that expose the binding site for the E2~Ub conjugate and allow access to the catalytic cysteine to promote formation of the E3~Ub intermediate.

Previous studies into the molecular features underpinning the mechanism of phosphorylation-dependent Parkin activation have provided a multi-faceted picture of the activation process including multiple atomic structures of Parkin along the activation pathway. In parallel, an alternative feedforward (open-cycle) mechanism for Parkin activation has been suggested that does not require its phosphorylation.

This study investigates the molecular details of this phosphorylation-independent mechanism and shows that it involves the the pUbl-binding site in the RING0 domain, which has higher affinity for pUb than for pUbl, which the authors suggest increases the robustness of the pathway for the clearance of damaged mitochondria. The authors also investigate the effect of poly-Ub chains on Parkin activation, which they suggest mimics the mitochondrial surface modified with multiple pUb molecules.

While the authors present some interesting observations about the effect of phosphorylated ubiquitin on Parkin activation it is not clear how much these data further the body of knowledge already available and how physiologically relevant the activation of Parkin by pUb is. Similarly, it is not clear what the experiments aimed at testing the effect of different poly-Ub chain types on Parkin activation tells us about its activation in a cellular context. Overall, the Discussion section of this paper is very speculative leaving the reader to wonder what precisely to take away from the data presented in this paper and how the new insight gained changes our understanding of Parkin activation.

Specific comments

1) How were the models of tetra-Ub chains bound to Parkin obtained? This needs to be explained otherwise the models shown are just an artist's impression. Why would the K48 chain have the most freedom? Have the authors taken intrinsic conformational flexibility of the different chains into account when creating the models?
The authors need to explain why their assays were done using K6-linked chains, when K48 and K63 chains are more efficient (Fig 4E).
Overall, this section needs to be put into context as to what type of chains are involved in Parkin activation and how a given chain type may alter the level or kinetics of activation. As it currently stands it doesn't add much information into the function of Parkin.

2) Based on their NMR experiments the authors estimate pUb to be 5-10 more effective in activating Parkin in functional assays and bind with higher affinity. This interaction needs be explored in much more detail to support this statement as this interaction is at the centre of this study.

Minor comments:

1) It would be helpful for readers not intimately familiar with Parkin structure and mechanism of activation to add a cartoon to Figure 2 showing the structure of autoinhibited Parkin and indicating the domains and binding sites discussed in the text to set the scene. Overall, it might be helpful to add a cartoon next to each experiment to illustrate what interactions are being interrogated.
Figure 2B: R0RBR K211N appears as a double band and running higher on the SDS gel. What is the reason for this? Please add a sentence explaining why the autoubiquitination assays were done with GST-fusion proteins.

2) The authors should specify which amino acid residues are covered in the constructs used in this study and not just refer to previous publication. This is important as there are multiple reports in the literature describing how the inclusion of N-terminal fusion proteins and the precise nature of the construct under investigation affect catalytic activity.

Referee #3 (Report for Author)

This paper reports an attractive iterative analysis of Parkin's activation mechanism in the context of the PINK1/Parkin pathway. The authors describe that phosphorylated ubiquitin can bind to two different sites on Parkin, RING0 (low affinity) and RING1 (high affinity). The authors then characterize in more detail the properties and possible function of the RING0 binding site in the context of Parkin's feedforward activation using various in-vitro and cell culture assays. Overall, I think that the observations made in the first figures are interesting, and the following in-vitro data and interpretation are convincing. Therefore, I believe overall the study is well conducted and presented. The data will be of interest to the PINK1/Parkin community, warranted that possible limitation to the alternative activation model described here is better highlighted in the manuscript.

General comments:

The foundation of this manuscript is based on the report that multiple studies have shown that deletion of the Ubl domain or loss of Ser65 phosphorylation of the Ubl (via Ser65 to Ala mutation) strongly impairs but does not entirely abolish Parkin recruitment to damaged mitochondria. One thing to keep in mind is that all these studies involve various levels of overexpression of Parkin mutant in cells (from transient over-expression to more controlled stable over-expression). I think that overall work in the past few years from several labs, including the authors, has clearly demonstrated that PINK1/Parkin signaling is a finely tuned system, where overexpression of one component would easily create an imbalance in the observed signaling output. In fact, one could argue that several recent studies have demonstrated the essential and critical role for Ubl Ser65 phosphorylation in vivo (mouse model, pmid: 30404819), an in-vitro neuronal model with endogenous CRISPR editing (Ser65Ala, PMID: 29656925; 32142685), or in the context of patient mutation S65N (pmid: 30404819), which would indicate that the feedforward activation mechanism is entirely dependent on Parkin's Ubl phosphorylation. That said, I think the work presented in this manuscript is an interesting and valid one, especially from the evolutionary perspective, but the manuscript would benefit from putting the finding in perspective, perhaps in a "study limitation" paragraph where the findings and relevance of the alternative activation model proposed here could be put into a broader endogenous context.

- Related to Figure 1: the authors should perform westernblot (Parkin antibody) of the transfected cells used here, to measure fist how much WT Parkin is overexpressed compared to endogenous Parkin present in un-transfected U2OS cells, and second to assess that the level of expression of each mutants is equal and similar to over-express WT Parkin.

- Related to Supplemental Figures S2 and S3 and the graphical representation of mito-mKeima data. A suggestion to the authors, I think these two figures would gain clarity for non-expert readers if the data was presented alongside (or only) as a single bar chart per figure, instead of 42 gated FACS plots (x-axis 405nm; y-axis 561nm), with the gating manually defined.

In a bar chart, the y-axis would be the 561nm/405nm average ratio of 100,000 events (calculated in FlowJo). All triplicate experiments can then be used simultaneously in a single bar chart plot. Authors would then be able to provide an error bar with S.D.. X-axis would be the different cell lines and treatment. In addition, there seems to be a typo in the current FACS representation in both figures, y-axis is labeled as 407nm, when the method section indicates 405nm, and similarly for the y-axis figure indicates 532nm, while the method indicates 561nm.

Specific comments:

-Page 5, line 15, (Wauer, 2015, Nature) should be added to citations about K211N that disrupt the phospho-serine binding site.

- Page 7, line 10, regarding the mention that pUb interferes with E2 discharging and Parkin activity, I would suggest to also add (pmid: 25284222) that initially reported the inhibitory effect of pUB on Parkin at high concentration.

- Figue 2C, 2E, 4C, are using GST-tag fused truncated version of Parkin R0RBR, while most of the other figures use an untagged version of R0RBR. Unless there is a specific reason for it, I would suggest for consistency across the paper, these three experiments are done with the R0RBR version that does not contain the GST tag. I don't think this is the case here, but in addition, GST is prone to forming dimers, so out of caution, performing these assays without possible interference from the GST tag would be preferable.

- Page 8, bottom of the page. The authors mention that poly-ubiquitin chains can be poly-phosphorylated at Ser65 positions, not only in-vitro but also in cells upon mitochondrial depolarization. While this is true, it would be informative for the reader that the authors also comment here on more recent work published using the Ub-clipping method, which reported that while these poly-phosphorylated chains are detectable, they, in fact, represent a very small portion of the phosphorylated Ubiquitin proteoforms (pmid: 31413367 and 32142685). Indeed, phospho-ubiquitin is mainly present in species that are primarily mono- or endcap ubiquitin (e.g., not inside a poly-Ub chain).

- Page 10, end of first paragraph, related to Figure 5C. The authors mention the dual phospho-ubiquitin conformation, and their experiment highlights that both conformations led to the detectable binding. Still, rapid exchange of the two conformations prevents the measurement of individual conformation. Have the authors tried to use either Ub L67S mutant or Ub TVLN mutant reported in pmid: 29133469, which stabilized the equilibrium to the C-term-retracted conformation?

Referee #4 (Report for Author)

The major activation pathway of the ubiquitin (Ub) ligase parkin depends on its high-affinity interaction with PINK1-phosphorylated Ub (pUb) that triggers dissociation of the Ub-like (Ubl) domain from the E2 binding RING1 and subsequent phosphorylation of the Ubl by PINK1. Phopho-Ubl (pUbl) interaction with the RING0 in turn releases the RING2 carrying the catalytic Cys to fully activate parkin. However, unphosphorylated / DeltaUbl parkin still possesses residual activity when bound to pUb (Kazlauskaite et al., 2014). Building on these results, Sauve et al. identify in their manuscript "Structural basis for feedforward control in the PINK1/parkin pathway" a mechanism for phosphorylation-independent activation of

parkin using localization and mitophagy assays, enzyme activity and NMR interaction studies. The authors show that the RING0 domain can interact with pUb as a monomer or as part of a poly-pUb chain in a manner similar to pUbl and activate parkin thereby circumventing the need for parkin phosphorylation.

The data are of high quality and support the authors' conclusions. Parkin is an important drug target and understanding its regulation is of high importance. However, the relevance of the discovered phosphorylation-independent activation mechanism is not explored to an extent that would justify publication in EMBO Journal in its current form.

Major issues:
The high affinity pUb interaction and release of the Ubl for phosphosphorylation occurs already at low pUb concentrations. The identified phosphorylation-independent activation, however, can only be relevant, if the rather high pUb or poly-pUb concentrations required for saturating the RING0 binding site were reached FASTER than Ubl phosphorylation by PINK1. The question as to how relevant the identified mechanism is in a natural setting, i.e. with WT full-length parkin being present, should therefore be addressed as detailed in the following points:

1) What is the order of Ub and parkin phosphorylation events in vivo? Does (poly-)pUb accumulate much faster at the mitochondrial membrane than phosphorylated parkin?

2) Stating that the affinity of pUb for the RING0 is stronger than the pUbl interaction in trans may be true, but it is doubtful that this would be the case in cis as in wild-type phosphorylated parkin. First, the authors should make clear at each instance whether the pUbl or pUb interactions that are described are in cis or in trans. Second, the authors should explore whether pUb or poly-pUb can compete for the RING0 when the pUbl is bound to the RING0 in cis (full-length phospho-parkin).

3) All in vitro experiments were carried out with parkin constructs lacking the Ubl domain or with the high affinity pUb binding site being mutated. These experiments support the conclusion that parkin can be activated to some degree in a phosphorylation-independent manner. However, to address the relevance of the mechanism and potential competition effects on parkin activity it would be important to include unphosphorylated and phosphorylated full-length parkin in these experiments. Also, a quantification of the levels of unconjugated (not Ub-modified) parkin in the in vitro activity assays would help to consolidate the authors' conclusions.

4) The authors conclude from their Ub charging and ubiquitination assays that pUb has a higher affinity for the RING0 domain than the pUbl in trans. In contrast, in the NMR competition experiments (Fig. 5E) the signals of the 15N pUbl are still broadened by complex formation with the R0BR/pUb and the signal of pS65 is not fully recovered even at a two-fold excess of pUb showing that the affinity of pUb cannot be much higher than for the pUbl. It would therefore be nice to get Kd-values for these interactions either by methyl-NMR or other biophysical methods and to discuss this issue.

Minor points:
1) It would be very helpful to the general readership to show a schematic overview of parkin activation steps highlighting the domain organization of parkin, positions / effects of mutations used in this study and catalytically important sites (catalytic Cys and E2 binding

surface) in the introduction or at least in the supplement.

2) Legend Figure 2: "C. Inhibition of" should be "B. Inhibition of"

3) Fig. 5B: There seems to be a general loss of signal intensity upon complex formation. The authors should show a quantification of signal decrease for all residues in the supplement.

4) Methods:
- Phos-tag SDS-PAGE is not explained.
- References to software packages are missing for ImageJ and SPARKY
- Delaglio et al. have developed NMRpipe
- Which software packages were used for statistical analysis, structure modeling and structure representation?
- Why do the authors use R0RBR constructs from different organisms for the NMR experiments?
- Which types of 1H-15N correlation spectra were recorded?
- Why were the spectra in Fig. 5B/C recorded with human R0BR and at 25 C, while those in Fig. 5E were acquired with rat R0BR and at 5 C?

Referee #1 (Report for Author)

Previous studies have shown that Parkin either lacking its PINK1 phosphorylation site (Ser65) or lacking its entire Ubl domain still retained mitophagic activity, albeit at a reduced rate. However, the mechanism behind how Parkin is activated in this context remained unknown. In this manuscript, Sauve et al discover that pUb can bind the RING0 domain within Parkin (where pUbl binds) and that this enables exposure of the catalytic cysteine in RING2 and therefore activation of Parkin. Parkin can therefore bind pUb at two separate sites which explains why S65A mutants of Parkin can still be activated. By having both pUbl and pUb activation modalities via RING0, the authors propose increased robustness of the pathway. Overall, the data in the manuscript are predominantly clear and convincing, and the discoveries help to close the loop on our understanding of Parkin activation mechanisms. The work will be of interest to researchers in multiple fields including Parkin biology, mitophagy and ubiquitin ligases.

1. Figure 1C: Despite not being active in mitophagy, can the authors show whether the mutants, alone or in combination can translocate to mitochondria? How do the mutant protein expression levels compare to WT?

The failure of the C431S and T240R mutants to translocate to mitochondria when expressed individually has been well documented in previous publications, e.g. Ordureau et al., Mol Cell, 2014. Given the failure of the two mutants to complement each other in autoubiquitination assays (Sauvé et al, NSMB, 2018) and in the mtKeima assay as shown here, it seems very unlikely that they would show complementation in the recruitment assay. We would prefer to leave this experiment to the one or two research groups that continue to believe parkin functions as a dimer.

To show equal protein expression, we will add a supplemental figure showing the mean GFP and CFP fluorescence intensity measured during flow cytometry.

2. Figure 1C: It would be beneficial to show whether the T240R mutant can efficiently expose its active site cysteine in RING2 using the Ub-vinyl sulfone experiments?

This experiment can easily be done.

3. Given that the trans activity mechanism maybe slow/less efficient with two mutant proteins, can the authors also confirm that following a longer CCCP treatment has evidence of mitophagy e.g. 8h and 12h?

We will redo the MtKeima complementation assay with longer CCCP treatments.

4. Figure 2: In the text the authors refer to pUb or pUbl concentrations required for Parkin activation e.g. "Addition of pUbΔG76 to wild-type R0RBR led to detectable crosslinking at 20 µM and 50% at 200 µM." Can the authors clarify how many times these experiments were repeated or were the conclusions drawn from single experiments? It would be more convincing to provide some quantitative data and n=3 of the Ub-VS crosslinking experiments to strongly support the data in this figure.

The paper currently shows two sets of experiments (main and supplemental figures) that were performed by two different researchers with different (independent) protein preparations. The experiments with pUb and pUbl have been performed at least three times with highly similar results each time.

5. Figure 3A: It would be interesting to determine whether the Ub-Parkin chimera can drive mitophagy in cells. This would demonstrate that it is functional and active within a cellular context.

The experiments with the chimera used a mutation, A320R, that prevents pUb binding to the RING1 site. Since that site is essential for parkin recruitment to mitochondria, the chimera with the mutation is very unlikely to be active in driving mitophagy in cells. Similarly, without the A320R mutation, the N-terminal pUb will bind to RING1 and prevent recruitment.

6. Figure 4 and the statement "Polyubiquitin chains can be phosphorylated by PINK1 in vitro and polyphosphorylated ubiquitin chains have been detected in cells following mitochondrial depolarization (Ordureau et al, 2014; Wauer et al, 2015b).": It should be noted that typically only the terminal ubiquitin is phosphorylated in cells (Swatek et al (2019) Nature). Therefore, it may be unlikely that Parkin encounters a poly-phosphorylated ubiquitin chain. This does not invalidate the in vitro experiments since they are still highly informative. However, it is unclear whether the pUb chain type and therefore its length will be an important factor in cells. What I think the in vitro data indicates is that Parkin may become more strongly activated on substrates that have two surface lysines at the right distance apart to bridge the two binding sites on Parkin. But, by having its UBL domain Parkin can circumvent this and add more robustness to its activation and expand its activation/recruitment substrate repertoire. It would be worthwhile to discuss these possibilities (at the author's discretion), however, the finding that terminal Ub molecules are typically phosphorylated must be referred to in the context of the pUb chain data.

The reviewer raises a good point. We will add the reference suggested and revise the discussion to clarify that in vivo the two pUb are likely on separate chains attached to different surface lysines.

Minor:

1. The following sentence should be modified to include the discovery of Parkin auto-inhibition by Chaugule et al (2011) EMBO J: "X-ray structures of parkin have revealed that it adopts an autoinhibited conformation in basal cell conditions (Riley et al, 2013; Trempe et al, 2013; Wauer & Komander, 2013)"

We will include the reference in the revised text.

2. Although only a discussion piece, given that it was also raised in the abstract, are there species along the evolutionary path which lack the Parkin UBL domain?

Yes, there are parkin homologs that lack the Ubl domain. We will clarify this upon revision.

Referee #2 (Report for Author)

Parkin is an RBR-type E3 ligase that plays a central role in mitochondrial quality control. The mechanism of Parkin activation has been an area of intense research due to the observation that autosomal mutations in the PARK2 gene which codes for Parkin are associated with early-onset Parkinson's disease. Parkin is cytosolic and autoinhibited under basal cell conditions. Upon sensing of mitochondrial damage, the kinase PINK1 phosphorylates ubiquitin molecules on the mitochondrial surface, which induces accumulation of Parkin on mitochondria and its subsequent activation. This is a multi-step process that involves binding of phosphorylated ubiquitin and phosphorylation by PINK1, which in turn induce structural rearrangements within Parkin that expose the binding site for the E2~Ub conjugate and allow access to the catalytic cysteine to promote formation of the E3~Ub intermediate.

Previous studies into the molecular features underpinning the mechanism of phosphorylation-dependent Parkin activation have provided a multi-faceted picture of the activation process including multiple atomic structures of Parkin along the activation pathway. In parallel, an alternative feedforward (open-cycle) mechanism for Parkin activation has been suggested that does not require its phosphorylation.

This study investigates the molecular details of this phosphorylation-independent mechanism and shows that it involves the the pUbl-binding site in the RING0 domain, which has higher affinity for pUb than for pUbl, which the authors suggest increases the robustness of the pathway for the clearance of damaged mitochondria. The authors also investigate the effect of poly-Ub chains on Parkin activation, which they suggest mimics the mitochondrial surface modified with multiple pUb molecules.

While the authors present some interesting observations about the effect of phosphorylated ubiquitin on Parkin activation it is not clear how much these data further the body of knowledge already available and how physiologically relevant the activation of Parkin by pUb is. Similarly, it is not clear what the experiments aimed at testing the effect of different poly-Ub chain types on Parkin activation tells us about its activation in a cellular context. Overall, the Discussion section of this paper is very speculative leaving the reader to wonder what precisely to take away from the data presented in this paper and how the new insight gained changes our understanding of Parkin activation.

We show that pUb can function both in recruiting parkin to mitochondria and in activating its ubiquitin ligase activity. This addresses the long-standing question of the mechanism responsible for feedforward activation of parkin, a process first described seven years ago. Not only does the mechanism provide insight into the evolutionary development of the PINK1/parkin system, it is highly relevant for active, on-going studies of parkin control of mitophagy in neurons and its activation at synaptic vesicles. While it is true that many questions about parkin remain unanswered, the identification of a novel activation mechanism is clearly relevant and newsworthy.

Specific comments

1) How were the models of tetra-Ub chains bound to Parkin obtained? This needs to be explained otherwise the models shown are just an artist's impression. Why would the K48 chain have the most freedom? Have the authors taken intrinsic conformational flexibility of the different chains into account when creating the models?
The authors need to explain why their assays were done using K6-linked chains, when K48 and K63 chains are more efficient (Fig 4E).
Overall, this section needs to be put into context as to what type of chains are involved in Parkin

activation and how a given chain type may alter the level or kinetics of activation. As it currently stands it doesn't add much information into the function of Parkin.

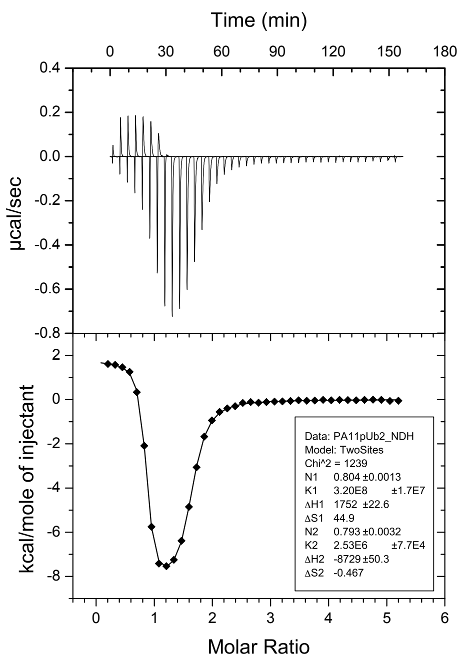
The models in the paper were generated by hand and not intended to be more than visual guides. They were generated using the phospho-parkin delta REP-RING2: pUb structure as a template. The two internal pUb molecules of the tetra-pUb chain were manually placed to bridge the pUb molecules at tetra-pUb extremities without clashing with R0RBR structure.

Our preference would be to remove the models from the supplementary material. If the reviewer feels it is important, we could do more rigorous modeling to generate atomic models of tetra-pUb binding to R0RBR. However, given that poly-phosphorylated ubiquitin chains are only a model of pUb on mitochondria, we'd prefer to remove the models altogether.

K6 pUb chains were used because they were the first ones available from commercial supplier. The assays with other chain types were done after the characterization of activation by K6 pUb chains. Our main point was not to discriminate which type of chains are the best in activating parkin, especially since poly-pUb chains are not very common in cells, but to make sure that we had a chain that could bridge the two binding sites and mimic the cooperativity / avidity of multiple pUb molecules on the mitochondrial surface.

We would suggest revising the description of the chain-type experiments to clarify the points raised by the review. We can also repeat some of the experiments with K48 pUb chains.

2) Based on their NMR experiments the authors estimate pUb to be 5-10 more effective in activating Parkin in functional assays and bind with higher affinity. This interaction needs be explored in much more detail to support this statement as this interaction is at the centre of this study.



We are currently carrying out isothermal titration calorimetry (ITC) experiments to quantify both pUb and pUbl binding to RING0 binding site. The preliminary experiment, shown on the left, clearly shows the existence of two pUb-binding sites on parkin. The opposite enthalpies of binding and hundred-fold difference in affinities allows the two sites to be clearly distinguished. Within a month or so, we should have completed all the experiments for a new figure. We are using mutations in the RING1 and RING0 sites to confirm the identification binding sites. The experiments will directly compare the relative binding affinities of pUb and pUbl as request by the reviewer.

Fig legend. ITC experiment of 379 µM pUb binding to 16.6 µM parkin without the Ubl and RING0 domains (i.e. RING0-RING1-IBR). The curve was fit using a model of two sites with affinities of 3 nM and 395 nM.

Minor comments:

1) It would be helpful for readers not intimately familiar with Parkin structure and mechanism of activation to add a cartoon to Figure 2 showing the structure of autoinhibited Parkin and indicating the domains and binding sites discussed in the text to set the scene. Overall, it might be helpful to add a cartoon next to each experiment to illustrate what interactions are being interrogated.

As suggested by both referees 2 and 4, we will add a cartoon of parkin inactive structure showing the position of key- or mutated residues referred to in our manuscript. We can also include schematic drawings as requested.

Figure 2B: R0RBR K211N appears as a double band and running higher on the SDS gel. What is the reason for this? Please add a sentence explaining why the autoubiquitination assays were done with GST-fusion proteins.

It is not clear why the K211N R0RBR parkin band is more diffuse in Fig 2B. The protein appears as a signal sharp band in Fig 2D and the Supplemental Fig 4. The gel showed some "smiling" which may make it appear that the mutant ran more slowly that wildtype protein. We can rerun the gel if necessary.

2) The authors should specify which amino acid residues are covered in the constructs used in this study and not just refer to previous publication. This is important as there are multiple reports in the literature describing how the inclusion of N-terminal fusion proteins and the precise nature of the construct under investigation affect catalytic activity.

We will add additional information about our constructs to our materials and methods section.

Referee #3 (Report for Author)

This paper reports an attractive iterative analysis of Parkin's activation mechanism in the context of the PINK1/Parkin pathway. The authors describe that phosphorylated ubiquitin can bind to two different sites on Parkin, RING0 (low affinity) and RING1 (high affinity). The authors then characterize in more detail the properties and possible function of the RING0 binding site in the context of Parkin's feedforward activation using various in-vitro and cell culture assays. Overall, I think that the observations made in the first figures are interesting, and the following in-vitro data and interpretation are convincing. Therefore, I believe overall the study is well conducted and presented. The data will be of interest to the PINK1/Parkin community, warranted that possible limitation to the alternative activation model described here is better highlighted in the manuscript.

We will describe the limitations to the alternative activation model.

General comments:

The foundation of this manuscript is based on the report that multiple studies have shown that deletion of the Ubl domain or loss of Ser65 phosphorylation of the Ubl (via Ser65 to Ala mutation) strongly impairs but does not entirely abolish Parkin recruitment to damaged mitochondria. One thing to keep in mind is that all these studies involve various levels of overexpression of Parkin mutant in cells (from transient over-expression to more controlled stable over-expression). I think that overall work in the past few years from several labs, including the authors, has clearly demonstrated that PINK1/Parkin signaling is a finely tuned system, where overexpression of one component would easily create an imbalance in the observed signaling output. In fact, one could argue that several recent studies have demonstrated the essential and critical role for Ubl Ser65 phosphorylation in vivo (mouse model, pmid: 30404819), an in-vitro neuronal model with endogenous CRISPR editing (Ser65Ala, PMID: 29656925; 32142685), or in the context of patient mutation S65N (pmid: 30404819), which would indicate that the feedforward activation mechanism is entirely dependent on Parkin's Ubl phosphorylation. That said, I think the work presented in this manuscript is an interesting and valid one, especially from the evolutionary perspective, but the manuscript would benefit from putting the finding in perspective, perhaps in a "study limitation" paragraph where the findings and relevance of the alternative activation model proposed here could be put into a broader endogenous context.

We will add a "study limitation" paragraph as requested.

- Related to Figure 1: the authors should perform westernblot (Parkin antibody) of the transfected cells used here, to measure fist how much WT Parkin is overexpressed compared to endogenous Parkin present in un-transfected U2OS cells, and second to assess that the level of expression of each mutants is equal and similar to over-express WT Parkin.

We can show the mean fluorescence intensity of GFP/CFP from flow cytometry data (also requested by referee #1). Endogenous Parkin expression is very low in U2OS cells and doesn't contribute to CCCP-induced mitophagy. We can provide a western blot as supplemental figure but the best evidence is the lack of mitophagy in the negative controls (e.g. cells transfected with parkin with the C431S mutation).

- Related to Supplemental Figures S2 and S3 and the graphical representation of mito-mKeima data. A suggestion to the authors, I think these two figures would gain clarity for non-expert readers if the data was presented alongside (or only) as a single bar chart per figure, instead of 42 gated FACS plots (x-axis 405nm; y-axis 561nm), with the gating manually defined.
In a bar chart, the y-axis would be the 561nm/405nm average ratio of 100,000 events (calculated in FlowJo). All triplicate experiments can then be used simultaneously in a single bar chart plot. Authors would then be able to provide an error bar with S.D.. X-axis would be the different cell lines and treatment. In addition, there seems to be a typo in the current FACS representation in both figures, y-axis is labeled as 407nm, when the method section indicates 405nm, and similarly for the y-axis figure indicates 532nm, while the method indicates 561nm.

We will show the ‘ratio-gating’ bar graphs as requested. We thank the reviewer for identifying the error in the wavelengths indicated in the figure. These should be changed to match those in the method section, e.g. 405 nm and 561 nm.

Specific comments:

-Page 5, line 15, (Wauer, 2015, Nature) should be added to citations about K211N that disrupt the phospho-serine binding site.

We apologize for the oversight and will include this reference in the revised manuscript.

- Page 7, line 10, regarding the mention that pUb interferes with E2 discharging and Parkin activity, I would suggest to also add (pmid: 25284222; Ordureau, Mol.Cell. 2014) that initially reported the inhibitory effect of pUB on Parkin at high concentration.

We apologize for the oversight and will include this reference in the revised manuscript.

- Figue 2C, 2E, 4C, are using GST-tag fused truncated version of Parkin R0RBR, while most of the other figures use an untagged version of R0RBR. Unless there is a specific reason for it, I would suggest for consistency across the paper, these three experiments are done with the R0RBR version that does not contain the GST tag. I don't think this is the case here, but in addition, GST is prone to forming dimers, so out of caution, performing these assays without possible interference from the GST tag would be preferable.

GST-tagged parkin has more activity in auto-ubiquitination assays which is generally attributed to the additional lysine residues in the tag acting as substrates. We agree with the review that the presence of the tag is unlikely to have an effect on the activation by pUb or pUbl. That said, it is easy to repeat the assays with parkin without the GST tag.

- Page 8, bottom of the page. The authors mention that poly-ubiquitin chains can be poly-phosphorylated at Ser65 positions, not only in-vitro but also in cells upon mitochondrial depolarization. While this is true, it would be informative for the reader that the authors also comment here on more recent work published using the Ub-clipping method, which reported that while these poly-phosphorylated chains are detectable, they, in fact, represent a very small portion of the phosphorylated Ubiquitin proteoforms (pmid: 31413367 and 32142685). Indeed, phospho-ubiquitin is mainly present in species that are primarily mono- or endcap ubiquitin (e.g., not inside a poly-Ub chain).

We will clarify the text that tetra-pUb was used as a tool to investigate pUb binding to RING0 in a context mimicking the presence of pUb molecules at mitochondrial surface. The use of multiple chain types might give a wrong message. We didn’t aim to investigate the efficiency of parkin activation by low-abundant poly pUb chains.

- Page 10, end of first paragraph, related to Figure 5C. The authors mention the dual phospho-ubiquitin conformation, and their experiment highlights that both conformations led to the detectable binding. Still, rapid exchange of the two conformations prevents the measurement of individual conformation. Have the authors tried to use either Ub L67S mutant or Ub TVLN mutant reported in pmid: 29133469, which stabilized the equilibrium to the C-term-retracted conformation?

We will do Ub-VS assays with the pUb TVLN mutant to test which pUb conformation is binding to RING0 binding site.

Referee #4 (Report for Author)

The major activation pathway of the ubiquitin (Ub) ligase parkin depends on its high-affinity interaction with PINK1-phosphorylated Ub (pUb) that triggers dissociation of the Ub-like (Ubl) domain from the E2 binding RING1 and subsequent phosphorylation of the Ubl by PINK1. Phopho-Ubl (pUbl) interaction with the RING0 in turn releases the RING2 carrying the catalytic Cys to fully activate parkin. However, unphosphorylated / DeltaUbl parkin still possesses residual activity when bound to pUb (Kazlauskaite et al., 2014). Building on these results, Sauve et al. identify in their manuscript "Structural basis for feedforward control in the PINK1/parkin pathway" a mechanism for phosphorylation-independent activation of parkin using localization and mitophagy assays, enzyme activity and NMR interaction studies. The authors show that the RING0 domain can interact with pUb as a monomer or as part of a poly-pUb chain in a manner similar to pUbl and activate parkin thereby circumventing the need for parkin phosphorylation.

The data are of high quality and support the authors' conclusions. Parkin is an important drug target and understanding its regulation is of high importance. However, the relevance of the discovered phosphorylation-independent activation mechanism is not explored to an extent that would justify publication in EMBO Journal in its current form.

We thank the reviewer for the appreciation of the quality of the data but we disagree with the conclusion that the relevance is not sufficient to justify publication in EMBO. As mentioned above, there are many aspects of parkin function which are still not yet understood. Work from our laboratory and others have shown that parkin is activated at synaptic vesicles in a phosphorylation dependent manner. Knowing that parkin can be activated without direct contact with PINK1 is highly relevant when considering models for parkin recruitment to synaptosomes. Further, as the referee points out, our study is highly significant for on-going efforts to develop small-molecule activators of parkin.

Major issues:
The high affinity pUb interaction and release of the Ubl for phosphosphorylation occurs already at low pUb concentrations. The identified phosphorylation-independent activation, however, can only be relevant, if the rather high pUb or poly-pUb concentrations required for saturating the RING0 binding site were reached FASTER than Ubl phosphorylation by PINK1. The question as to how relevant the identified mechanism is in a natural setting, i.e. with WT full-length parkin being present, should therefore be addressed as detailed in the following points:

1) What is the order of Ub and parkin phosphorylation events in vivo? Does (poly-)pUb accumulate much faster at the mitochondrial membrane than phosphorylated parkin?

In Tang et al, Nature comm. 2017 (PMCID: PMC5347139), it was shown that pUb molecules are present at mitochondria surface before parkin is recruited.

2) Stating that the affinity of pUb for the RING0 is stronger than the pUbl interaction in trans may be true, but it is doubtful that this would be the case in cis as in wild-type phosphorylated parkin. First, the authors should make clear at each instance whether the pUbl or pUb interactions that are described are in cis or in trans. Second, the authors should explore whether pUb or poly-pUb can compete for the RING0 when the pUbl is bound to the RING0 in cis (full-length phospho-parkin).

We apologize for the confusion and will make it more obvious in the revised manuscript whether we are referring to interactions *in cis* or *in trans*.

We don't claim that pUb binding to RING0 overtakes pUbl binding to RING0, but that it occurs in parallel. In the absence of pUbl (e.g. experiments with the delta Ubl parkin mutant), pUb-binding to RING0 provides an alternative pathway for parkin activation

3) All in vitro experiments were carried out with parkin constructs lacking the Ubl domain or with the high affinity pUb binding site being mutated. These experiments support the conclusion that parkin can be activated to some degree in a phosphorylation-independent manner. However, to address the relevance of the mechanism and potential competition effects on parkin activity it would be important to include unphosphorylated and phosphorylated full-length parkin in these experiments. Also, a quantification of the levels of unconjugated (not Ub-modified) parkin in the in vitro activity assays would help to consolidate the authors' conclusions.

As discussed in the paper, intramolecular (*in cis*) activation of full-length parkin by pUbl -binding will be much more efficient than intermolecular (*in trans*) activation. We estimate that the effective local intramolecular concentration of pUbl (*in cis*) is about 10 mM, which is much higher than can be obtained when added *in trans*. We'd be happy to include autoubiquitination assays with full-length wild-type parkin to confirm that there is more activation *in cis* than *in trans*.

We will quantify the level of unconjugated parkin in the reactions loaded on SDS-PAGE.

4) The authors conclude from their Ub charging and ubiquitination assays that pUb has a higher affinity for the RING0 domain than the pUbl in trans. In contrast, in the NMR competition experiments (Fig. 5E) the signals of the 15N pUbl are still broadened by complex formation with the R0BR/pUb and the signal of pS65 is not fully recovered even at a two-fold excess of pUb showing that the affinity of pUb cannot be much higher than for the pUbl. It would therefore be nice to get Kd-values for these interactions either by methyl-NMR or other biophysical methods and to discuss this issue.

As described above, we are currently carrying out ITC experiments to quantify both pUb and pUbl binding to RING0 binding site.

Minor points:
1) It would be very helpful to the general readership to show a schematic overview of parkin activation steps highlighting the domain organization of parkin, positions / effects of mutations used in this study and catalytically important sites (catalytic Cys and E2 binding surface) in the introduction or at least in the supplement.

As suggested by both referees 2 and 4, we will add a cartoon of parkin inactive structure showing the position of key- or mutated residues referred to in our manuscript.

2) Legend Figure 2: "C. Inhibition of" should be "B. Inhibition of"

Thank you for pointing out this mistake. We will change it in our revised version.

3) Fig. 5B: There seems to be a general loss of signal intensity upon complex formation. The authors should show a quantification of signal decrease for all residues in the supplement.

We can add a quantification of signal decrease in the supplement.

4) Methods:
- Phos-tag SDS-PAGE is not explained.
- References to software packages are missing for ImageJ and SPARKY
- Delaglio et al. have developed NMRpipe
- Which software packages were used for statistical analysis, structure modeling and structure representation?

We will add references and more details in our material and methods section.

- Why do the authors use R0RBR constructs from different organisms for the NMR experiments?

The choice of human or rat parkin was a matter of convenience. Rat was chosen for the Ubl titration, since the pS65 peak of rat Ubl is better separated from other residues peaks than that of human Ubl. .

- Which types of 1H-15N correlation spectra were recorded?
- Why were the spectra in Fig. 5B/C recorded with human R0BR and at 25 C, while those in Fig. 5E were acquired with rat R0BR and at 5 C?

Details about the types of 1H-15N correlation spectra will be added to the Materials and Methods section. The spectra in Fig 5E were recorded at the lower temperature to enhance detection of the weak binding by pUbl.

1st Editorial Decision **7th Oct 2021**

Thank you for tentative response to the referee reports, and proposal for revising this work for The EMBO Journal. I have now had a chance to consider these plans, and realize that your plans for addressing the concrete experimental concerns especially of the structural referees may be able alleviate many of the major concerns; I also appreciate your responses to most of the conceptual concerns of the biological referees. In conclusion, I shall be happy to consider a revised manuscript further for EMBO Journal publication, and would like to herewith invite you to modify the study as proposed in your draft response. In this respect, please do take note of the comments I added to your responses and suggestions in the attached document.

Referee #1 (Report for Author)

Previous studies have shown that Parkin either lacking its PINK1 phosphorylation site (Ser65) or lacking its entire Ubl domain still retained mitophagic activity, albeit at a reduced rate. However, the mechanism behind how Parkin is activated in this context remained unknown. In this manuscript, Sauve et al discover that pUb can bind the RING0 domain within Parkin (where pUbl binds) and that this enables exposure of the catalytic cysteine in RING2 and therefore activation of Parkin. Parkin can therefore bind pUb at two separate sites which explains why S65A mutants of Parkin can still be activated. By having both pUbl and pUb activation modalities via RING0, the authors propose increased robustness of the pathway. Overall, the data in the manuscript are predominantly clear and convincing, and the discoveries help to close the loop on our understanding of Parkin activation mechanisms. The work will be of interest to researchers in multiple fields including Parkin biology, mitophagy and ubiquitin ligases.

We thank the reviewer for the kind appreciation of our work.

1. Figure 1C: Despite not being active in mitophagy, can the authors show whether the mutants, alone or in combination can translocate to mitochondria? How do the mutant protein expression levels compare to WT?

The failure of the C431S and T240R mutants to translocate to mitochondria when expressed individually has been well documented in previous publications, e.g. Ordureau et al., Mol Cell, 2014. Given the failure of the two mutants to complement each other in autoubiquitination assays (Sauvé et al, NSMB, 2018) and in the mtKeima assay as shown here, it seems very unlikely that they would show complementation in the recruitment assay.

To show equal protein expression, we have added supplemental figures showing western blots and the mean GFP and CFP fluorescence intensity measured during flow cytometry.

2. Figure 1C: It would be beneficial to show whether the T240R mutant can efficiently expose its active site cysteine in RING2 using the Ub-vinyl sulfone experiments?

We have added Appendix Figure S3 that shows that the catalytic cysteine of the parkin becomes exposed upon phosphorylation and in the presence of pUb.

3. Given that the trans activity mechanism maybe slow/less efficient with two mutant proteins, can the authors also confirm that following a longer CCCP treatment has evidence of mitophagy e.g. 8h and 12h?

We have redone the MtKeima complementation assay with longer CCCP treatments and now present the results at 12 h in Figure 1D. We thank the reviewer for the suggestion.

4. Figure 2: In the text the authors refer to pUb or pUbl concentrations required for Parkin activation e.g. "Addition of pUbΔG76 to wild-type R0RBR led to detectable crosslinking at 20 µM and 50% at 200 µM." Can the authors clarify how many times these experiments were repeated or were the conclusions drawn from single experiments? It would be more convincing to provide some quantitative data and n=3 of the Ub-VS crosslinking experiments to strongly support the data in this figure.

The paper currently shows two sets of experiments (main and supplemental figures) that were performed by two different researchers with different (independent) protein preparations. The experiments with pUb and pUbl have been performed at least three times with highly similar results each time. We have added quantification of the autoubiquitination results.

5. Figure 3A: It would be interesting to determine whether the Ub-Parkin chimera can drive mitophagy in cells. This would demonstrate that it is functional and active within a cellular context.

The experiments with the chimera used a mutation, A320R, that prevents pUb binding to the RING1 site. Since that site is essential for parkin recruitment to mitochondria, the chimera with the mutation is very unlikely to be active in driving mitophagy in cells. Similarly, without the A320R mutation, the N-terminal pUb will bind to RING1 and prevent recruitment.

6. Figure 4 and the statement "Polyubiquitin chains can be phosphorylated by PINK1 in vitro and polyphosphorylated ubiquitin chains have been detected in cells following mitochondrial depolarization (Ordureau et al, 2014; Wauer et al, 2015b).": It should be noted that typically only the terminal ubiquitin is phosphorylated in cells (Swatek et al (2019) Nature). Therefore, it may be unlikely that Parkin encounters a poly-phosphorylated ubiquitin chain. This does not invalidate the in vitro experiments since they are still highly informative. However, it is unclear whether the pUb chain type and therefore its length will be an important factor in cells. What I think the in vitro data indicates is that Parkin may become more strongly activated on substrates that have two surface lysines at the right distance apart to bridge the two binding sites on Parkin. But, by having its UBL domain Parkin can circumvent this and add more robustness to its activation and expand its activation/recruitment substrate repertoire. It would be worthwhile to discuss these possibilities (at the author's discretion), however, the finding that terminal Ub molecules are typically phosphorylated must be referred to in the context of the pUb chain data.

The reviewer raises a good point. We have added the reference suggested and revised the discussion to clarify that in vivo the two pUb are likely on separate chains attached to different surface lysines (as shown in Fig 7.)

Minor:

1. The following sentence should be modified to include the discovery of Parkin auto-inhibition by Chaugule et al (2011) EMBO J: "X-ray structures of parkin have revealed that it adopts an autoinhibited conformation in basal cell conditions (Riley et al, 2013; Trempe et al, 2013; Wauer & Komander, 2013)"

We have included the reference in the revised text.

2. Although only a discussion piece, given that it was also raised in the abstract, are there species along the evolutionary path which lack the Parkin UBL domain?

There is a parkin homolog that reportedly lacks the Ubl domain but it is not clear if this is a sequencing artifact or an ancestral form of parkin.

Referee #2 (Report for Author)

Parkin is an RBR-type E3 ligase that plays a central role in mitochondrial quality control. The mechanism of Parkin activation has been an area of intense research due to the observation that autosomal

mutations in the PARK2 gene which codes for Parkin are associated with early-onset Parkinson's disease. Parkin is cytosolic and autoinhibited under basal cell conditions. Upon sensing of mitochondrial damage, the kinase PINK1 phosphorylates ubiquitin molecules on the mitochondrial surface, which induces accumulation of Parkin on mitochondria and its subsequent activation. This is a multi-step process that involves binding of phosphorylated ubiquitin and phosphorylation by PINK1, which in turn induce structural rearrangements within Parkin that expose the binding site for the E2~Ub conjugate and allow access to the catalytic cysteine to promote formation of the E3~Ub intermediate.

Previous studies into the molecular features underpinning the mechanism of phosphorylation-dependent Parkin activation have provided a multi-faceted picture of the activation process including multiple atomic structures of Parkin along the activation pathway. In parallel, an alternative feedforward (open-cycle) mechanism for Parkin activation has been suggested that does not require its phosphorylation.

This study investigates the molecular details of this phosphorylation-independent mechanism and shows that it involves the the pUbl-binding site in the RING0 domain, which has higher affinity for pUb than for pUbl, which the authors suggest increases the robustness of the pathway for the clearance of damaged mitochondria. The authors also investigate the effect of poly-Ub chains on Parkin activation, which they suggest mimics the mitochondrial surface modified with multiple pUb molecules.

While the authors present some interesting observations about the effect of phosphorylated ubiquitin on Parkin activation it is not clear how much these data further the body of knowledge already available and how physiologically relevant the activation of Parkin by pUb is. Similarly, it is not clear what the experiments aimed at testing the effect of different poly-Ub chain types on Parkin activation tells us about its activation in a cellular context. Overall, the Discussion section of this paper is very speculative leaving the reader to wonder what precisely to take away from the data presented in this paper and how the new insight gained changes our understanding of Parkin activation.

> *We show that pUb can function both in recruiting parkin to mitochondria and in activating its ubiquitin ligase activity. This addresses the long-standing question of the mechanism responsible for feedforward activation of parkin, a process first described seven years ago. Not only does the mechanism provide insight into the evolutionary development of the PINK1/parkin system, it is highly relevant for active, on-going studies of parkin control of mitophagy in neurons and its activation at synaptic vesicles. While it is true that many questions about parkin remain unanswered, the identification of a novel activation mechanism is clearly relevant and newsworthy.*

Specific comments

1) How were the models of tetra-Ub chains bound to Parkin obtained? This needs to be explained otherwise the models shown are just an artist's impression. Why would the K48 chain have the most freedom? Have the authors taken intrinsic conformational flexibility of the different chains into account when creating the models?

> *We have chosen to remove the models to avoid misconceptions about the occurrence of tetra-pUb in cells or the nature of the models show in the previous supplemental figure. The models were generated by hand using the phospho-parkin delta REP-RING2: pUb structure as a template. The two internal pUb molecules of the tetra-pUb chain were manually placed to bridge the pUb*

molecules at tetra-pUb extremities without clashes. The models were not intended to be more than visual guides.

The authors need to explain why their assays were done using K6-linked chains, when K48 and K63 chains are more efficient (Fig 4E).

K6 pUb chains were used because they were the first ones available from a commercial supplier. The assays with other chain types were done after the characterization of activation by K6 pUb chains. Our point was not to discriminate which type of chains are the best in activating parkin, especially since poly-pUb chains are not very common in cells, but to make sure that we had a chain that could bridge the two binding sites and mimic the cooperativity / avidity of multiple pUb molecules on the mitochondrial surface.

Overall, this section needs to be put into context as to what type of chains are involved in Parkin activation and how a given chain type may alter the level or kinetics of activation. As it currently stands it doesn't add much information into the function of Parkin.

We agree that the comparison of different chain types doesn't add much information. For simplicity and consistency, we now only show experiments with K48 and K63 pUb chains.

2) Based on their NMR experiments the authors estimate pUb to be 5-10 more effective in activating Parkin in functional assays and bind with higher affinity. This interaction needs be explored in much more detail to support this statement as this interaction is at the centre of this study.

We have carried out isothermal titration calorimetry (ITC) experiments to quantify both pUb and pUbl binding to RING0 binding site. The experiments, presented in a new Figure 6, clearly show the existence of two pUb-binding sites on parkin. The binding reactions show opposite enthalpies of binding and a hundred-fold difference in affinities, which allows the two sites to be clearly distinguished. We used mutations in the RING1 and RING0 sites to confirm the identification binding sites. The experiments allowed the direct comparison of the binding affinities of pUb and pUbl and confirm that pUb has higher affinity.

Minor comments:

1) It would be helpful for readers not intimately familiar with Parkin structure and mechanism of activation to add a cartoon to Figure 2 showing the structure of autoinhibited Parkin and indicating the domains and binding sites discussed in the text to set the scene. Overall, it might be helpful to add a cartoon next to each experiment to illustrate what interactions are being interrogated.

We thank the reviewer for the suggestion, which was also mentioned by referee 4. We have added a schematic of key residues and mutations in parkin as Fig 1A and cartoons of the inactive and active conformations as Extended View Fig 1.

Figure 2B: R0RBR K211N appears as a double band and running higher on the SDS gel. What is the reason for this? Please add a sentence explaining why the autoubiquitination assays were done with GST-fusion proteins.

It is not clear why the K211N R0RBR parkin band is more diffuse in Fig 2B. The protein appears as a signal sharp band in Fig 2D and the Supplemental Fig 4. The gel showed some "smiling" which may make it appear that the mutant ran more slowly that wildtype protein.

We have added a sentence to explain why the autoubiquitination assays used GST-parkin and, as requested by reviewer 3, we have replicated our results with untagged parkin.

2) The authors should specify which amino acid residues are covered in the constructs used in this study and not just refer to previous publication. This is important as there are multiple reports in the literature describing how the inclusion of N-terminal fusion proteins and the precise nature of the construct under investigation affect catalytic activity.

We have added additional information about our constructs to the Materials and Methods section.

Referee #3 (Report for Author)

This paper reports an attractive iterative analysis of Parkin's activation mechanism in the context of the PINK1/Parkin pathway. The authors describe that phosphorylated ubiquitin can bind to two different sites on Parkin, RING0 (low affinity) and RING1 (high affinity). The authors then characterize in more detail the properties and possible function of the RING0 binding site in the context of Parkin's feedforward activation using various in-vitro and cell culture assays. Overall, I think that the observations made in the first figures are interesting, and the following in-vitro data and interpretation are convincing. Therefore, I believe overall the study is well conducted and presented. The data will be of interest to the PINK1/Parkin community, warranted that possible limitation to the alternative activation model described here is better highlighted in the manuscript.

General comments:

The foundation of this manuscript is based on the report that multiple studies have shown that deletion of the Ubl domain or loss of Ser65 phosphorylation of the Ubl (via Ser65 to Ala mutation) strongly impairs but does not entirely abolish Parkin recruitment to damaged mitochondria. One thing to keep in mind is that all these studies involve various levels of overexpression of Parkin mutant in cells (from transient over-expression to more controlled stable over-expression). I think that overall work in the past few years from several labs, including the authors, has clearly demonstrated that PINK1/Parkin signaling is a finely tuned system, where overexpression of one component would easily create an imbalance in the observed signaling output. In fact, one could argue that several recent studies have demonstrated the essential and critical role for Ubl Ser65 phosphorylation in vivo (mouse model, pmid: 30404819), an in-vitro neuronal model with endogenous CRISPR editing (Ser65Ala, PMID: 29656925; 32142685), or in the context of patient mutation S65N (pmid: 30404819), which would indicate that the feedforward activation mechanism is entirely dependent on Parkin's Ubl phosphorylation. That said, I think the work presented in this manuscript is an interesting and valid one, especially from the evolutionary perspective, but the manuscript would benefit from putting the finding in perspective, perhaps in a "study limitation" paragraph where the findings and relevance of the alternative activation model proposed here could be put into a broader endogenous context.

We agree with the reviewer that PINK1/Parkin signaling is a finely tuned system. It is important to understand the different mechanism of parkin activation - both major and minor. As requested by

Reviewer 4, we have added experiments with full-length parkin that show that the parkin is more efficiently activated by the phosphorylation of the Ubl domain in the same polypeptide chain (in cis) than in trans.

- Related to Figure 1: the authors should perform westernblot (Parkin antibody) of the transfected cells used here, to measure fist how much WT Parkin is overexpressed compared to endogenous Parkin present in un-transfected U2OS cells, and second to assess that the level of expression of each mutants is equal and similar to over-express WT Parkin.

We have added western blots showing the expression levels of GFP- and CFP-parkin proteins in the mt-Keima assay as Appendix Figures 2A and 3B. We have also added the mean fluorescence intensity of GFP/CFP from flow cytometry data (also requested by referee #1) as Appendix Figures 2B and 3C. Endogenous Parkin expression is very low in U2OS cells and doesn't contribute to CCCP-induced mitophagy. This can be seen from the lack of mitophagy in the negative controls (e.g. cells transfected with parkin with the C431S mutation.)

- Related to Supplemental Figures S2 and S3 and the graphical representation of mito-mKeima data. A suggestion to the authors, I think these two figures would gain clarity for non-expert readers if the data was presented alongside (or only) as a single bar chart per figure, instead of 42 gated FACS plots (x-axis 405nm; y-axis 561nm), with the gating manually defined.
In a bar chart, the y-axis would be the 561nm/405nm average ratio of 100,000 events (calculated in FlowJo). All triplicate experiments can then be used simultaneously in a single bar chart plot. Authors would then be able to provide an error bar with S.D.. X-axis would be the different cell lines and treatment. In addition, there seems to be a typo in the current FACS representation in both figures, y-axis is labeled as 407nm, when the method section indicates 405nm, and similarly for the y-axis figure indicates 532nm, while the method indicates 561nm.

We have replaced the graphical representation of mito-mKeima data with violin plots in Appendix Figures 2 and 3. We chose not to combine the plots to allow assessment of the reproducibility of the results.

We thank the reviewer for identifying the error in the wavelengths indicated in the figure. These have been changed to match those in the method section, e.g. 405 nm and 561 nm.

Specific comments:

-Page 5, line 15, (Wauer, 2015, Nature) should be added to citations about K211N that disrupt the phospho-serine binding site.

We apologize for the oversight and have included this reference in the revised manuscript.

- Page 7, line 10, regarding the mention that pUb interferes with E2 discharging and Parkin activity, I would suggest to also add (pmid: 25284222; Ordureau, Mol.Cell. 2014) that initially reported the inhibitory effect of pUB on Parkin at high concentration.

We apologize for the oversight and have included this reference in the revised manuscript.

- Figue 2C, 2E, 4C, are using GST-tag fused truncated version of Parkin R0RBR, while most of the other figures use an untagged version of R0RBR. Unless there is a specific reason for it, I would suggest for consistency across the paper, these three experiments are done with the R0RBR version that does not contain the GST tag. I don't think this is the case here, but in addition, GST is prone to forming dimers, so out of caution, performing these assays without possible interference from the GST tag would be preferable.

GST-tagged parkin has more activity in auto-ubiquitination assays which is generally attributed to the additional lysine residues in the tag acting as substrates. We agree with the reviewer that the presence of the tag is unlikely to affect activation by pUb or pUbl; however, we have repeated the assays with parkin without the GST tag to confirm this. The results with untagged R0RBR are shown in Figure EV1B.

- Page 8, bottom of the page. The authors mention that poly-ubiquitin chains can be poly-phosphorylated at Ser65 positions, not only in-vitro but also in cells upon mitochondrial depolarization. While this is true, it would be informative for the reader that the authors also comment here on more recent work published using the Ub-clipping method, which reported that while these poly-phosphorylated chains are detectable, they, in fact, represent a very small portion of the phosphorylated Ubiquitin proteoforms (pmid: 31413367 and 32142685). Indeed, phospho-ubiquitin is mainly present in species that are primarily mono- or endcap ubiquitin (e.g., not inside a poly-Ub chain).

We thank the reviewer for pointing out these important studies. We have specified in the text that tetra-pUb was used as a tool to investigate pUb binding to RING0. Tetra-pUb was used to mimic the presence of multiple pUb molecules at mitochondrial surface rather than be representative of typical phosphorylated ubiquitin chains. These could be part of ubiquitin chains or even single monoubiquitin molecules on mitochondrial outer membrane proteins.

- Page 10, end of first paragraph, related to Figure 5C. The authors mention the dual phospho-ubiquitin conformation, and their experiment highlights that both conformations led to the detectable binding. Still, rapid exchange of the two conformations prevents the measurement of individual conformation. Have the authors tried to use either Ub L67S mutant or Ub TVLN mutant reported in pmid: 29133469, which stabilized the equilibrium to the C-term-retracted conformation?

We thank the reviewer for the interesting suggestion. We performed Ub-VS assays with the pUb TVLN mutant and observed no activation. These suggest that the minor pUb conformation is unable to bind to RING0 binding site and that the loss of signal intensity for the minor form in the NMR experiments is due to exchange between the two conformations.

Referee #4 (Report for Author)

The major activation pathway of the ubiquitin (Ub) ligase parkin depends on its high-affinity interaction with PINK1-phosphorylated Ub (pUb) that triggers dissociation of the Ub-like (Ubl) domain from the E2 binding RING1 and subsequent phosphorylation of the Ubl by PINK1. Phopho-Ubl (pUbl) interaction with the RING0 in turn releases the RING2 carrying the catalytic Cys to fully activate parkin. However, unphosphorylated / DeltaUbl parkin still possesses residual activity when bound to pUb (Kazlauskaite et

al., 2014). Building on these results, Sauve et al. identify in their manuscript "Structural basis for feedforward control in the PINK1/parkin pathway" a mechanism for phosphorylation-independent activation of parkin using localization and mitophagy assays, enzyme activity and NMR interaction studies. The authors show that the RING0 domain can interact with pUb as a monomer or as part of a poly-pUb chain in a manner similar to pUbl and activate parkin thereby circumventing the need for parkin phosphorylation.

The data are of high quality and support the authors' conclusions. Parkin is an important drug target and understanding its regulation is of high importance. However, the relevance of the discovered phosphorylation-independent activation mechanism is not explored to an extent that would justify publication in EMBO Journal in its current form.

> *We thank the reviewer for the appreciation of the quality of the data but we disagree with the conclusion that the relevance is not sufficient to justify publication in EMBO. As mentioned above, there are many aspects of parkin function which are still not yet understood. Work from our laboratory and others have shown that parkin is activated at synaptic vesicles in a phosphorylation dependent manner. Knowing that parkin can be activated without direct contact with PINK1 is highly relevant when considering models for parkin recruitment to synaptosomes. Further, as the referee points out, the study is highly significant for on-going efforts to develop small-molecule activators of parkin.*

Major issues:
The high affinity pUb interaction and release of the Ubl for phosphosphorylation occurs already at low pUb concentrations. The identified phosphorylation-independent activation, however, can only be relevant, if the rather high pUb or poly-pUb concentrations required for saturating the RING0 binding site were reached FASTER than Ubl phosphorylation by PINK1. The question as to how relevant the identified mechanism is in a natural setting, i.e. with WT full-length parkin being present, should therefore be addressed as detailed in the following points:

1) What is the order of Ub and parkin phosphorylation events in vivo? Does (poly-)pUb accumulate much faster at the mitochondrial membrane than phosphorylated parkin?

> *The reviewer is correct that the mechanisms occurring in a natural setting are key questions for future study. In particular, the order of Ub and parkin phosphorylation has been widely debated. In our opinion, it appears most likely that pUb molecules are present at mitochondria surface before parkin is recruited as shown by Tang et al, Nature comm. 2017 (PMCID: PMC5347139). Regardless, we feel that the identification of the mechanism responsible for feedforward activation is an important step forward toward understanding the processes naturally occurring in cells.*

2) Stating that the affinity of pUb for the RING0 is stronger than the pUbl interaction in trans may be true, but it is doubtful that this would be the case in cis as in wild-type phosphorylated parkin. First, the authors should make clear at each instance whether the pUbl or pUb interactions that are described are in cis or in trans. Second, the authors should explore whether pUb or poly-pUb can compete for the RING0 when the pUbl is bound to the RING0 in cis (full-length phospho-parkin).

> *We apologize for the confusion and will make it more obvious in the revised manuscript whether we are referring to interactions in cis or in trans. We don’t claim that pUb binding to RING0*

overtakes pUbl binding to RING0, but rather that it occurs in parallel. In the absence of pUbl (e.g. experiments with the delta Ubl parkin mutant), pUb-binding to RING0 provides an alternative pathway for parkin activation.

Whether pUb or poly-pUb can compete for the RING0 when the pUbl is bound is an interesting question but the result wouldn't be detectable in assays of parkin activity since both pUb or pUbl binding to RING0 should lead to activation. As pUbl is available intramolecularly (in cis), we would expect that phosphorylated parkin would generally not have pUb or poly-pUb bound to the RING0 domain.

3) All in vitro experiments were carried out with parkin constructs lacking the Ubl domain or with the high affinity pUb binding site being mutated. These experiments support the conclusion that parkin can be activated to some degree in a phosphorylation-independent manner. However, to address the relevance of the mechanism and potential competition effects on parkin activity it would be important to include unphosphorylated and phosphorylated full-length parkin in these experiments. Also, a quantification of the levels of unconjugated (not Ub-modified) parkin in the in vitro activity assays would help to consolidate the authors' conclusions.

As discussed in the paper, intramolecular (cis) activation of full-length parkin by pUbl -binding is much more efficient than intermolecular (trans) activation. We estimate that the effective local intramolecular concentration of pUbl (in cis) is about 10 mM, which is much higher than can be obtained when added in trans. Nonetheless, to avoid any misconceptions, we have included autoubiquitination assays with full-length wild-type parkin to confirm that there is more activation by pUbl (or pUb) when present in cis than in trans.

We have added quantification of the levels of unconjugated parkin in the reactions loaded on SDS-PAGE. In our experience, loss of the unmodified protein is more accurate and reproducible than measurements of the broad smear of ubiquitinated proteins.

4) The authors conclude from their Ub charging and ubiquitination assays that pUb has a higher affinity for the RING0 domain than the pUbl in trans. In contrast, in the NMR competition experiments (Fig. 5E) the signals of the 15N pUbl are still broadened by complex formation with the R0BR/pUb and the signal of pS65 is not fully recovered even at a two-fold excess of pUb showing that the affinity of pUb cannot be much higher than for the pUbl. It would therefore be nice to get Kd-values for these interactions either by methyl-NMR or other biophysical methods and to discuss this issue.

As described above, we have added ITC experiments to quantify pUb and pUbl binding to RING0 binding site. The NMR titrations with labeled pUb and pUbl in Fig 5 are not directly comparable due to the lower temperature using for the experiments with labeled pUbl. Experiments done at the same temperature (RT) confirmed the weaker binding of pUbl. We chose not to show those experiments since the ITC experiments are quantitative and more directly address the question of the relative affinities.

Minor points:
1) It would be very helpful to the general readership to show a schematic overview of parkin activation steps highlighting the domain organization of parkin, positions / effects of mutations used in this study and catalytically important sites (catalytic Cys and E2 binding surface) in the introduction or at least in the supplement.

We thank the reviewers for the excellent suggestion. We have added a schematic of the domain organization and key residues mutated in Fig 1A and models of the active and inactive conformations in EV Fig 1. In addition, the new ITC figure with the ITC data (Fig 6) shows a schematic models of the regulatory R0BR core and the RING1 and RING0 domain showing the binding sites for pUb and pUbl.

2) Legend Figure 2: "C. Inhibition of" should be "B. Inhibition of"

We thank the reviewer for pointing out the error, which has been corrected in the revised version.

3) Fig. 5B: There seems to be a general loss of signal intensity upon complex formation. The authors should show a quantification of signal decrease for all residues in the supplement.

The reviewer is correct. The titrations involve a dilution of the labeled sample, which causes a decrease in signal intensity for all the residues.

4) Methods:
- Phos-tag SDS-PAGE is not explained.
- References to software packages are missing for ImageJ and SPARKY
- Delaglio et al. have developed NMRpipe
- Which software packages were used for statistical analysis, structure modeling and structure representation?

We have added the requested references along with additional details to the Materials and Methods section.

- Why do the authors use R0RBR constructs from different organisms for the NMR experiments?

The choice of human or rat parkin was a matter of convenience. Rat was chosen for the Ubl titration, since the pS65 peak of rat Ubl is better separated from other residues peaks than that of human Ubl. An explanation has been added to the Materials and Methods section.

- Which types of 1H-15N correlation spectra were recorded?
- Why were the spectra in Fig. 5B/C recorded with human R0BR and at 25 C, while those in Fig. 5E were acquired with rat R0BR and at 5 C?

Details about the types of 1H-15N correlation spectra have added to the Materials and Methods section. The spectra in Fig 5E were recorded at the lower temperature to enhance detection of the weak binding by pUbl.

Thank you again for submitting your revised manuscript for our consideration. It has now been re-reviewed by the original referees 3 and 4, whose comments are copied below. As you will see, while referee 3 is satisfied with the revisions, referee 4 still retains a few reservations that I feel should be answered to in an additional round of minor revision. The majority of their points seem to be addressable by textual/presentational changes, but point 2 would require complementation of the ITC assays with two additional constructs. On the other hand, point 3 (pUb concentration estimation via NMR or FRET) would appear beyond the scope of this second revision for me, but I would nevertheless be interested in your response to this request.

I am therefore returning the manuscript to you for a second round of revision, hoping you will be able to address the remaining scientific and editorial concerns in a timely manner. Should you have any questions/comments with regard to the requirements/options for addressing the remaining reviewer points, please do not hesitate to contact me for further discussion.

Referee #3:

The authors have accurately answered the questions I posed and reflected them in the text of the paper, so I consider this revised paper acceptable.

Referee #4:

Review EMBO-J-2021-109460R

In the revised manuscript the authors have addressed some of the points raised by the reviewers and consolidated their finding of a second pUb binding site in parkin with additional ITC experiments and in vitro assays.

While pUb binding to the RING0 domain and its potential to activate parkin is very well supported by the data, the authors still fail to make sufficiently clear that the addition of pUbl to the parkin R0RB(R) constructs is an entirely artificial situation. Free pUbl does not exist in cells and therefore it is in a sense misleading to compare pUb and pUbl binding in trans. The questions that are relevant in an in vivo setting are rather:

i) Can pUb displace the RING2 domain from the RING0 in unphosphorylated parkin and activate the enzyme? The authors have addressed this question showing that rather high concentrations of pUb are required for activation which may be achieved in poly-pUb chains. Such high concentrations of pUb would however require a high degree of PINK1 activity. This in turn would entail the question as to how large the fraction of unphosphorylated parkin would be in a situation of high PINK1 activity given that PINK1 phosphorylates both Ub and the parkin Ubl.

ii) Can pUb compete with the pUbl bound in cis in phosphorylated parkin? Given that the concentrations of (poly-)pUb (chains) can be high at the mitochondrial surface it may be possible that the second pUb binding site is not only relevant in unphosphorylated parkin.

In this sense, the authors should address the following points:

1) As already pointed out in the previous review it should be made clear throughout the text when comparing pUb and pUbl binding that the pUbl is added in trans, as opposed to binding in cis as in full length parkin. Therefore, the following sentences should be modified to:
- In the abstract: "RING0 site has higher affinity for phospho-ubiquitin than phosphorylated Ubl" in trans.
- p. 4: "pUb can bind to the pUbl-binding site and, in fact, has higher affinity than the pUbl domain " in trans.
- p. 11: "to directly measure the affinity of pUb and pUbl" in trans.
"Comparison of pUbl and pUb binding shows that pUbl binds RING0 with slightly weaker (four-fold) affinity" in trans.
- p. 13: "the RING0 site is not strongly selective although it exhibits a slightly higher affinity for pUb" than the pUbl added in trans "in functional assays"

2) The ITC experiments should be complemented with measurements of the WT and mutant R0RBR constructs. This will take into account the displacement of the RING2 and thus more closely reflect the in vivo situation.
Together with point 3), this would also add experimental data to the speculation in the discussion for which it is unclear where the numbers 99% and 10 mM stem from and the Kd of 1.3 µM refers to a pUbl interaction in trans ("We can deduce that pUbl has higher intrinsic affinity than RING2 for RING0 since pUbl is able to displace RING2 when both are present in the same polypeptide chain. If we assume that i) autoinhibited parkin is 99% inactive, ii) the local concentration of RING2 is 10 mM, and iii) phosphorylated parkin is 99% active, then the intrinsic affinity for binding pUbl should be 1 µM. This estimate is remarkably close to the 1.3 µM affinity measured by ITC (Figure 6).").

3) As requested in the previous review it would be important to get an estimate on the pUb concentrations required for pUbl displacement in a construct containing the pUbl in cis. While I agree that this question will be difficult to address in activity assays, NMR spectroscopy or FRET would be very well suited.

4) The Discussion section should contain a more rigorous discussion of the two questions (i and ii) outlined above.

5) p. 7: Fig. 2 is very dense. It would be nice to refer to the panels in Fig. 2 directly (e.g. Fig. 2A, upper left panel). The entire description of the experiments shown in Fig. 2 on p. 6/7 would benefit from more references to the panels in Fig. 2.

6) p. 8: GST-tagged "phosphorylated full-length parkin".

7) p. 8: "Only 24% of unmodified parkin band was still present in the in cis reaction compared to 83% for the in trans reaction". Please refer to the corresponding panels in the figure so that it is obvious where these numbers stem from.

8) p. 10: Fig. 5A is mentioned after Fig. 5B and C. Fig. EV4A is actually Fig. S5 in the Appendix. It would be nice to have it as Fig. EV4A though.

9) p. 13: The last paragraph on p. 13 lacks a discussion that pUb activation is less efficient than pUbl activation in cis.

10) The authors should describe the ITC measurements in more detail in the Methods section. What sample concentrations and injection volumes were used etc.?

Referee #4:

Review EMBO-J-2021-109460R

In the revised manuscript the authors have addressed some of the points raised by the reviewers and consolidated their finding of a second pUb binding site in parkin with additional ITC experiments and in vitro assays.

While pUb binding to the RING0 domain and its potential to activate parkin is very well supported by the data, the authors still fail to make sufficiently clear that the addition of pUbl to the parkin R0RB(R) constructs is an entirely artificial situation. Free pUbl does not exist in cells and therefore it is in a sense misleading to compare pUb and pUbl binding in trans. The questions that are relevant in an in vivo setting are rather:

> The reviewer appears to be missing the point. *In vitro* studies are artificial, simplified systems used to dissect and understand the *in vivo* situation. The true tests of the relevance of pUb activation are the *in vivo* assays (Fig. 1) that show that loss of pUbl leads to only a partial loss of recruitment and mitophagy in cells. The purpose of the *in vitro* assays is not to reproduce the situation of parkin on the mitochondrial membrane. They are tools for the dissecting and understanding the *in vivo* situation.
>
> The reviewer is correct that free pUbl is unlikely to exist in any significant concentrations in cells; however, the same is true for pUb. Ub only becomes phosphorylated when on mitochondria (see, for example, Okatsu et al, 2015 J Cell Biol 209:111-28). Indeed, the presence of significant amounts free pUb would prevent parkin localization to mitochondria. When tethered to parkin as tetra-pUb, pUb is able to activate at micromolar concentrations (Fig. 4). As shown in the model of Fig. 7, we are not suggesting that free pUb binds to parkin on mitochondria. Rather, the presence of multiple pUb molecules on mitochondria can act both in the recruitment and activation of parkin.

i) Can pUb displace the RING2 domain from the RING0 in unphosphorylated parkin and activate the enzyme? The authors have addressed this question showing that rather high concentrations of pUb are required for activation which may be achieved in poly-pUb chains. Such high concentrations of pUb would however require a high degree of PINK1 activity. This in turn would entail the question as to how large the fraction of unphosphorylated parkin would be in a situation of high PINK1 activity given that PINK1 phosphorylates both Ub and the parkin Ubl.

> As the reviewer points out, the recruitment and mitophagy assays in Figure 1 show that pUb can in fact displace the RING2 domain and activate unphosphorylated parkin *in vivo*. The efficiency of this activation mechanism appears to be 20 to 40% compared to parkin phosphorylation (Figure 1C). Unfortunately, we don't currently have a way to

selectively inactivate the feed-forward activation to measure its contribution when both activation mechanisms are present.

Parkin is recruited to mitochondria by binding pUb so the bound parkin is always in the presence of at least one pUb molecule. There is no reason to believe that the feed-forward mechanism requires a high degree of PINK1 activity. More likely, the basal level of ubiquitin present on the mitochondrial surface plays a larger role. The feed-forward mechanism requires multiple pUb molecules to be present in proximity while parkin activation by phosphorylation requires proximity of PINK1 molecule and an adjacent pUb. It would be interesting to explore if the two activation mechanisms are differentially sensitive to the basal level of mitochondrial ubiquitination but, hopefully, the reviewer will agree that those experiments are out of the scope of the current study.

ii) Can pUb compete with the pUbl bound in cis in phosphorylated parkin? Given that the concentrations of (poly-)pUb (chains) can be high at the mitochondrial surface it may be possible that the second pUb binding site is not only relevant in unphosphorylated parkin.

We agree that it is possible that pUb could displace pUbl bound to RING0. It is unclear how to test this with parkin on mitochondria since the result of either pUb or pUbl binding is activation of the ligase. In NMR experiments, we observed competition between free pUbl and pUb for binding to RING0 (Fig. 5E). This is unlikely to occur in solution with pUbl bound *in cis* due to the effective, high local concentration.

In this sense, the authors should address the following points:

1) As already pointed out in the previous review it should be made clear throughout the text when comparing pUb and pUbl binding that the pUbl is added in trans, as opposed to binding in cis as in full length parkin. Therefore, the following sentences should be modified to:
- In the abstract: "RING0 site has higher affinity for phospho-ubiquitin than phosphorylated Ubl" in trans.

- p. 4: "pUb can bind to the pUbl-binding site and, in fact, has higher affinity than the pUbl domain " in trans.

- p. 11: "to directly measure the affinity of pUb and pUbl" in trans.
"Comparison of pUbl and pUb binding shows that pUbl binds RING0 with slightly weaker (four-fold) affinity" in trans.

- p. 13: "the RING0 site is not strongly selective although it exhibits a slightly higher affinity for pUb" than the pUbl added in trans "in functional assays"

We have modified the text to specify the pUbl was "in trans".

2) The ITC experiments should be complemented with measurements of the WT and mutant R0RBR constructs. This will take into account the displacement of the RING2 and thus more closely reflect the in vivo situation.

As requested, we performed an ITC experiment to measure pUb binding to the RING0 domain in the WT R0RBR construct. We expected that the RING2 *in cis* should strongly compete with the pUb *in trans*, preventing binding. In agreement with expectations, we saw only a small amount heat released upon pUb addition, suggesting little or no binding. It is impossible to make a definitive conclusion as it possible that the enthalpy of RING2 release matched the enthalpy of pUb binding but a fitting of the peaks in the thermogram is consistent with near millimolar affinity. This weak binding agrees with published ITC experiments with full-length and R0RBR parkin that did not detect a second pUb binding site (Sauvé et al, EMBO J, 2015; Kumar et al, EMBO J, 2015).

We again point out that free pUb does not reflect the *in vivo* situation. pUb is found on mitochondria where it acts to recruit and, to a certain degree, activate parkin.

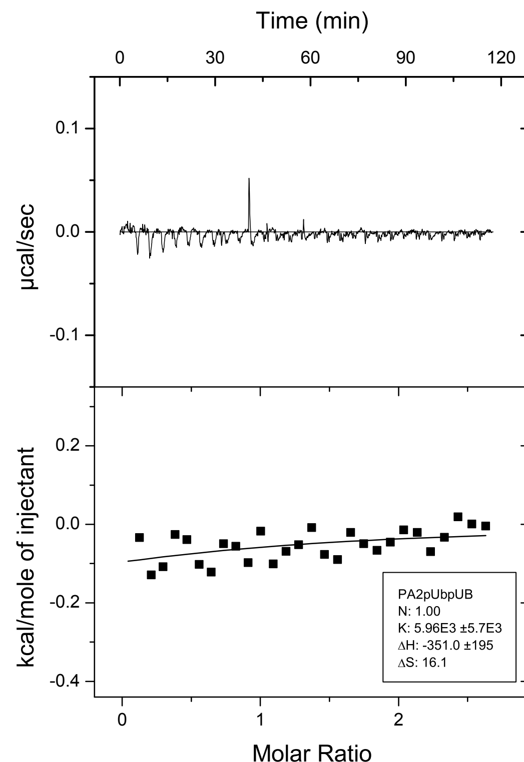


Figure legend. Isothermal titration calorimetry of a one-to-one complex of R0RBR and pUb (63 µM R0RBR:pUb) titrated with pUb (730 µM) in 50mM Tris-HCl; 150 mM NaCl, 1 mM TCEP, pH 7.4 at 20°C (one injection of 5 µl followed by 28 injections of 10 µl). The integrated heats of binding were fit with one-to-one binding stoichiometry and an affinity of ~200 µM.

Together with point 3), this would also add experimental data to the speculation in the discussion for which it is unclear where the numbers 99% and 10 mM stem from and the Kd of 1.3 µM refers to a pUbl interaction in trans ("We can deduce that pUbl has higher intrinsic affinity than RING2 for RING0 since pUbl is able to displace RING2 when both are present in the

same polypeptide chain. If we assume that i) autoinhibited parkin is 99% inactive, ii) the local concentration of RING2 is 10 mM, and iii) phosphorylated parkin is 99% active, then the intrinsic affinity for binding pUbl should be 1 μM. This estimate is remarkably close to the 1.3 μM affinity measured by ITC (Figure 6).").

The discussion has been expanded to explain the origin of the estimates. The estimation of the intramolecular concentration was based on the volume accessible to the tethered domain. Assuming the linker restricts the binding partner to a sphere with diameter of 100 Å, the average concentration can be calculated to be of 3.2 mM or roughly in the range of 1 to 10 mM. The higher value was used in the discussion since the volume of the sphere is not equally sampled - the effective concentration is not equal throughout. The estimates of 1% and 99% for parkin activity were based on the parsimonious assumption that the levels of repression or activation will be no larger than required.

3) As requested in the previous review it would be important to get an estimate on the pUb concentrations required for pUbl displacement in a construct containing the pUbl in cis. While I agree that this question will be difficult to address in activity assays, NMR spectroscopy or FRET would be very well suited.

We agree that it would be interesting to know this but, as the reviewer points out, the actual measurements will be difficult. It is also not clear what the biological significance of the displacement would be since parkin is active in both conformations.

4) The Discussion section should contain a more rigorous discussion of the two questions (i and ii) outlined above.

The discussion has been expanded to elaborate on the points raised above.

5) p. 7: Fig. 2 is very dense. It would be nice to refer to the panels in Fig. 2 directly (e.g. Fig. 2A, upper left panel). The entire description of the experiments shown in Fig. 2 on p. 6/7 would benefit from more references to the panels in Fig. 2.

We thank the reviewer for the suggestion and have added more detailed references to the panels in Fig. 2.

6) p. 8: GST-tagged "phosphorylated full-length parkin".

The experiment shows a comparison of GST-tagged R0RBR + equimolar pUb in trans (Fig. 2D) activity with that of untagged full-length parkin in cis (Fig. EV2A).

7) p. 8: "Only 24% of unmodified parkin band was still present in the in cis reaction compared to 83% for the in trans reaction". Please refer to the corresponding panels in the figure so that it is obvious where these numbers stem from.

> We thank the reviewer for the suggestion and have added more detailed references to the panels in Fig. 2.

8) p. 10: Fig. 5A is mentioned after Fig. 5B and C. Fig. EV4A is actually Fig. S5 in the Appendix. It would be nice to have it as Fig. EV4A though.

> We thank the review for the corrections. We have changed the order of the panels in Figure 5 and moved Fig. S5 to the EV figure.

9) p. 13: The last paragraph on p. 13 lacks a discussion that pUb activation is less efficient than pUbl activation in cis.

> The discussion has been expanded to elaborate on the points raised above.

10) The authors should describe the ITC measurements in more detail in the Methods section. What sample concentrations and injection volumes were used etc.?

> We apologize for the oversight. The sample concentrations were given in the Figure EV5 but the injection volumes were removed from the Materials section during editing. The number of injections and volumes are now included.

2nd Revision - Editorial Decision 8th Apr 2022

Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ➔

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Prof. Kalle Gehring
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2021-109460R1

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.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- ➔ the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ➔ figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- ➔ graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- ➔ if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- ➔ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

- ➔ a specification of the experimental system investigated (eg cell line, species name).
- ➔ the assay(s) and method(s) used to carry out the reported observations and measurements
- ➔ an explicit mention of the biological and chemical entity(ies) that are being measured.
- ➔ an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- ➔ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- ➔ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- ➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
- ➔ definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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.

USEFUL LINKS FOR COMPLETING THIS FORM

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B- Statistics and general methods

Please fill out these boxes ➔ (Do not worry if you cannot see all your text once you press return)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size for the assays was preset to three.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	One set of recruitment assays was excluded because of small response observed in both the experimental and controls samples.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	NA
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	NA

Is the variance similar between the groups that are being statistically compared?	One-way ANOVA with Tukey's post-test was used to account for multiple comparisons in the mt-Keima assays.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Rabbit anti-Parkin antibody (Ab15954, AbCam) - https://www.abcam.com/parkin-antibody-ab15494.html; PRK8 antibody (Ab77924, AbCam) - https://www.abcam.com/parkin-antibody-prk8-ab77924.html
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The U2OS cells used were the same as in the previous publication: Tang MY, Vranas M, Krahn AI, Pundlik S, Trempe JF, Fon EA (2017) Structure-guided mutagenesis reveals a hierarchical mechanism of Parkin activation. Nat Commun 8: 14697. The cells were used without further characterization.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No