EMBO reports

PINK1 autophosphorylation is required for ubiquitin recognition

Shafqat Rasool, Naoto Soya, Luc Truong, Nathalie Croteau, Gergely L Lukacs, Jean-François Trempe

Review timeline:

Submission date:	14 August 2017
Editorial Decision:	2 October 2017
Revision received:	3 January 2018
Editorial Decision:	19 January 2018
Revision received:	26 January 2018
Accepted:	1 February 2018

Editor: Martina Rembold

Transaction Report:

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

1st Editorial Decision 2 October 2017

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge the potential interest of the findings. However, referees 1 and 2 also point out several technical concerns and have a number of suggestions for how the study should be strengthened, and I think that all of them should be addressed. Referee 1 suggests to test experimentally if poly-ubiquitin chains are a better substrate for PINK1 than mono-ubiquitin. Referee 2 suggests to validate the proposed role of S205/S228 phosphorylation for human PINK1 and to validate the NMR results and the data obtained with the truncated TcPINK1 fragment.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix

includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Regarding data quantification, please ensure to specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in all respective figure legends.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

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REFEREE REPORTS

Referee #1:

PINK1 is the first kinase identified that is capable of phorphorylating ubiquitin (on Ser65). It is a mitochondrially targeted kinase that in addition to phosphorylating f ubiquitin phosphorylates Parkin on its Ubl domain. Both of these events are required for activation of Parkin, an E3 ligase that plays an important role in mitochondrial quality control.
The discovery that PINK1 can act as a ubiquitin kinase caused a lot of excitement and there is a lot of interest in characterizing its role on a molecular level and understand the features that mediate specificity for ubiquitin. Unfortunately, human PINK1 has been difficult to study so far due to problems with protein expression and activity issues. Hence all studies in this field have been carried out using PINK1 orthologs from insect species that express well and are active.

In this manuscript, the authors use Tribolium castaneum PINK1 to study its mechanism and show that autophosphorylation on residue Ser205 is required for substrate recognition. This is a carefully executed study of high technical quality that should be of interest to a broad audience. I have no major concerns about the work but there are a range of points that should be addressed to make the manuscript more easily accessible and clarify some of the conclusions drawn.

Based on their data, the authors suggest a model in which Ub chains on mitochondrial proteins are the main substrate and which once phosphorylated serve to localize Parkin to mitochondria and trigger its activation. This implies that Ub chains would be phosphorylated faster than mono Ub, which should be experimentally testable. On a more general level, it is not clear to me where these chains come from and how their synthesis is triggered. And does their topology matter? The authors should discuss this. This model also implies that the real activator of Parkin are phosphorylated

polyUb chains and not mono Ub. Is there any evidence for this? Please discuss.

Minor points

- Please always indicated if you are talking about hPINK1 or TcPink1. This is rather confusing, especially in the discussion.

- Page 4 last paragraph, "Using 2-dimensional NMR and phosphorylation we established... " Please be explicit - "phosphorylation assays"?

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- Page 7, please provide references for the statement "the ability of kinases to interact with their substrates.....autophosphorylation".

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- Figure S3 is very difficult to follow, maybe the authors could clearly state what is what with different colours as they did in the main manuscript in figure 4. Moreover, is the label KD referring to the mutant D337N? If yes this should just be indicated as "D337N" for clarity.

- The Appendix Figure 5 should also contain: q range of the registered SAXS curves and values for I(0), Rg and Dmax.

- Page 11 second paragraph, the reference to "Figure 3 A,C" is wrong.

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- Figure 3D is very difficult to understand. Maybe provide a more lengthy description in the figure legend.

- Regions of the HSQC spectra containing the resonances used to obtain the Relative Chemical shift graphs reported in figure 4 should be reported in the supporting information.

- Page 16, first sentence - please rephrase, it's not clear at all what this sentence means.

- Page 18, where are the molecular weights reported estimated using Vc reported?

- The authors should show a gel in the Appendix that demonstrates the linearity of the phos-tag gels

as all their quantification of phosphorylation assays relies on this.

- Some sentences are clunky or have missing words. Please check.

- This manuscript describes a nice story but I'm not convinced that the discussion does the story justice.

Referee #2:

This paper addresses fundamental questions regarding how the Parkinson's disease kinase PINK1 recognises its ubiquitin substrates. Due to technical challenges posed by the human PINK1 enzyme, the authors study a homolog of PINK1, Tribolium castaneum (TribPINK1) that shares over 40% identity with the human kinase domain. The major findings of the paper are identification of the surface interface on the Ubl domain (of Parkin) and ubiquitin (Ub) through which TribPINK1 binds. They also reveal a role for TribPINK1 autophosphorylation at residue Serine 205 towards substrate binding and phosphorylation. They show by hydrogen-deuterium exchange mass spec that phosphorylation of Serine 205 leads to major changes in the conformation of TribPINK1 especially in regions important for kinase activation and catalysis. Their mass spec analysis combined with SAX also reveal some novel insights into the structure of TribPINK1.

Overall the findings represent a significant advance in understanding the molecular regulation of ubiquitin by its upstream kinase PINK1. That said some of the analyses lack controls that would bolster the conclusions. Serine 205 is also conserved in human PINK1 (Serine 228) yet no analysis has been undertaken with the human enzyme to validate the mechanism of PINK1 regulation they have uncovered for Serine 205 phosphorylation. This could be addressed by straight forward cell based assays of human PINK1. This is expanded below.

MAJOR POINTS:

1. A major finding in the paper is that TribPINK1 autophosphorylates at Ser205 in trans. I am not an expert in mass spectrometry but in my opinion inspecting the mass spec analysis in Figure 3 and EV4 does not completely rule out that another Serine 2 residues down (Serine 207) also comprises a phosphorylation site. It seems that at least one other paper has suggested that Ser207 in addition to Ser205 is a phosphorylation site. The authors should show the MS/MS fragment spectrum for the phospho-Ser205 peptide that demonstrates that's the site and not Ser207. They could also repeat their mass spec analysis using a S205A or S205N version of the kinase dead TribPINK1 substrate to show that the phosphorylation is indeed absent.

2. Ser205 is conserved in human PINK1 (Ser228). Whilst a previous study has reported that human PINK1 is autophosphorylated at Ser228, no previous studies have examined the role of this site for substrate phosphorylation. The authors should compare the ability of wild-type and S228A/N human PINK1 to phosphorylate Parkin and ubiquitin in standard cell based assays using readily available phospho-ubiquitin and phospho-Parkin antibodies.

3. The authors undertake kinetic analysis of parkin Ubl and Ub phosphorylation with TribPINK1 and show that Ubl has a 10 fold lower Km than Ub (Fig 1). However, all of these assays have been performed with truncated TribPINK1 fragments (121-570 or 143-570). Perhaps removal of the N-terminus may disrupt the recognition of Ub but not Ubl by TribPINK1. It would be important to confirm this analysis with the full length TribPINK1 enzyme. It would also be interesting for the authors to consider at some stage repeating the analysis with Ub chains - dimers and tetramers - as they speculate in the Discussion that these Ub molecules are likely to be phosphorylated in cells rather than monomeric Ub (however this study is not required for this paper).

4. The NMR studies of TribPINK1 have been undertaken using a GST-fusion version of the enzyme. This would induce artificial dimerization of the protein and may alter the interaction with Ubl and Ub. Furthermore, their NMR data reveals very little overlap between the Ubl and Ub residues whose NMR signal is lost upon PINK1 interaction. The binding site mutation data shown is not wholly convincing with only I44A and R72A showing reduced phosphorylation by phostag of Ubl. In my opinion, the data would be further strengthened if the authors could address some of the

below points.

• Check that the Ubl/Ub surface mutants studied in Fig 1D and EV3 are not destabilising as an alternative explanation for reduced phosphorylation
• Engineer appropriate mutations in the human Parkin protein for cell based analysis by human PINK1 to determine whether Ubl surface mutants impair Parkin phosphorylation by human PINK1 after mitochondrial uncoupler treatment.
• They show in Figure 3 that the binding of TribPINK1 is mutually exclusive with the RING1 domain of Parkin and the SH3 domain of Endophilin. Whilst this is likely, how can the exclude that the NMR binding conditions permit non-specific interactions with any protein at these particular Ubl surface residues. Therefore it would be better if the authors showed GST alone or an unrelated protein does not lead to loss of signal loss by NMR of Ubl and/or Ub at residues mediated by TribPINK1 interaction.

MINOR POINTS:

1. They report using densitometry to measure the degree of phosphorylation of Ubl and Ub by phostag analysis. The signals in Appendix Figure S1 look extremely saturated and it is unclear whether the densitometry software used is actually quantitative.

2. In Figure 4, the conclusions of how Ser205 phosphorylation may alter the TribPINK1 structure by HDX-MS analysis would be significantly bolstered if they study a non-phosphorylatable mutant (S205A/N) were studied in parallel.

3. In Figure 4C, the authors show that mutation of Serine disrupts binding of the kinase dead trans substrate to the Ubl domain by NMR analysis. However, their kinase analysis in Figure 4A shows that the Ubl domain can still be phosphorylated albeit reduced whereas the Ub phosphorylation is abolished. It would be useful to show the NMR analysis of binding of the S205A/N mutants with Ub.

4. On page 6 the final paragraph should be rewritten to explain more clearly the interactions between Ubl, TribPINK1, RING1 domain of Parkin and SH3 domain. I am unable to follow what is written in this section.

5. On page 9 line 2 the authors state that Fig 3B shows that the transphosphorylation activity is retained by S205A/N mutants however this data is not shown. It is critical that this data is included.

6. On page 10 they refer to a figure in Appendix Fig S4 as showing expression of the isolated C-terminal region of TribPINK1. However, the figure does not show this and instead shows kinase domain containing fragments of TribPINK1 that lack the C-terminus.

1st Revision - authors' response 3 January 2018

Point-by-point response to each reviewer's comments (in italic) are highlighted in bold.

Reviewer #1

PINK1 is the first kinase identified that is capable of phorphorylating ubiquitin (on Ser65). It is a mitochondrially targeted kinase that in addition to phosphorylating f ubiquitin phosphorylates Parkin on its Ubl domain. Both of these events are required for activation of Parkin, an E3 ligase that plays an important role in mitochondrial quality control.
The discovery that PINK1 can act as a ubiquitin kinase caused a lot of excitement and there is a lot of interest in characterizing its role on a molecular level and understand the features that mediate specificity for ubiquitin. Unfortunately, human PINK1 has been difficult to study so far due to problems with protein expression and activity issues. Hence all studies in this field have been carried out using PINK1 orthologs from insect species that express well and are active.

In this manuscript, the authors use Tribolium castaneum PINK1 to study its mechanism and show that autophosphorylation on residue Ser205 is required for substrate recognition. This is a carefully

executed study of high technical quality that should be of interest to a broad audience. I have no major concerns about the work but there are a range of points that should be addressed to make the manuscript more easily accessible and clarify some of the conclusions drawn.

We thank the reviewer for the encouraging and positive comments.

Based on their data, the authors suggest a model in which Ub chains on mitochondrial proteins are the main substrate and which once phosphorylated serve to localize Parkin to mitochondria and trigger its activation. This implies that Ub chains would be phosphorylated faster than mono Ub, which should be experimentally testable. On a more general level, it is not clear to me where these chains come from and how their synthesis is triggered. And does their topology matter? The authors should discuss this. This model also implies that the real activator of Parkin are phosphorylated polyUb chains and not mono Ub. Is there any evidence for this? Please discuss.

We acknowledge that a more in-depth look at how topology of polyUb chains in general affects its phosphorylation would have been in order, and we have addressed this issue both experimentally and in the discussion. First, we have performed phosphorylation time-course of diubiquitin (Ub_2) linked via Lys6, Lys48 and Lys63 (p. 6 in the results section, new Figs 1E and Appendix Fig S3, and p. 12 in the discussion). Based on our NMR titration, we inferred that those three lysines would likely be implicated in PINK1 binding, and thus formation of an isopeptide linkage at these side-chains might interfere with phosphorylation of the proximal subunit (the distal would be unaffected). We observe that all Ub_2 chains are phosphorylated only slightly faster than monoUb at equal molar concentration, which might be caused by an avidity effect (the molar concentration of Ub *subunits* being double that of monoUb). However, there is a marked preference for a single phosphorylation event, in particular for K6-Ub_2 where no doubly phosphorylated species could be observed. This experiment confirms the PINK1 binding site on Ub, and suggests that Ub chains are preferentially phosphorylated on the distal subunit.

Regarding the origin and topology of the chains that serve to recruit Parkin on mitochondria, we believe that the word "chain" is somewhat misleading and we have re-worded our discussion and now write about tethered ubiquitin. Indeed, we and others have observed that phospho-ubiquitinated proteins appear as a ladder of bands above 75 kDa by immunoblotting (new Fig 4D; Tang et al. 2017; Puschmann et al. 2017). These bands could come from mitochondrial outer membrane proteins such as mitofusin (75 kDa) with one or more pUb linked to it, which we call "tethered" ubiquitin, to distinguish from "chains" that imply formation of isopeptide bond between two ubiquitin moieites. While we know that mitochondria undergoing Parkin-mediated autophagy do have polyubiquitin chains of various linkages including K6, K48 and K63 (Durcan et al. 2014; Ordureau et al. 2014), it is not clear whether the initial pUb that appear on mitochondria prior to Parkin activation is on the distal end of a polyUb chains or single pUb tethered to OMM proteins. Given that pUb alone has high affinity for Parkin, both would be efficient in recruiting Parkin. This is now discussed on p. 12.

Minor points

- Please always indicated if you are talking about hPINK1 or TcPink1. This is rather confusing, especially in the discussion.

We have clarified this point by indicating "hPINK1" or "TcPINK1" when referring to specific experiments. However, when talking more generally about the mechanism of PINK1 regulation, which would be applicable to all animals, we then simply use "PINK1".

- Page 4 last paragraph, "Using 2-dimensional NMR and phosphorylation we established... " Please be explicit - "phosphorylation assays"?

Corrected

- Page 5: What is the effect of PD mutations on Ubl phosphorylation?

This has already been investigated by Iguchi et al. (2013) in the context of full-length Parkin and hPINK1. The mutations E240K and G309D abolish Parkin phosphorylation. More recently, Kumar et al (2017) showed that E217K in TcPINK1 abolish Ubl phosphorylation. Here, the data shown was a validation of our system, rather than an extensive study.

- Page 6 first paragraph, "We attribute this primarily to the oligomerization that takes place in TcPINK1 at concentration above 100 uM, ..." Do the authors have a citation for this or this based on their own observations? If yes, these data should be included.

The size-exclusion chromatogram of TcPINK1 shown in Appendix Fig S8 shows that there is oligomerization. We have also observed that GST-TcPINK1 tends to precipitate over time at room temperature at concentrations above 5 mg/mL. Moreover, given the new observation that Ub may adopt a minor conformation that makes it suitable for phosphorylation by PINK1 (Gladkova et al. 2017), it is also possible that the observed effect arises from conformational exchange in ubiquitin. We have updated the text on p.6, and refer to an entirely new discussion paragraph (p. 13) for the explanation.

- Page 6 second paragraph, the authors talk of "parkinized" Ub mutants. Why not just say "we mutated ubiquitin to the corresponding residues in the Ubl of PARKIN"?

Corrected

- Page 7 second paragraph, what is the biological relevance of the Parkin Ubl- Endophilin-A1 SH3 domain interaction? Please explain and also comment on the competition between the SH3 domain and PINK1 for the Ubl. Under what physiological circumstances does such an competition take place?

Here, we have used the SH3 domain as a mean of estimating the K_d of the PINK1:Ubl interaction, as well as to confirm the binding site on the Ubl, which overlaps with that of PINK. However, given that the biological function of the endophilin-A1 SH3 :Parkin Ubl interaction remains unclear, we are unsure whether the competition has any biological relevance. We have updated the discussion on p. 13 (2nd paragraph) to explain why we used the SH3 domain, and what the biological consequences of the competition might be.

- Page 7, please provide references for the statement "the ability of kinases to interact with their substrates.....autophosphorylation".

We have inserted (Endicott et al. 2012) as a reference (now on top of p.8).

- Values of the dissociation constants derived from NMR experiments for the Ubl-SH3 complex and for the Ubl-PINK1 complex should be reported in Figure 2D for clarity.

Corrected

- Page 8 last paragraph. Recombinant TcPINK1 S205A and S205N are still catalytically active but according to the authors they have lost the ability to phosphorylate Ubl and Ub. From Figure 4A this seems to be true only for Ub, for Ubl phosphorylation is still clearly visible. The authors should rephrase and state that they have lower kinase activity for the Ubl than the wild type protein and discuss this in their interpretation of the data

We have replaced the word “lost” by “are impaired”, and “loss” by “reduced”, and added a sentence to improve interpretation. The results can easily be explained by the fact that Ubl has a lower K_m (higher affinity) than Ub for TcPINK1. Thus, at the same concentration, the Ubl is completely phosphorylated after 5 min, whereas only 50% of ubiquitin is phosphorylated after 30 min (Fig 4A, lanes 2, 5). With the S205A/N mutants, about 20% phosphorylation remains for Ubl, and undetectable levels for Ub. Here, it is worth emphasizing that the assay was performed at 30 µM Ub/Ubl, i.e. near the K_m for Ubl.

- Figure S3 is very difficult to follow, maybe the authors could clearly state what is what with different colours as they did in the main manuscript in figure 4. Moreover, is the label KD referring to the mutant D337N? If yes this should just be indicated as "D337N" for clarity.

We agree. Labels were added to this figure (now Fig S6) and KD was replaced by D337N.

- The Appendix Figure 5 should also contain: q range of the registered SAXS curves and values for I(0), Rg and Dmax.

The q-range is shown on top of the scattering profile in this figure (now Fig S8A). R_g values were already shown on the SEC plot (top-left graph in S8A, right axis) and on the extracted scattering profile (top-right graph in S8A). The I_0 values were added to the latter graph. Both R_g and I_0 are also indicated on the Guinier plot for the monomer (Fig S8B). The D_{max} value used to compute the *P(r)* function is shown in Fig S8B.

- Page 11 second paragraph, the reference to "Figure 3 A,C" is wrong.

We corrected for "Fig 2A".

- Please indicate the program used for the primary sequence alignment in Figure EV1.

We used the MUSCLE server to perform the sequence alignment for the segment TcPINK1$^{121-570}$. This is now indicated in the legend of Fig EV1.

- Reports the PDB codes for both structures reported in Figure 2A.

The PDB codes were already indicated in the figure legend. We have added them to the main figure for clarity.

- Figure 3D is very difficult to understand. Maybe provide a more lengthy description in the figure legend.

We have upgraded the diagram to indicate that the kinase-dead D337N cannot phosphorylate in *cis*. We have also improved the description in the figure legend. Finally, we have added a couple of sentences in the discussion to explain how this experiment and the structure strongly suggest the implausibility of *cis* phosphorylation.

- Regions of the HSQC spectra containing the resonances used to obtain the Relative Chemical shift graphs reported in figure 4 should be reported in the supporting information.

Given that each graph is the average of 10 chemical shifts perturbations in 7 experiments, the resulting figure would have 70 panels, which would be a bit overwhelming. Instead we have decided to provide a link to high-resolution pictures of the entire spectra, superposed with the same color-coding as in Appendix Fig. S6. The hyperlink is provided in the legend of the Fig. S6.

- Page 16, first sentence - please rephrase, it's not clear at all what this sentence means.

We have rephrased this sentence, which now appears on the end of p. 18.

- Page 18, where are the molecular weights reported estimated using Vc reported?

They were reported in Appendix Fig. S8A (top right). This is now clearly indicated in the methods section.

- The authors should show a gel in the Appendix that demonstrates the linearity of the phos-tag gels as all their quantification of phosphorylation assays relies on this.

See new panel in Appendix Fig. S1B, which shows the linearity of the response.

- Some sentences are clunky or have missing words. Please check.

Corrected

- This manuscript describes a nice story but I'm not convinced that the discussion does the story justice.

Thank you again. We have added many paragraphs to improve our discussion, regarding all the points raised by the reviewers. We hope this is now satisfactory.

Referee #2:

This paper addresses fundamental questions regarding how the Parkinson's disease kinase PINK1 recognises its ubiquitin substrates. Due to technical challenges posed by the human PINK1 enzyme, the authors study a homolog of PINK1, Tribolium castaneum (TribPINK1) that shares over 40% identity with the human kinase domain. The major findings of the paper are identification of the surface interface on the Ubl domain (of Parkin) and ubiquitin (Ub) through which TribPINK1 binds. They also reveal a role for TribPINK1 autophosphorylation at residue Serine 205 towards substrate binding and phosphorylation. They show by hydrogen-deuterium exchange mass spec that phosphorylation of Serine 205 leads to major changes in the conformation of TribPINK1 especially in regions important for kinase activation and catalysis. Their mass spec analysis combined with SAX also reveal some novel insights into the structure of TribPINK1.

Overall the findings represent a significant advance in understanding the molecular regulation of ubiquitin by its upstream kinase PINK1. That said some of the analyses lack controls that would bolster the conclusions. Serine 205 is also conserved in human PINK1 (Serine 228) yet no analysis has been undertaken with the human enzyme to validate the mechanism of PINK1 regulation they have uncovered for Serine 205 phosphorylation. This could be addressed by straight forward cell based assays of human PINK1. This is expanded below.

We thank the reviewer for their positive and constructive comments. As you will read below, we have addressed all of the points raised.

MAJOR POINTS:

1. A major finding in the paper is that TribPINK1 autophosphorylates at Ser205 in trans. I am not an expert in mass spectrometry but in my opinion inspecting the mass spec analysis in Figure 3 and EV4 does not completely rule out that another Serine 2 residues down (Serine 207) also comprises a phosphorylation site. It seems that at least one other paper has suggested that Ser207 in addition to Ser205 is a phosphorylation site. The authors should show the MS/MS fragment spectrum for the phospho-Ser205 peptide that demonstrates that's the site and not Ser207. They could also repeat their mass spec analysis using a S205A or S205N version of the kinase dead TribPINK1 substrate to show that the phosphorylation is indeed absent.

The MASCOT analysis provided in Table EV2 unambiguously show that *trans* phosphorylation of TcPINK1-D337N occurs only at Ser205 (99.6% confidence). We now provide the MS/MS spectrum of this peptide to further reinforce the strength of the finding (new Fig EV4B). However, we would like to point out that we can also detect Ser207 phosphorylation in the WT protein, in the form of a 205,207-doubly phosphorylated peptide (Table EV1). Moreover, S207 phosphorylation is resistant to CIP treatment, and therefore appears as a minor earlier-eluting peak in the extracted ion chromatogram (EIC) of ^{15}N-WT phospho-197-212 (see Fig 3E, bottom). MS/MS data for the re-phosphorylated protein (new Fig EV4D) unambiguously shows that these two isobaric peptides have distinct phosphorylation sites. Finally, the reviewer should note that the S207 phosphorylation EIC is absent from the ^{14}N-D337N sample (Fig 3E, top), suggesting S207 phosphorylation is non-specific and only takes place after several hour overexpression in *E. coli*.

2. Ser205 is conserved in human PINK1 (Ser228). Whilst a previous study has reported that human PINK1 is autophosphorylated at Ser228, no previous studies have examined the role of this site for

substrate phosphorylation. The authors should compare the ability of wild-type and S228A/N human PINK1 to phosphorylate Parkin and ubiquitin in standard cell based assays using readily available phospho-ubiquitin and phospho-Parkin antibodies.

We have followed the reviewer's recommendations and performed the experiments suggested. We obtained PINK1-KO U2OS cells from the lab of Edward Fon and transfected with hPINK1 expressed from a modified promoter for low expression. In agreement with our phosphorylation assays with TcPINK1 WT and S205A/N, cells transfected with PINK1 S228A show a drastic decrease in the level of phosphoSer65-Ub compared to PINK1 WT following treatment with CCCP (shown in figure 4D). We performed immunoblots against phospho-Parkin using a polyclonal antibody (Abcam; ab154995) however we were unable to detect endogenous phospho-Parkin in either WT or S228A transfected cells (data not provided). Multiple studies have shown that only a small fraction of the total cellular parkin is phosphorylated (Tang and Vranas 2017, Iguchi et al. 2013) and is sufficient to drive the pathway. In our experimental setup that involved endogenous Parkin, we were not able to detect this fraction. Nonetheless, the striking difference in the levels of phospho-Ub between WT and S228A is consistent with our experiments with TcPINK1 that show that the role of S228 phosphorylation is conserved in both species.

3. The authors undertake kinetic analysis of parkin Ubl and Ub phosphorylation with TribPINK1 and show that Ubl has a 10 fold lower Km than Ub (Fig 1). However, all of these assays have been performed with truncated TribPINK1 fragments (121-570 or 143-570). Perhaps removal of the N-terminus may disrupt the recognition of Ub but not Ubl by TribPINK1. It would be important to confirm this analysis with the full length TribPINK1 enzyme. It would also be interesting for the authors to consider at some stage repeating the analysis with Ub chains - dimers and tetramers - as they speculate in the Discussion that these Ub molecules are likely to be phosphorylated in cells rather than monomeric Ub (however this study is not required for this paper).

We have succeeded in expressing and purifying full-length TcPINK1, and obtained sufficient amounts to conduct phosphorylation assays. The time-course was performed with equal concentrations of Ub and Ubl, and clearly shows that Ubl is phosphorylated faster than Ub (Appendix Fig. S1D). Thus, the N-terminal residues do not significantly affect the preference for the Ubl.

Moreover, since reviewer #1 also asked us how chains behaved as TcPINK1 substrates, we have performed phosphorylation time course with K6, K48 and K63-Ub_2 (new Figs 1E and Appendix Fig S3). As explained above, we observe that all Ub_2 chains are phosphorylated only slightly faster than monoUb at equal molar concentration, which might be caused by an avidity effect (the molar concentration of Ub *subunits* being double that of monoUb). However, there is a marked preference for a single phosphorylation event, in particular for K6-Ub_2 where no doubly phosphorylated species could be observed. This experiment confirms the PINK1 binding site on Ub, and suggests that Ub chains are preferentially phosphorylated on the distal subunit. We have also added a substantial discussion on that topic (p.12).

4. The NMR studies of TribPINK1 have been undertaken using a GST-fusion version of the enzyme. This would induce artificial dimerization of the protein and may alter the interaction with Ubl and Ub. Furthermore, their NMR data reveals very little overlap between the Ubl and Ub residues whose NMR signal is lost upon PINK1 interaction. The binding site mutation data shown is not wholly convincing with only I44A and R72A showing reduced phosphorylation by phostag of Ubl. In my opinion, the data would be further strengthened if the authors could address some of the below points.

• Check that the Ubl/Ub surface mutants studied in Fig 1D and EV3 are not destabilising as an alternative explanation for reduced phosphorylation

The reviewer's concern about the stability Ubl mutants is indeed valid. We report 1D proton NMR on GST-fusion Ub WT and mutants to address this issue. We have used the characteristic chemical shift Cδ2 Leu61 (a hydrophobic core residue) in Ubl as a reporter of the folding status of Ubl mutants (Fig EV3C). The identical position of this chemical group across WT Ubl and mutants spectra indicates that mutants are folded similarly to WT Ubl. A

previous study has already shown that the K48A mutation has no significant effect on the stability of Ubl (Safadi et al. 2007). The same study also showed that the AR-JP mutation R42P results in protein unfolding and can be observed in the form drastic changes in the NMR chemical shifts of hydrophobic core residues. We have chosen the Leu61 peak specifically for this analysis, as it lies in a very distinct region of the 1-D spectrum. Since untagged-Ubl displays the same peak, this peak cannot originate from GST.

• Engineer appropriate mutations in the human Parkin protein for cell based analysis by human PINK1 to determine whether Ubl surface mutants impair Parkin phosphorylation by human PINK1 after mitochondrial uncoupler treatment.

Because the phospho-Parkin antibody was not effective in our hands, we did not carry out this assay. Moreover, some of the mutants would release Ubl from RING1, and this would give a confounding result, where a mutation could increase access to PINK1, but reduce its affinity, and the magnitude of the opposing effects would be difficult to predict. However, we would like to point that we can achieve a consistent reduction is phosphorylation of Ubl mutants by using GST-TcPINK1$^{121-570}$ (Fig EV3B), as well as GST-TcPINK1$^{143-570}$ (EV3A), showing the phenomenon is reproducible in different experimental settings.

• They show in Figure 3 that the binding of TribPINK1 is mutually exclusive with the RING1 domain of Parkin and the SH3 domain of Endophilin. Whilst this is likely, how can the exclude that the NMR binding conditions permit non-specific interactions with any protein at these particular Ubl surface residues. Therefore it would be better if the authors showed GST alone or an unrelated protein does not lead to loss of signal loss by NMR of Ubl and/or Ub at residues mediated by TribPINK1 interaction.

As suggested by the reviewer, we performed our TROSY NMR experiment with both ^{2}H,^{15}N-Ubl and ^{2}H,^{15}N-Ub with GST to rule out the possibility that these molecules bind GST (Appendix Fig S2A). No significant signal loss was observed, and therefore the signal loss we observe in Fig 1B is specific to TcPINK1 WT. To improve the visual rendering of this titration, we have decided to superpose the spectra and shift them on the y-axis. In addition, we have also performed an additional titration of ^{2}H,^{15}N-Ubl with GST-TcPINK1 D337N (Appendix Fig S2B), which show only minor peak loss, consistent with the reduced affinity measured in our NMR competition assay (Fig. 4C).

MINOR POINTS:

1. They report using densitometry to measure the degree of phosphorylation of Ubl and Ub by phostag analysis. The signals in Appendix Figure S1 look extremely saturated and it is unclear whether the densitometry software used is actually quantitative.

The densitometry is quantitative. As shown in the new Appendix Fig. S1B, the densitometry measurements are linear with ubiquitin concentration.

2. In Figure 4, the conclusions of how Ser205 phosphorylation may alter the TribPINK1 structure by HDX-MS analysis would be significantly bolstered if they study a non-phosphorylatable mutant (S205A/N) were studied in parallel.

Unfortunately, this was not possible because the S205A/N are heterogeneously phosphorylated when purified from *E. coli* (see Appendix Fig S5A). This leads to considerable variability in exchange rates for the same peptide in different states, which makes interpretation difficult. We will leave it as is for now, and will further investigate the dynamics in future studies when we are capable of producing homogenous WT TcPINK1 with no phosphorylation.

3. In Figure 4C, the authors show that mutation of Serine disrupts binding of the kinase dead trans substrate to the Ubl domain by NMR analysis. However, their kinase analysis in Figure 4A shows that the Ubl domain can still be phosphorylated albeit reduced whereas the Ub phosphorylation is abolished. It would be useful to show the NMR analysis of binding of the S205A/N mutants with Ub.

We appreciate the comment, but unfortunately this experiment is not possible because the SH3 domain of endophilin-A1 does not bind Ub (see Trempe et al. 2009). However, we do not believe that the S205A/N mutants affect more Ub than Ubl. As requested by reviewer #1, we have re-worded this section (p. 9) to explain that the results can easily be explained by the fact that Ubl has a lower K_m (higher affinity) than Ub for TcPINK1. Thus, at the same concentration, the Ubl is completely phosphorylated after 5 min, whereas only 50% of ubiquitin is phosphorylated after 30 min (Fig. 4A, lanes 2, 5). With the S205A/N mutants, about 20% phosphorylation remains for Ubl (100%->20%), and undetectable levels for Ub (50%->0%). Here, it is worth emphasizing that the assay was performed at 30 µM Ub/Ubl, i.e. near the K_m for Ubl. To better understand this, let's assume phosphorylation at S205 changes the K_m by 10-fold; then the K_m of S205A for the Ubl would be approx. 400 µM. In this case, the TcPINK1^{S205A}-Ubl kinetics would be similar to TcPINK1^{WT}-Ub, which is what we observe. For Ub, the S205A mutant would have a K_m of 4 mM, which is 100X greater than the concentration, and thus no phosphorylation is observed.

4. On page 6 the final paragraph should be rewritten to explain more clearly the interactions between Ubl, TribPINK1, RING1 domain of Parkin and SH3 domain. I am unable to follow what is written in this section.

We have re-worded this paragraph, now on p. 7. We hope this is clearer.

5. On page 9 line 2 the authors state that Fig 3B shows that the transphosphorylation activity is retained by S205A/N mutants however this data is not shown. It is critical that this data is included.

The 3rd panel in Fig 3B actually shows that GST-TcPINK1-S205N can phosphorylate untagged D337N at a level similar to WT (2nd panel). The data does show that S205N can trans-phosphorylate.

6. On page 10 they refer to a figure in Appendix Fig S4 as showing expression of the isolated C-terminal region of TribPINK1. However, the figure does not show this and instead shows kinase domain containing fragments of TribPINK1 that lack the C-terminus.

This comment is accurate. However, we have decided to remove this figure, as we deemed it unnecessary in the context of the new crystal structures of insect PINK1, which show that the C-terminal extension is indeed part of the C-lobe.

<u>To both reviewers</u>

We would like to point out that we re-analyzed our SAXS and HDX data in the light of the new crystal structures of PINK1 that were recently published (Kumar et al. 2017; Schubert et al. 2017). While our initial manuscript was submitted before these papers were published, we felt it was essential to incorporate this new data in our analysis. The availability of coordinates for both TcPINK1$^{151-570}$ and PhPINK1$^{148-575}$ allowed us to make a better use of our SAXS data and explore the solution conformation of TcPINK1$^{121-570}$. In brief, we used the two structures to make high-quality models of TcPINK1, and then perform rigid-body fit with flexible linkers analysis using the software CORAL. We found that the PhPINK1 structure fits rather well the SAXS data, mostly because its insert 3 is ordered. We then used this model to map the HDX results. In this way, our HDX results are more relevant, and are rather consistent with the position of different structural elements. The new analysis is shown in Fig 5A-D, as well as Appendix Fig S8C-D.

2nd Editorial Decision 19 January 2018

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see both referees are positive about the study and support publication in EMBO reports with only a minor revision.

Browsing through the manuscript myself, I noticed a few minor editorial things that we need before we can proceed with the acceptance of your study.

We look forward to seeing a final version of your manuscript as soon as possible. Please let me know if you have questions or comments regarding the revision.

****
REFEREE REPORTS

Referee #1

All my concerns have been adequately addressed.
The authors have done a good job re-analyzing their SAXS and HDX data in light of the recently published crystal structures of Pink1 and adjusting the Discussion accordingly.

Minor point: The reference Kaiser et al. 2011, which is mentioned in the Discussion, is missing in References.

Referee #2

The authors have significantly revised this study and addressed the bulk of the Reviewers concerns to the best of their ability. I recommend that the paper be accepted for publication

2nd Revision - authors' response 26 January 2018

The authors performed all minor editorial changes.

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: Jean-Francois Trempe
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2017-44981V2

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 $\chi 2$ 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.

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
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?	NA
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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.	NA
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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?	NA

C- Reagents

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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).	All antibodies cat# and provider are provided in the methods section p. 17
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	U2OS PINK1 KO cell line generated by CRISPR/Cas9 in Edward Fon's lab at the Montreal Neurological Institute. Recently tested for mycoplasma contamination. See Methods section p.16

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

D- Animal Models

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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
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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
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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
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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

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