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Mitochondrial Processing Peptidase Regulates PINK1 Processing, Import and Parkin Recruitment

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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. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 17 August 2011

Thank you for the submission of your research manuscript to EMBO reports. It has been sent to three referees, and so far we have received reports from two of them, which I copy below. As both referees feel that the manuscript is interesting and recommend that you should be given a chance to revise it, I would like to ask you to begin revising your manuscript according to the referees' comments. Please note that this is a preliminary decision made in the interest of time, and that it is subject to change should the third referee offer very strong and convincing reasons for this. As soon as we receive the third report it will be forwarded to you.

In his/her report, referee #1 expresses several concerns that should be discussed in the manuscript. However, s/he also suggests some experiments regarding mitochondrial integrity and considers that a direct link between MPPbeta reduction and mitochondrial clearance should be provided, a concern shared with referee #2 (point 4). S/he also remarks, in partial agreement with referee #2, that Fig. 4 needs to include further controls of proteinase K activity.

Referee #2, besides the points already mentioned, is concerned with the experiments performed using RNAi techniques. S/he particularly points out to the experiments regarding MMPbeta siRNAs

and suggests the addition of more controls. As the model suggested by your manuscript is that mitochondria depolarization leads to PINK1 stabilization, s/he also suggests that it should be tested whether these proteases are affected or not under depolarization conditions. Finally, s/he considers that data in Fig. 3 must be further discussed and a quantitative analysis of PINK1 levels should be provided.

Given the reviewers constructive comments and the potential interest of your study, I would like to give you the opportunity to revise your manuscript, with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

I look forward to seeing a revised version of your manuscript when it is ready.

Yours sincerely,

Editor
EMBO reports

REFEREE REPORTS:

Referee #1:

Mitochondrial processing of the important recessive Parkinson's disease gene product PINK1 is a timely topic that is currently receiving much well deserved attention, in part because of its regulation mitophagy, a mechanism potentially clearing damaged mitochondria and hence of great possible pathogenic importance. A number of very recent reports (all cited here) point to PARL as an important PINK1 processing enzyme. The authors have for the first time performed an unbiased RNAi screen of practically all mitochondrial proteases. They confirm a role of PARL in the second PINK1 cleavage step. Moreover, they discover that knockdown of the m-AAA protease AFG3L2 has a similar effect on PINK1 processing as siPARL. In addition, the catalytic subunits of endopeptidase Clp and particularly MPP are identified as potential primary PINK1 processing enzymes. In the validations, most focus is on MPPβ. Partial knockdown of MPPβ leads to dramatic accumulation of unprocessed, full-length PINK1. It appears that reduction of its MTS cleavage leads to an accumulation of full-length PINK1 at the outer mitochondrial membrane (OMM) of healthy mitochondria, possibly by impairment of import. This causes the anticipated recruitment of parkin, known to occur via accumulated OMM PINK1 during mitophagy.

This good manuscript could be improved by addressing a few points:
1. It is noteworthy that the paraplegin-like protein AFG3L2 involved in PINK1 processing similar to PARL is linked to spinocerebellar ataxia type 28. This additional neurodegeneration link could be mentioned perhaps.
2. Mitochondrial integrity was determined biochemically. Morphological photomicrographs are required to state that mitochondrial network in regard to fusion and fission is not affected. Moreover it would be nice to assess the mitochondrial clearance via the parkin recruitment after siMPPβ treatment. In this regard, the study ends somewhat abruptly.
3. One major concern is the remarkable specificity for PINK1. Should MPP knockdown not prevent processing and import of all mitochondrial proteins? Why is the effect so exceptionally prominent for PINK1?
4. The PK digests in Fig. 4A must be shown side-by-side on a single immunoblot. Complete with additional mitochondrial markers (e.g. for the matrix, IMM and OMM).
5. Specify the used antibodies exactly, the companies often offer several ones.

Referee #2:

Comments to Authors

This is an interesting study by Greene and colleagues, who found that besides the reported PARL mitochondrial protease, MPP, m-AAA and ClpXP are three other proteases that are apparently capable of PINK1 processing. In particular, MPP-mediated PINK1 processing appears to be the crucial step for subsequent PINK1 import and further processing. Importantly, Si-RNA mediated MPP silencing leads to PINK1 accumulation on the surface of the mitochondria and the concomitant recruitment of parkin. Given the current intense interest in the mechanics of parkin/PINK1 pathway in mitochondrial quality control, the novel findings presented by Greene and colleagues in this study are important and timely. Moreover, the experiments are well thought of and systematically performed.

Notwithstanding the above, I do have a couple of comments about the report in its present form, which I have discussed below.

1. This is largely a RNAi-based study and a formal concern of such a mode of gene silencing unlike overt genetic ablation is the presence of off-target effects. To the authors' credit, they have acknowledged and tried to exclude this concern but perhaps it might be helpful in this case to perform a "rescue" experiment via overexpression of MPP-beta (RNAi-refractory if needed) in otherwise MPP-beta-silenced cells (notwithstanding that overexpression studies also have their own set of problems). Related to this, MPP-alpha overexpression in MPP-beta knockdown cells might also shed some useful insights on its proposed regulatory role in PINK1 processing.

2. Taken together, the results indirectly suggest that mitochondrial depolarization leads to the downregulation (or inactivation) of MPP-beta and relevant mitochondrial proteases, which collectively promotes the accumulation of PINK1 on the mitochondria. From a physiological point of view, it would be important to examine if this is indeed true, i.e. is the level and/or activity of the various implicated proteases (particularly MPP-beta) affected in the presence of mitochondrial depolarization?

3. The results in Fig. 3 suggest that both CCCP treatment and MPP-beta silencing can retard PINK1 cleavage into the 52 kD product. Accordingly, a corresponding increase in full length PINK1 is seen in both cases (Fig. 3A). Curiously, neither full length nor the presumed MPP-cleaved PINK1 (that migrates just under the full length protein) accumulates appreciably in the mitochondrial-enriched fractions of PARL and Clp knockdown cells. The authors should discuss this. It will be helpful to provide quantitative data of full length and MPP-cleaved PINK1 alongside the 52kD cleavage product data shown in Fig. 3B.

4. Finally, while it is good to note that parkin recruitment to the mitochondria occurs following mitochondrial PINK1 stabilization in MPP-beta-silenced cells, it would be interesting to determine if parkin localization to the mitochondria under such conditions is sufficient to trigger mitophagy.

Minor:

- It is intriguing to note that the level of Rieske is visibly affected (i.e. decrease) in the presence of both MPP-alpha and -beta silencing

- A time-dependent study of the action of proteinase K (at low concentration) would serve as a useful accompaniment to Fig. 4A.

Correspondence 22 August 2011

Thank you again for the submission of your research manuscript to EMBO reports. As I mentioned in my previous letter, your manuscript was sent to three referees and we have just received the third report, which I copy below. As all three referees agree in that the manuscript is interesting and recommend that you should be given a chance to revise it, I would like to ask you to continue revising your manuscript according to the referees' comments.

As you can see below, in his/her report, referee #3 expresses three main concerns besides other minor points. Two of them relate to further clarification of some results: s/he thinks that the possible role of CLPP in PINK1 processing and the difference between MPPbeta KD and CCCP treatment should be addressed. The third point, regarding the role of MPPbeta in PD progression, falls outside the scope of this manuscript and therefore, it would not need to be addressed for publication. However, if you already have some experimental evidence, as suggested by the referee, connecting MPPbeta to PD, its addition would only add to the general interest of the manuscript.

I look forward to seeing a revised form of your manuscript when it is ready.

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS:

Referee #3

In this interesting manuscript Greene et al. describe the processing of the PARK6-associated kinase PINK1 primarily by the mitochondrial processing peptidase (MPP). It is shown quite convincingly that reduction of MPP by knockdown results in accumulation of endogenous full-length PINK1 and loss of a shorted PINK1 fragment of about 60 kDa (most probably PINK1 without the mitochondrial targeting sequence as suggested by Jin et al. (J Cell Biol. 191(5):933-42)). The accumulation of full-length PINK1 after MPP knockdown results in the recruitment of the E3 ubiquitin ligase Parkin. Furthermore, MPP seems to be required for the PARL-mediated formation of the 52 kDa PINK1 isoform.
This report adds nicely to the ongoing molecular work on PINK1 processing. However the manuscript does not elucidate any link from MPP to the development and progression of Parkinson's disease (PD) and would therefore appeal only to a limited audience. For a greater impact it would be useful to demonstrate the biological/physiological/functional relevance (see major point 3).

Major points
1. In Figure 1A and 1C and Figure 2A it seems that the knockdown of the mitochondrial protease CLPP reduced the "MPP-cleaved" PINK1 form, which would mean that also CLPP is involved in the PINK1 processing at this step. I would like to see a quantification of the blots to clarify this matter.

2. The Parkin recruitment shown in Fig. 4C is very interesting, it is clearly induced by MPPβ knockdown but is weaker compared to CCCP treatment. Does that mean there is less Parkin/mitochondrion or are only some mitochondria tagged with Parkin? A fluorescence microscopy approach perhaps even with overexpressed GFP-Parkin would answer this question.

3. The authors claim rightly that knowledge about PINK1 cleavage and degradation is crucial "for gaining insight into mitochondrial quality-control in PD". Thus it would by very interesting to get an insight into the role of MPPβ in PD. Possible experiments could include a screen of MPPβ levels in PD patients or PD model systems or functional assays where the effects of MPPβ modulation (knockdown and overexpression) on mitochondrial functionality and cellular survival/apoptosis after stress are investigated to strenghen their hypothesis that "tonic, low-level inhibition of MPPβ activity could induce moderate increases in PINK1 levels, Parkin recruitment, and mitophagy of defective organelles. This could represent an exciting therapeutic opportunity for slowing the progression of PD by reducing the accumulation of mitochondrial defects." As stressors would be apart from CCCP also more PD related

stressors like oxidative stress or 6-hydroxydopamine or MPTP useful.

Minor points
1. Although the reviewer is aware of the difficulties in analyzing the endogenous levels of PINK1 by western blot the PINK1 signal in Figure 2A in the MPPβ lane is somewhat unclear, are there additional bands under the full length PINK1? Figure 2A could be also moved to supplementary data.

2. Does the quantification in Figure 3B relate to the MG132-treated samples? If yes this should be included in the figure legend.

3. Figure 3C is a bit confusing: why is there a strong full-length PINK1 band in lane 3 without CCCP treatment?

4. For readers who are not very familiar with the processing of PINK1 it would be helpful if a diagram would be added, where the different PINK1 cleavage products are shown.

5. In the Material and Methods part some minor corrections are necessary:
Cell Culture: are the sequences of the siRNAs available?
Cell Culture: 2 mM instead of 2 mm
Cell fractionation: Which protease inhibitors were used?

1st Revision - authors' response 23 December 2011

Response to the reviewer's comments:

Re: Manuscript EMBOR-2011-35287V1
Revised manuscript submitted to EMBO reports, entitled "Mitochondrial processing Peptidase Regulates PINK1 Processing, Import, and Parkin Recruitment".

Reviewer #1

We thank Reviewer #1 for the insightful comments on our manuscript. We were happy to hear that the referee felt that "*This good manuscript could be improved by addressing a few points*". We agree and have addressed the points below, providing new data for the manuscript.

1. It is noteworthy that the paraplegin-like protein AFG3L2 involved in PINK1 processing similar to PARL is linked to spinocerebellar ataxia type 28. This additional neurodegeneration link could be mentioned perhaps.

We agree completely with the reviewer, and in the conclusion we mentioned the link with SCA28 as follows: "*Mutations in the AFG3L2 subunit of m-AAA have been linked to the neurodegenerative disorder dominant hereditary ataxia SCA28 (Di Bella et al, 2010), and it will be interesting to examine whether altered PINK1 cleavage plays a role in this disease.*"

2a. Mitochondrial integrity was determined biochemically. Morphological photomicrographs are required to state that mitochondrial network in regard to fusion and fission is not affected.

This is a very good point. As requested, we now provide confocal photomicrographs of live cells showing that mitochondrial integrity is largely intact after transient knockdown of the mitochondrial proteases (new Fig. 2A). Indeed, mitochondrial membrane potential (MitoTracker Deep Red/MitoTracker Green ratio) is reduced as expected after CCCP but not after silencing the proteases (images in Fig. 2A and quantified in new Fig. 2B). This is consistent with our original findings using cytofluorometric analysis of the JC-1 ratio (now moved to the supplemental Fig. 3B). The interconnectivity of the mitochondrial network (area/perimeter ratio) also seems largely intact in the siRNA-treated but not in the CCCP-treated cells (new Fig. 2C).

2b. Moreover it would be nice to assess the mitochondrial clearance via the parkin recruitment

after siMPPβ treatment. In this regard, the study ends somewhat abruptly.

The referee asks about the effects of silencing the proteases on clearance of mitochondria. We agree that this is a logical extension of our study. Using MitoTracker Green staining followed by flow cytometry, we examined the effects of the siRNAs on mitochondrial mass. As shown in new Fig. 5D, mitochondrial mass was reduced by ~40% in the MPPβ knockdown cells compared to control NT siRNA-transfected cells. CCCP treatment also significantly reduced mitochondrial mass, whereas silencing PARL or AFG3L2 did not. Together, these findings indicate that both CCCP-treatment and MPPβ knockdown lead to a reduction of mitochondrial mass, consistent with an enhancement in mitochondrial clearance, likely via the induction of mitophagy. It has been reported that, in cells over-expressing parkin, prolonged treatment with CCCP can lead to a complete elimination of mitochondria from cells [Narendra et al. 2008 JCB]. It is worth noting that we did not observe this dramatic phenotype after silencing MPPβ. We speculate that this may be due to the relatively gradual and incomplete knockdown achieved with the MPPβ siRNA compared to the massive and synchronous depolarization achieved with CCCP. It must also be kept in mind that the partial clearance of mitochondria that we observe with CCCP and MPPβ siRNA (new Fig. 5D) occurred in the context of endogenous parkin levels whereas the complete elimination of mitochondria requires parkin over-expression. Thus, while more subtle, the mitochondrial clearance achieved by silencing MPPβ may more closely mimic the physiological function of the parkin-PINK1-mitophagy pathway in mitochondrial quality control.

3. One major concern is the remarkable specificity for PINK1. Should MPP knockdown not prevent processing and import of all mitochondrial proteins? Why is the effect so exceptionally prominent for PINK1?

We were also struck by the remarkable specificity for PINK1 and believe this is one of the main findings of our paper. We agree that interfering with MPPβ should affect all mitochondrial proteins that require cleavage of their mitochondrial targeting sequence (MTS). However, it is likely that most of these proteins are quite stable with long half-lives once they are imported into mitochondria and processed into their mature forms by MPP. We therefore believe that the ***partial*** and ***transient*** knockdown achieved in our system is much more likely to affect proteins that are turned over extremely rapidly such as PINK1. In fact, the essential function of MPP may have been the reason we were unable to achieve a higher degree of MPPβ silencing (compared to the other proteases; Fig. 1D). Indeed, we believe that more complete silencing of MPPβ would have affected the processing of a broader range of proteins, which would have been toxic. Our data fully supports this hypothesis by showing that the *partial* MPPβ knockdown that we obtained had only modest effects on the processing and levels of other known MPP substrates (new Supp. Fig. 3A; moved to the supplemental section as requested by reviewer #3). Indeed, we mention, "*We found little effect on the levels and processing of most mitochondrial proteins (Supp Fig 3A), consistent with a good overall preservation of mitochondrial integrity, at least for the degree of silencing and time scale (96 hrs) of the experiment. In particular, even the processing of established substrates of MPP such as MnSOD, OPA1, and Rieske was affected only marginally or not at all by the MPPβ siRNA, presumably because the knockdown was incomplete*". We also provide extensive new data showing that mitochondrial membrane potential (new Fig. 2B; Supp. Fig. 3B), morphology and network integrity (new Fig. 2A and 2C), and oxygen consumption (except for ClpP; Fig. 2D) are not overtly affected by the *partial* knockdown of MPPβ.

4. The PK digests in Fig. 4A must be shown side-by-side on a single immunoblot. Complete with additional mitochondrial markers (e.g. for the matrix, IMM and OMM).

This experiment has been repeated using IMM (Tim23), IMS (Cytochrome C) and OMM (Mfn2) markers as suggested by the reviewer. It now more clearly shows that like CCCP, MPPβ siRNA leads to increased sensitivity of full-length PINK1 to PK compared to the processed PINK1 species that accumulate with PARL and AFG3L2 silencing. The concentrations of PK required to digest PINK1 in the CCCP-treated and MPPβ siRNA mitochondria are similar to the concentrations required to digest the OMM protein Mfn2, suggesting that PINK1 is localized to the OMM in these cells. In contrast, the pattern of PINK1 digestions in the PARL and AFG3L2 silenced cells is similar to cytochrome C and Tim23, suggesting it is internalized within mitochondria, where it is protected from PK digestion.

5. Specify the used antibodies exactly, the companies often offer several ones.

This information is now provided at the beginning of the supplemental section with the exact catalog numbers.

Reviewer 2:

We thank reviewer 2 for stating, "… *the novel findings presented by Greene and colleagues in this study are important and timely. Moreover, the experiments are well thought of and systematically performed*". The referee's comments are addressed point by point below.

1. This is largely a RNAi-based study and a formal concern of such a mode of gene silencing unlike overt genetic ablation is the presence of off-target effects. To the authors' credit, they have acknowledged and tried to exclude this concern but perhaps it might be helpful in this case to perform a "rescue" experiment via overexpression of MPP-beta (RNAi-refractory if needed) in otherwise MPP-beta-silenced cells (notwithstanding that overexpression studies also have their own set of problems). Related to this, MPP-alpha overexpression in MPP-beta knockdown cells might also shed some useful insights on its proposed regulatory role in PINK1 processing.

We agree that this is an important experiment. We now provide new data showing that rat MPPβ (resistant to the siRNAs directed against human MPPβ) can rescue the accumulation of PINK1 induced by silencing endogenous MPPβ in HEK293T cells (new Supp. Fig. 2C). These results, together with our original findings in Supp. Fig. 2A, argue strongly that the accumulation of PINK1 in the MPPβ knockdowns is not due to an off-target effect. We have not tried overexpressing MPPα in the MPPβ knockdown, but agree with the referee that this would be an interesting avenue to explore in future work.

2. Taken together, the results indirectly suggest that mitochondrial depolarization leads to the downregulation (or inactivation) of MPP-beta and relevant mitochondrial proteases, which collectively promotes the accumulation of PINK1 on the mitochondria. From a physiological point of view, it would be important to examine if this is indeed true, i.e. is the level and/or activity of the various implicated proteases (particularly MPP-beta) affected in the presence of mitochondrial depolarization?

The referee makes an interesting point. In fact, we did not intend to imply that depolarization downregulates or inactivates the mitochondrial proteases. Rather, we believe that depolarization leads to an accumulation of PINK1 because it blocks PINK1 import (see the bottom of first paragraph of the introduction, as well as the model presented as new Fig. 6). In our model, when import is blocked PINK1 is retained at the outer mitochondrial membrane, where it is sequestered/protected from the proteases, which are intra-mitochondrial. However, the referee is right to point out that we cannot exclude the possibility that mitochondrial depolarization downregulates protease levels or activity. Therefore, as recommended by the referee, we examined if depolarization affects the various proteases. We find that CCCP-induced depolarization does not appreciably change protease levels (new Supp. Fig. 3C). Given the known effects of CCCP on protein import, we feel that it is less likely that it would act by *inactivating* the proteases and are not sure how we would test for this directly.

3. The results in Fig. 3 suggest that both CCCP treatment and MPP-beta silencing can retard PINK1 cleavage into the 52 kD product. Accordingly, a corresponding increase in full length PINK1 is seen in both cases (Fig. 3A). Curiously, neither full length nor the presumed MPPcleaved PINK1 (that migrates just under the full length protein) accumulates appreciably in the mitochondrial-enriched fractions of PARL and Clp knockdown cells. The authors should discuss this. It will be helpful to provide quantitative data of full length and MPP-cleaved PINK1 alongside the 52kD cleavage product data shown in Fig. 3B.

Our original intent in Fig. 3A, was to characterize the role of the proteases in the processing of the mature 52 kDa PINK1 fragment (specifically in that it gets exported to the cytosol and

degraded by the proteasome) rather than to focus on the MPP-cleaved PINK1 fragment. We now provide new quantitative data, as requested by the referee, also showing the effects of the various knockdowns on the MPP-cleaved PINK1 fragment (new Supp. Fig. 1B). Consistent with observations from the blots in Figures 1A, 1C, 3A and Supp. Fig. 2C, knockdown of PARL or AFG3L2 greatly increases the abundance of this species. Two additional points are worth noting: First, in contrast to the mature 52 kDa fragment, this MPP-cleaved PINK1 fragment does not get exported to the cytosol (Fig. 3A). Second, the combined knockdown of PARL and AFG3L2 has an even more dramatic effect on the accumulation of the MPP-cleaved fragment, suggesting that these two proteases may be partially redundant in PINK1 processing (Supp. Fig. 1C). We agree with the reviewer that the MPP-cleaved fragment does not significantly accumulate in the ClpP knockdown. In contrast, despite some blot-to-blot variability, full-length PINK1 does accumulate in the ClpP siRNA cells as shown in Fig. 1B. However, it should be kept in mind that our results with ClpP siRNA must be interpreted with caution given its effects on cell viability and oxygen consumption (Fig. 2D), as stated in the manuscript.

4. Finally, while it is good to note that parkin recruitment to the mitochondria occurs following mitochondrial PINK1 stabilization in MPP-beta-silenced cells, it would be interesting to determine if parkin localization to the mitochondria under such conditions is sufficient to trigger mitophagy

This is an important point and, as discussed above in our reply to point 2b from Reviewer #1, we now provide data showing that MPPβ silencing can trigger mitophagy (new Fig. 5D).
Minor points:

- It is intriguing to note that the level of Rieske is visibly affected (i.e. decrease) in the presence of both MPP-alpha and -beta silencing

This point is similar to point 3 from Reviewer #1. While we predicted that silencing MPPβ should affect the processing of many mitochondrial proteins, we were surprised at how disproportionately the silencing affected PINK1 compared to other MPP substrates. We do agree, however, that certain mitochondrial proteins, Rieske being the most prominent, were moderately affected by the MPP knockdown. A reduction in Rieske levels was to be expected, as Rieske is a known MPP substrate, and uncleaved forms of imported proteins are often unstable.

- A time-dependent study of the action of proteinase K (at low concentration) would serve as a useful accompaniment to Fig. 4A.

This point is similar to point 4 from Reviewer #1. We repeated these experiments with additional controls as shown in new Fig. 4A and discussed in detail above. Although we did not use a time course experiment, we feel that the modifications we put in place significantly strengthen the figure.

Reviewer 3:

We thank Reviewer 3 for mentioning that "*This report adds nicely to the ongoing molecular work on PINK1 processing*".

1. In Figure 1A and 1C and Figure 2A it seems that the knockdown of the mitochondrial protease CLPP reduced the "MPP-cleaved" PINK1 form, which would mean that also CLPP is involved in the PINK1 processing at this step. I would like to see a quantification of the blots to clarify this matter.

This point is similar to point 3 from Reviewer #2. As discussed in detail above, we now provide quantification of all 3 major PINK1 bands for all the protease knockdowns. These are shown in Fig. 1B (full-length), Fig. 3B (mature 52 kDa band) and new Supp. Fig. 1B (MPP-cleaved fragment). As mentioned above, the results with ClpP siRNA show some blot-to-blot variability and must be interpreted with caution given the effects of ClpP silencing on cell viability and oxygen consumption (Fig. 2D). Thus, we have not pursued the results with ClpP in as much detail as the other three proteases obtained in our screen.

2. The Parkin recruitment shown in Fig. 4C is very interesting, it is clearly induced by MPPβ knockdown but is weaker compared to CCCP treatment. Does that mean there is less Parkin/mitochondrion or are only some mitochondria tagged with Parkin? A fluorescence microscopy approach perhaps even with overexpressed GFP-Parkin would answer this question.

As requested by the referee, we now provide representative confocal fluorescence micrographs showing that transfected GFP-parkin is recruited to OCT-DsRed-expressing mitochondria after MPPβ knockdown (or CCCP treatment) but not after transfection with MPPα, PARL, AFG3L2, ClpP, or non-targeting (NT) siRNA (new Fig. 5A). A significantly greater proportion of MPPβ knockdown cells displayed a punctate mitochondrial pattern of GFP-parkin fluorescence compared to control (NT) siRNA-transfected cells (new Fig. 5B). This proportion was only slightly lower than what was observed in CCCP-treated cells. While cannot say with certainty whether there are differences in the number of mitochondria per cell that are tagged with parkin, the pattern with CCCP is clearly different from that observed with MPPβ siRNA. In particular, parkin-positive mitochondria appear clustered in the perinuclear region after CCCP, as reported previously (Okatsu et al; *Genes Cells*; 2010). In contrast, the parkin-positive mitochondria appear smaller, more dispersed, and display heterogeneous parkin recruitment in the MPPβ knockdown cells. We provide a representative image of these differences in Fig. 5A, and comment in the second to last paragraph of the results and discussion section. Importantly, these morphological findings are completely consistent with our original biochemical results with endogenous parkin (now Fig. 5C).

3. The authors claim rightly that knowledge about PINK1 cleavage and degradation is crucial "for gaining insight into mitochondrial quality-control in PD". Thus it would by very interesting to get an insight into the role of MPPβ in PD. Possible experiments could include a screen of MPPβ levels in PD patients or PD model systems or functional assays where the effects of MPPβ modulation (knockdown and overexpression) on mitochondrial functionality and cellular survival/apoptosis after stress are investigated to strenghen their hypothesis that "tonic, low-level inhibition of MPPβ activity could induce moderate increases in PINK1 levels, Parkin recruitment, and mitophagy of defective organelles. This could represent an exciting therapeutic opportunity for slowing the progression of PD by reducing the accumulation of mitochondrial defects." As stressors would be apart from CCCP also more PD related stressors like oxidative stress or 6-hydroxydopamine or MPTP useful.

We couldn't agree more that the ultimate goal will be to decipher how MPPβ might impact PD pathogenesis. We thank the reviewer for the very good suggestions, but feel that these experiments would be beyond the scope of the current manuscript. We are excited to pursue them in future work.

Minor points:

1. Although the reviewer is aware of the difficulties in analyzing the endogenous levels of PINK1 by western blot the PINK1 signal in Figure 2A in the MPPβ lane is somewhat unclear, are there additional bands under the full length PINK1? Figure 2A could be also moved to supplementary data.

We agree that, when compared with some of the other PINK1 blots in the manuscript, Fig. 2A is not ideal. However, the main point of this blot was not so much to look at PINK1 but to survey a panel of other mitochondrial proteins (thus the gel was run to optimize these). We have moved this panel to Supp. Fig. 3A, as suggested by the referee.

2. Does the quantification in Figure 3B relate to the MG132-treated samples? If yes this should be included in the figure legend.

Yes it does. We have made the changes in the legend.

3. Figure 3C is a bit confusing: why is there a strong full-length PINK1 band in lane3 without CCCP treatment?

We agree that the original labeling of this figure was confusing. We have now added “in vivo” and “in vitro” CCCP treatment, corresponding to the CCCP treatment of the cells and the isolated mitochondria respectively.

4. For readers who are not very familiar with the processing of PINK1 it would be helpful if a diagram would be added, where the different PINK1 cleavage products are shown.

We agree that a diagram of PINK1 cleavage by the diverse mitochondrial protease would help readers to understand our model and improve the manuscript. We added this model as Figure 6.

5. In the Material and Methods part some minor corrections are necessary:
Cell Culture: are the sequences of the siRNAs available?

All the siRNAs were Dharmacon smartPools, which contain a combination of four siRNAs. The combinations of sequences used in the pool are proprietary but can be obtain from Dharmacon upon purchase (I am not sure if we would infringe copyright or patent by listing them in the paper).

Cell Culture: 2 mM instead of 2 mm

The change has been made

Cell fractionation: Which protease inhibitors were used?

Benzamidine, PMSF, Aprotinin and Leupeptin. This was included in the methods section.

Editorial Decision 25 January 2012

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #2:

I am happy to note that the authors have adequately addressed the majority of my concerns in their revised manuscript. The current version is a considerably improved one. I also like to reiterate that this is an important and timely study that helps to clarify the mechanism underlying parkin/pink1-mediated mitophagy.

Referee #3:

I am satisfied the authors have addressed my previous comments concerning the article. I am happy to approve it for publication in EMBO Reports without further changes.

