USP8 Regulates Mitophagy by Removing K6-linked Ubiquitin Conjugates from Parkin

Thomas M. Duncan, Matthew Y. Tang, Joëlle R. Pérusse, Eman Dashti, Miguel A. Aguileta, Gian-Luca McLelland, Priti Gros, Thomas A. Shaler, Denis Faubert, Benoit Coulombe and Edward A. Fon

Corresponding author: Edward A. Fon, Montreal Neurological Institute, McGill University

Review timeline:

<table>
<thead>
<tr>
<th>Event</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Submission date</td>
<td>06 August 2014</td>
</tr>
<tr>
<td>Editorial Decision</td>
<td>15 August 2014</td>
</tr>
<tr>
<td>Revision received</td>
<td>20 August 2014</td>
</tr>
<tr>
<td>Accepted</td>
<td>21 August 2014</td>
</tr>
</tbody>
</table>

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.)

Editor: Andrea Leibfried

1st Editorial Decision 15 August 2014

Thank you for submitting your manuscript for consideration by the EMBO Journal. As your manuscript had been under consideration elsewhere before, it has now been seen by an arbitrating referee, who had access to the initial concerns raised as well as to your point-by-point response to them. I enclose the comments of this referee on the current version of your manuscript below. As you will see, the arbitrating referee finds your manuscript interesting and endorses publication here, but raises still some concerns that should be addressed. Additionally, I also involved another referee, and this second referee supports publication of your manuscript here.

Given the referees’ positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of referee #1. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

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

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the
conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Referee #1:

E. A. Fon et coworkers used an siRNA-based approach to search for DUBs that impair parkin-induced mitophagy. They identified USP8 as a modulator of both CCCP-induced parkin-mediated mitophagy and parkin stability. After one hour of CCCP treatment the amount of cells with mitochondrial parkin was significantly reduced in USP8-deficient U2OS cells stably expressing GFP-Parkin. After two hours of CCCP treatment there was no longer a difference in parkin recruitment to mitochondria between control and USP8-deficient cells, suggesting that USP8 silencing delays parkin translocation. Similarly, USP8 silencing caused a delay in parkin-induced removal of depolarized mitochondria, which was no longer detectable at 48 hours after CCCP treatment. By using in vitro ubiquitination assays the authors could show that USP8 can directly deubiquitinate parkin. Mass spectrometry indicated that CCCP increases ubiquitination of parkin at three lysines within the UBL domain, which was further increased in cells silenced for USP8 expression. USP8 seems to preferentially remove K6-linked polyubiquitin chains from parkin. From overexpressing different ubiquitin mutants (HA-ubi-K6 only, HA-ubi-K6R) the authors concluded that the removal of K6-linked ubiquitin from parkin promotes mitophagy.

This is a convincing study of high technical quality. The experiments were carefully designed and controlled. However, there are some points that need to be addressed.

1. CCCP has been reported to induce parkin activation by increasing PINK1-mediated phosphorylation of ubiquitin and the UBL domain of parkin. In this study the authors reported that CCCP induces K6-ubiquitination of the UBL domain of parkin, which inhibits parkin-induced mitophagy. The deubiquitinase USP8 is then needed to remove the K6-ubiquitin chains from parkin thereby restoring its effect on mitophagy. This appears a bit counterintuitive, how do the authors explain this complex regulation in a physiological context?

2. USP8 silencing only delays but does not impair parkin translocation and mitochondrial removal in CCCP-treated cells, since 2 or 48 hours after CCCP treatment there is no difference in parkin translocation or mitophagy in control and USP8-deficient cells. How does this fit to the model presented by the authors? Does it mean that there are other DUBs that can remove K6-linked ubiquitin chains from parkin or is there another modification that activates K6-ubiquitinated parkin? According to the siRNA screen, only UPS8 und USP2 have an impact on parkin autoubiquitination, but USP2 has no effect on parkin-induced mitophagy.

3. Figure 3 c: Given that parkin autoubiquitination involves K6-, K11-, K48-, and K63-linked chains, it is surprising that in the experiment shown only UPS8 and USP2 reduce parkin autoubiquitination.

4. The authors reported that neither steady state levels nor the ubiquitination of parkin substrates were affected by USP8 silencing in CCCP-treated cells. However, the blots shown in Figure 4 a and 4 d do show some effects, at least on Mfn2 ubiquitination.

5. Figure 6 c: Why is the HA (ubiquitin) signal only seen at the cell periphery in some images? What do the white arrows indicate? Why is the effect of HA-ubiquitin-K6 only overexpression on CCCP-induced mitochondrial translocation of parkin observed in Figure 7 a, b not seen in USP8-knockdown cells? Should USP8 silencing not increase this effect?

Minor points:
6. Figure 3 a: According to the figure legend, "WB: FLAG" should be an input control. The labeling of the figure is a bit confusing, because only "WB: USP8" and "WB: Actin" are marked as input.

7. TOM20 staining was used to determine the efficiency of mitophagy. This is somewhat problematic, since the disappearance of the TOM20 signal not necessarily correlates with the removal of mitochondria.

8. Which antibody was used to detect endogenous PINK1? This is not mentioned in Materials and Methods.

Response to Reviewer comments:

Referee #1:

E. A. Fon et coworkers used an siRNA-based approach to search for DUBs that impair parkin-induced mitophagy. They identified USP8 as a modulator of both CCCP-induced parkin-mediated mitophagy and parkin stability. After one hour of CCCP treatment the amount of cells with mitochondrial parkin was significantly reduced in USP8-deficient U2OS cells stably expressing GFP-Parkin. After two hours of CCCP treatment there was no longer a difference in parkin recruitment to mitochondria between control and USP8-deficient cells, suggesting that USP8 silencing delays parkin translocation. Similarly, USP8 silencing caused a delay in parkin-induced removal of depolarized mitochondria, which was no longer detectable at 48 hours after CCCP treatment. By using in vitro ubiquitination assays the authors could show that USP8 can directly deubiquitinate parkin. Mass spectrometry indicated that CCCP increases ubiquitination of parkin at three lysines within the UBL domain, which was further increased in cells silenced for USP8 expression. USP8 seems to preferentially remove K6-linked polyubiquitin chains from parkin. From overexpressing different ubiquitin mutants (HA-ubi-K6 only, HAubi-K6R) the authors concluded that the removal of K6-linked ubiquitin from parkin promotes mitophagy.

This is a convincing study of high technical quality. The experiments were carefully designed and controlled. However, there are some points that need to be addressed.

We were happy to hear that “is a convincing study of high technical quality” and that “The experiments were carefully designed and controlled”. Thank you. The reviewer raises several important issues with the manuscript that we address below in our detailed point-by-point response.

1. CCCP has been reported to induce parkin activation by increasing PINK1-mediated phosphorylation of ubiquitin and the UBL domain of parkin. In this study the authors reported that CCCP induces K6-ubiquitination of the UBL domain of parkin, which inhibits parkin-induced mitophagy. The deubiquitinase USP8 is then needed to remove the K6-ubiquitin chains from parkin thereby restoring its effect on mitophagy. This appears a bit counterintuitive, how do the authors explain this complex regulation in a physiological context?

This is an excellent point by the reviewer that perhaps was not clear enough in the original manuscript. To help address the reviewer’s concerns and further elaborate on the impact of USP8, taking into account the roles of PINK1 and phosphorylated Ub, we have expanded our concluding paragraph in our discussion to speculate further on how K6-linked Ub chains could impede mitophagy in a physiological context:

“Beyond the role of parkin, our work manipulating the ratios of different Ub linkages in cells suggests, for the first time, a role for K6 ubiquitination per se in mitochondrial quality control. The presence of these conjugates on parkin may impede it from interacting with substrates, PINK1 and/or phosphorylated Ub. The latter two have been shown to be essential for parkin activation and translocation onto the mitochondria (Kane et al, 2014; Kondapalli et al, 2012; Koyano et al, 2014). As parkin autoubiquitination appears to occur predominantly on the Ubl domain (K48, K76), it is also possible that K6-linked conjugates interfere with binding to Ubl-interacting proteins (i.e. endophilin, Eps15, ataxin-3) (Durcan & Fon, 2011; Fallon et al, 2006;
Trempe et al, 2013) or PINK1-mediated phosphorylation of the Ubl at S65 (Kondapalli et al, 2012). Moreover, the presence of increased levels of K6-linked Ub chains appears to impair parkin-mediated mitophagy, perhaps by interfering with ongoing parkin E3 Ub ligase activity or because of a reduced capacity of these chains to recruit p62, LC3 or other autophagy proteins. Regardless of the precise mechanism, our work uncovers a novel layer of regulation, mediated by USP8 and K6-linked auto-ubiquitination, critical for parkin-dependent mitophagy."

2. USP8 silencing only delays but does not impair parkin translocation and mitochondrial removal in CCCP-treated cells, since 2 or 48 hours after CCCP treatment there is no difference in parkin translocation or mitophagy in control and USP8-deficient cells. How does this fit to the model presented by the authors? Does it mean that there are other DUBs that can remove K6-linked ubiquitin chains from parkin or is there another modification that activates K6-ubiquitinated parkin? According to the siRNA screen, only UPS8 and USP2 have an impact on parkin auto-ubiquitination, but USP2 has no effect on parkin-induced mitophagy.

From our in vitro assays, none of the commercially available DUBs with the exception of USP2 and USP8 could deubiquitinate parkin. However, the DUB family of enzymes is comprised of over 90 members, and we cannot eliminate the possibility that other untested DUBs may be able to hydrolyze K6 linkages in parkin Ub-conjugates. Yet, in the context of our siRNA screen and CCCP-activated mitophagy no other DUBs had an effect, including USP2. As mentioned in the manuscript, in vitro, USP2 is a very promiscuous DUB with the ability to deubiquitinate conjugates indiscriminately. Thus, while USP2 can deubiquitinate parkin in vitro, it may not be so surprising that in cells it has little effect. Thus, based on our screen, we do not believe that other DUBs regulate parkin recruitment. However, we do acknowledge recent work showing that two others DUBs, USP15 and USP30 regulate mitophagy, albeit indirectly by deubiquitinating parkin substrates rather than parkin itself (Bingol et al, 2014; Cornelissen et al, 2014). The reviewer very correctly points out that silencing USP8 leads to a delay but not an absolute inhibition of recruitment and mitophagy. While we are not certain of the mechanisms, it must be kept in mind that parkin does not make K6-linked chains exclusively (see our AQUA mass spec results in Fig. 5G and the reviewer’s next point below). It is therefore plausible that other DUBs could eventually cleave mixed chains at other sites (i.e. K11, K48, K63) or even at K6, albeit perhaps with lower efficiency. Such a process might explain the observed delay in recruitment and mitophagy.

3. Figure 3 C: Given that parkin auto-ubiquitination involves K6-, K11-, K48-, and K63-linked chains, it is surprising that in the experiment shown only UPS8 and USP2 reduce parkin auto-ubiquitination.

Although these findings are surprising, it is becoming clearer that the architecture of Ub conjugates on a substrate protein is a lot more complex than previously imagined. For parkin Ub conjugates, we hypothesize that the predominance of K6 linkages within these chains may be causing these Ub chains to adopt a compact conformation, based on crystal structures of free K6-linked chains (Hospenthal et al, 2013). We speculate, as mentioned in the discussion, that by interacting with parkin, the active site of USP8 may be positioned in such a manner that it can access and hydrolyze these K6 linkages whereas the compact structure may hinder the access to most other DUBs.

4. The authors reported that neither steady state levels nor the ubiquitination of parkin substrates were affected by USP8 silencing in CCCP-treated cells. However, the blots shown in Figure 4 A and 4 D do show some effects, at least on Mfn2 ubiquitination.

We agree with the reviewer that subtle differences can be detected in certain mitochondrial proteins. However, we believe it is fair to say that one does not observe dramatic differences between these substrates (and in particular compared to the substantial effect on parkin itself). To address the reviewer’s point we have now modified the sentence to state: "known substrates of parkin such as the mitofusins and VDAC (Geisler et al, 2010; Lazarou et al, 2013; Sarraf et al, 2013) did not appear to be greatly affected by USP8 knockdown”.

5. Figure 6 C: Why is the HA (ubiquitin) signal only seen at the cell periphery in some images? What do the white arrows indicate? Why is the effect of HA-ubiquitin-K6 only overexpression on CCCP-induced
mitochondrial translocation of parkin observed in Figure 7a, b not seen in USP8-knockdown cells? Should USP8 silencing not increase this effect?

We agree that it was surprising to see Ub close to the periphery in certain cells, although we believe the high levels of Ub in the cytoplasm and the timing of the cell cycle may be causing it to migrate into these regions. These findings were consistent with previous findings from Geisler et al., in which they also observe staining of HA-Ub at the periphery in certain cells (Geisler et al, 2010). In the images, white arrows are pointing to cells expressing the indicated HA-Ub construct.

The reviewer also brings up an excellent point, why do we not see the same effect from overexpression of HA-UbK6 only when USP8 is knocked down. We speculate that when USP8 is knocked down, the level of K6-linked Ub conjugates that persist on parkin attains maximal threshold using the endogenous pool of Ub. Thus, although additional HA-UbK6 only is present at high levels, we speculate that parkin is unable to accommodate any additional K6-linked conjugates. This is in contrast to cells in which USP8 is present; as it is actively removing K6 conjugates that form. As a result, when HA-UbK6 only is overexpressed, this overwhelms USP8, resulting in an increased persistence of K6-linked conjugates on parkin that delay its mitochondrial recruitment.

Minor points:
6. Figure 3a: According to the figure legend, "WB: FLAG" should be an input control. The labeling of the figure is a bit confusing, because only "WB: USP8" and "WB: Actin" are marked as input.

We thank the reviewer for pointing out the confusing wording of our figure legend. We have corrected this in the updated manuscript by rewording the figure legend to “Lysates were immunoprecipitated with FLAG resin and analyzed by immunoblotting for HA and FLAG. Input lysates (5% of total input) were analyzed by immunoblotting for USP8 and actin”. The legend is now congruent with what is shown in Figure 3A.

7. TOM20 staining was used to determine the efficiency of mitophagy. This is somewhat problematic, since the disappearance of the TOM20 signal not necessarily correlates with the removal of mitochondria.

We agree with the reviewer’s assertion that normally TOM20 degradation via the proteasome precedes clearance of the mitochondria with prolonged CCCP treatment. However, the important finding of our paper is that silencing USP8 delays recruitment and mitophagy. Thus, the persistence of TOM20 staining is even further proof that one of the “earlier steps” (i.e. TOM20 removal) is delayed. Moreover, these TOM20 structures consistently colocalized with other mitochondrial proteins [COX1 and TIM23 (Supplementary Figure S4B)] following USP8 siRNA, again supporting a delay in mitophagy. Thus, we understand the reviewer’s concerns but given the specific questions being addressed in our work, we believe that TOM20 staining provides an accurate readout of mitochondrial clearance.

8. Which antibody was used to detect endogenous PINK1? This is not mentioned in Materials and Methods.

The antibody used to detect endogenous PINK1 was a rabbit polyclonal (Cat# BC100-494) from Novus Biologicals. We have now included the relevant information on the PINK1 antibody in our updated materials and methods.

REFERENCES


Fallon L, Belanger CM, Corera AT, Kontogiannea M, Regan-Klapisz E, Moreau F, Voortman J,


2nd Editorial Decision 21 August 2014

I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.