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Autoregulation of Parkin activity through its ubiquitin-like domain

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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 03 May 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received the reports from two expert reviewers, who both consider your findings on Parkin E3 ligase regulatory mechanisms potentially interesting and important. They nevertheless raise a certain number of issues, some of which I feel should be addressed experimentally to round off the study for publication in a general interest journal such as The EMBO Journal. In particular, this should include referee 1's point 2 on better defining the intramolecular Ubl-RING interaction, and referee 2's main point of testing the Ubl inhibitory effects not only for autoubiquitination but also for ubiquitination of some (model) Parkin substrate. With such additional insights, we could consider an eventual revised version further for ultimate publication in these pages.

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. We generally allow three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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://www.nature.com/emboj/about/process.html

Please don't hesitate to contact me should you have any further question regarding this decision or your revision! I look forward to your revision.

Yours sincerely,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Mutations if the E3 ubiquitin ligase parkin are the most common cause of recessive parkinsonism and may even confer risk for sporadic Parkinson's disease. Thus, elucidation of parkin catalytic activity and regulation are a very important topic. Surprisingly, divergent results on ubiquitinylation activities have been reported. In the present study, Chaugule et al. performed a most comprehensive biochemical investigation to demonstrate that parkin is auto-inhibited via the N-terminal ubiquitin-like (UBL) domain. Although this contrasts some previous publications, good explanations are provided and elegantly confirmed with an impressive range of biochemical and biophysical experiments. Moreover, UBL-binding polypeptides are shown to be potential allosteric parkin activators. The discussion aims at generalizing the present findings for E3 ubiquitin ligases, but the experimental evidence provided here is restricted to parkin. The paper is well written. One can follow the manuscript easily understanding the reasoning and conclusions about the mechanism of parkin auto-regulation.

A few minor things might be taken in consideration to further improve this excellent manuscript:

1) The entire study was done in vitro. Some key cell-based experiments would greatly increase relevance.

2) It would be interesting to map the UBL binding site within the RING configuration (loosely sketched in Fig. 3c). Also, is the lysine-48 within UBL ubiquitinylated? Would such a ubiquitinylation (by parkin itself, or maybe even an ubstream ligase) alter the auto-inhibitory properties of the parkin UBL?

3) On page 14-15 some references are not formatted.

Referee #2 (Remarks to the Author):

Walden et al. demonstrate that parkin's UBL domain interacts with the remaining portion of the protein and that this interaction prevents its autoubiquitination in vitro. The interaction is supported by NMR titration experiments and a binding constant is derived by ITC. K48, which is conserved in ubiquitin, appears to play a role in the interaction. Pathogenic mutations in the UBL domain or adding fusion tags to the wild-type sequence result in its inability to recapitulate the autoinhibition effect of the wild-type UBL domain. Finally, UIMs that bind parkin's UBL domain trigger parkin autoubiquitination, presumably by releasing the UBL domain from the catalytic region. These findings are convincing and interesting.

It would be good if the authors addressed how their observations of parkin intramolecular interaction affect parkin's E3 ligase activity towards its substrates. In addition does the tagging of parkin affect its ubiquitination of its substrates? Along the same lines, the authors showed that full length parkin binds to an E2, but is unable to receive ubiquitin from it. This finding should be discussed further. In particular, does the UBL domain prevent only parkin autoubiquitination in this context, or also ubiquitin transfer to substrates? If only autoubiquitination is affected then the UBL domain may be activating parkin towards its substrates. Finally, it is not clear how the parkin autoubiquitination for the pathogenic proteins will play out in the cellular context. Perhaps quality control pathways identify these before they catalyze their own ubiquitination. If not then the

question of whether the autoubiquitination triggers their own destruction is an interesting one versus their potential sequestering of ubiquitin receptors.

Some minor suggestions are that the presentation of Figure 1c could be improved by following the format of Figure 6e in which each component's presence is indicated by '+' and '-' symbols. Also, the effects of Figure 4a are difficult to see and zoomed in regions as well as a legend could be included for clarity.

1st Revision - authors' response 11 May 2011

We thank the reviewers for taking the time to evaluate our manuscript. We have performed several additional experiments to address the points raised. We include some cell-based data (supplementary figure 4c), data addressing the point of interaction and subsequent mechanism of inhibition (figures 7 and supplementary figure 8). We have also included a model summarising our findings (figure 8) as requested by the editor. A list of additional experiments is included for reference:

1. Cell-based experiment with the pathogenic mutants and deltaUblD (supplementary figure 4)
2. Peptide arrays showing interaction of UblD and ubiquitin with Parkin (supplementary figure 8)
3. Testing hydrophobic patch mutants of ubiquitin with all 8 Parkin species (figure 7a and 7b)
4. Expression and purification of I44A-Parkin and testing in an assay (figure 7c)
5. Cloned, expressed and purified to homogeneity I44A-UblD and performed ITC with deltaUblD Parkin (saw no evidence of interaction - figure 7d)

Below is point by point response to the comments.

Referee #1 (Remarks to the Author):

Mutations if the E3 ubiquitin ligase parkin are the most common cause of recessive parkinsonism and may even confer risk for sporadic Parkinson's disease. Thus, elucidation of parkin catalytic activity and regulation are a very important topic. Surprisingly, divergent results on ubiquitinylation activities have been reported. In the present study, Chaugule et al. performed a most comprehensive biochemical investigation to demonstrate that parkin is auto-inhibited via the N-terminal ubiquitin-like (UBL) domain. Although this contrasts some previous publications, good explanations are provided and elegantly confirmed with an impressive range of biochemical and biophysical experiments. Moreover, UBL-binding polypeptides are shown to be potential allosteric parkin activators. The discussion aims at generalizing the present findings for E3 ubiquitin ligases, but the experimental evidence provided here is restricted to parkin. The paper is well written. One can follow the manuscript easily understanding the reasoning and conclusions about the mechanism of parkin auto-regulation.

We are very grateful to this reviewer for their kind words and clear understanding of our study.

A few minor things might be taken in consideration to further improve this excellent manuscript:

1) The entire study was done in vitro. Some key cell-based experiments would greatly increase relevance.

We appreciate that some cell-based data would enhance the manuscript. In order to address this point, we have reproduced the experiment in figure 2b in a cell-based assay (supplementary figure 4). What we, and others (eg PMID:16049031) observe is that the Ubl-pathogenic mutants are more active than wild type Parkin in the cellular context, and also less stable, requiring proteasome inhibitor for stability (eg PMID:15606901).

2) It would be interesting to map the UBL binding site within the RING configuration (loosely sketched in Fig. 3c). Also, is the lysine-48 within UBL ubiquitinylated? Would such a ubiquitinylation (by parkin itself, or maybe even an ubstream ligase) alter the auto-inhibitory

properties of the parkin UBL?

To address the question of whether Lysine-48 is ubiquitinated, we performed a large-scale autoubiquitination assay using K48A, R42P and deltaUblD-parkin and performed mass spec analysis to identify the ubiquitination sites (in conjunction with the Protein Analysis Lab at Clare Hall Labs) See figure R1 for the gel. Lysine-48 was not modified in this system.

Figure R1. A scaled-up autoubiquitination assay to identify sites of ubiquitination on Parkin. K48 was not modified.

To address the mapping of the UBL binding site and further develop the mechanism of this interaction, we have identified a C-terminal Ubl-binding motif through peptide arrays, and demonstrated this binds ubiquitin as well. We have also shown that previously 'active' Parkin becomes inactive when faced with a hydrophobic patch mutant (I44A) of ubiquitin. In addition, the conserved I44 in the Ubl-domain when mutated gives rise to active full-length Parkin. All these data are in revised figure 7 and supplementary figure 8.

3) On page 14-15 some references are not formatted.

Thank you for pointing these out, we have made sure they are now formatted.

Referee #2 (Remarks to the Author):

Walden et al. demonstrate that parkin's UBL domain interacts with the remaining portion of the protein and that this interaction prevents its autoubiquitination in vitro. The interaction is supported by NMR titration experiments and a binding constant is derived by ITC. K48, which is conserved in ubiquitin, appears to play a role in the interaction. Pathogenic mutations in the UBL domain or adding fusion tags to the wild-type sequence result in its inability to recapitulate the autoinhibition effect of the wild-type UBL domain. Finally, UIMs that bind parkin's UBL domain trigger parkin autoubiquitination, presumably by releasing the UBL domain from the catalytic region. These findings are convincing and interesting.

We are also grateful to this reviewer for their clear summary and for finding the manuscript convincing and interesting.

It would be good if the authors addressed how their observations of parkin intramolecular interaction affect parkin's E3 ligase activity towards its substrates. In addition does the tagging of parkin affect its ubiquitination of its substrates?

This is a very important question. The substrates identified for Parkin remain somewhat controversial and there seems to be little consensus as to what defines a 'true' Parkin substrate and a lack of reproducibility of data lab to lab. In addition, although we have tried extensively, we have been unable to express and purify well-behaved, soluble putative substrates. Consequently, this makes addressing this point extremely difficult. (Please see Corti & Brice 2007, Drug discovery today: Disease Mechanisms, 2007, vol 4 121-127 and PMID:20187240 both of which contain some interesting discussions/arguments concerning substrates). Therefore, although we feel this is a very important question, we think it is one better addressed in a robust, independent study of Parkin substrates, one that I feel is beyond the scope of this study. Given our data, the way one would go about finding a substrate would be fundamentally different to the methods that have been used previously - for example one wouldn't tag Parkin and pull down putative substrates. We have included some points in the discussion.

Along the same lines, the authors showed that full length parkin binds to an E2, but is unable to receive ubiquitin from it. This finding should be discussed further.
In particular, does the UBL domain prevent only parkin autoubiquitination in this context, or also ubiquitin transfer to substrates? If only autoubiquitination is affected then the UBL domain may be activating parkin towards its substrates.

This reviewer clearly summarises the puzzle our data suggest. We have performed a number of experiments to address this point, and they are included in the revised figure 7 and supplementary figure 8 (please also see response to reviewer 1). What we observe is that there is an overlapping but non-equivalent Ubl/Ub-binding motif in the C-terminus of Parkin. In the context of the very active deltaUblD Parkin or pathogenic Ubl mutants, mutations in the hydrophobic patch of ubiquitin render Parkin 'inactive', suggesting Parkin needs to 'see' both the E2 and the charged ubiquitin in order to be active. A very recent paper from the Klevit group (Nature online, May 2011) indicates that the RBR family of ligases may be RING/HECT hybrids, forming a catalytic intermediate with ubiquitin. Although they were unable to show that Parkin truly does form a catalytic intermediate via a cysteine, we think the ability (and apparent requirement) to bind ubiquitin on the E2 for Parkin to have (self) ubiquitination activity constitutes a semi-catalytic intermediate. We think this explains the ability of Parkin to bind an E2 and yet not accept the activated ubiquitin from it. We have added this point to the discussion and summarised our data and discussion in a model (figure 8).

We present evidence towards a mechanism for Parkin autoregulation that requires effector binding to the Ubl domain. This could present an analogy to the cullin-RING ligases whereby the Ubl domain recruits the effector, which in turn recruits substrates. There is some support for this model in previous studies isolating endogenous Parkin as a large, heteromeric complex, and another showing Parkin interaction and function with components of CRLs. We have now included this in the discussion.

An alternative model could indeed be as this reviewer suggests, that the Ubl domain is activating towards substrates. There is certainly circumstantial evidence for this in the flurry of recent papers that describe Parkin 'activation' at the mitochondria. This is now emphasised in the discussion. The honest response is that we don't know which, if either, of these models is correct and we are addressing these and other questions in future experiments.

Finally, it is not clear how the parkin autoubiquitination for the pathogenic proteins will play out in the cellular context. Perhaps quality control pathways identify these before they catalyze their own ubiquitination. If not then the question of whether the autoubiquitination triggers their own destruction is an interesting one versus their potential sequestering of ubiquitin receptors.

This is a really key point. We have addressed this with our experiment laid out in supplementary figure 4 (please also see the response to reviewer 1). The evidence from us and others would certainly suggest that Parkin Ubl mutants are unstable - it is difficult to distinguish whether this is through degradation via protein quality control, or degradation due to autoubiquitination, or both. The idea of sequestering ubiquitin receptors is an intriguing one, and not one we had considered. We now mention this in the discussion.

Some minor suggestions are that the presentation of Figure 1c could be improved by following the format of Figure 6e in which each component's presence is indicated by '+' and '-' symbols. Also, the effects of Figure 4a are difficult to see and zoomed in regions as well as a legend could be included for clarity.

Thank you for the suggestions to improve presentation, we have done as requested.

Acceptance letter 24 May 2011

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by one of the original referees (see comments below), and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

There are only two issues with presentation in the figures (indicated in the referee comments below) - a minor one regarding the summary figure, and another one regarding Figure 4, where data points

appear to have been involuntarily cut off (in the upper right). I would therefore like to kindly ask you to revisit these two figures, and send us revised versions via email (if possible) - we would then replace them in our tracking system.

After that, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

Yours sincerely,

Editor
The EMBO Journal

Referee #2

(Remarks to the Author)
This reviewer continues to be enthusiastic towards this manuscript, which includes novel insights into parkin mediated function and dysfunction. One concern is that Fig 4A appears to have been cutoff in a strange manner for its expanded insert. In addition, proteasomal is spelled incorrectly in Fig 8. This manuscript will no doubt be well cited and enjoyed by scientists studying ubiquitin-mediated degradation and Parkinson's disease.

