THE EMBO JOURNAL

Manuscript EMBO-2016-94314

Ubiquitylation-dependent oligomerization regulates activity of Nedd4 ligases

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Review timeline:

Submission date:	13 March 2016
Editorial Decision:	18 April 2016
Revision received:	25 September 2016
Editorial Decision:	18 October 2016
Revision received:	25 November 2016
Accepted:	06 December 2016

Editor: Anne Nielsen

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 18 April 2016

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see from the reports, the referees all express interest in the findings reported in your manuscript although they also raise a number of concerns - mainly related to protein constructs used, further mutagenesis, and the size of the regulatory effect - that you will have to address experimentally before they can support publication of your study in The EMBO Journal.

Given the referees' overall positive recommendations, I would like to invite you to submit a revised version of the manuscript, in which you address the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance or rejection of your manuscript will therefore depend on the completeness of your responses in this revised version.

For the revised manuscript I would particularly ask you to focus your efforts on the following points:

-> Both ref #1 and #3 raise concerns about the choice of protein construct used for the analysis (truncation and presence of MBP tag) and it will therefore be important for you to extend these experiments using full-length and untagged protein.

-> In addition, please include additional mutagenesis work as requested by refs #1 and #2.

-> You will see that referee #2 is the most critical of the three and that this person would need to see the findings repeated using a more robust Rps5 activity assay. The referee outlines one potential strategy for doing this but we would also welcome your suggestions for an alternative way to approach this question. As you will see from the report, the referee finds further experimental data for this point to be a prerequisite for supporting publication in The EMBO Journal.

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. 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.

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

REFEREE REPORTS

Referee #1:

In this interesting manuscript, it is shown that NEDD4 ligases are able to oligomerize, specifically trimerize, a status in which these ligases are proposed to be inactive. The transition from a monomeric, active state, into the trimerization state is proposed to be controlled by ubiquitylation of lysines present in the alpha1 helix that is localized at the N-terminal end of the HECT domain. The evidence for such a mechanism is tested by yeast two-hybrid system, size exclusion chromatography, crosslinking experiments and sedimentation velocity experiments. Computer based modelling is also applied, as well as a number of other sophisticated techniques. The physiological relevance is tested in the yeast on the proteasome subunit RPN10, and in mammalian cells on NEDD4 dependent ubiquitylation of FGFR1, and on Iks channels.
Comments:
1. Based on previous crystallization studies it is proposed that the alpha1 helix present in the NEDD4 family members interferes with the transition from monomeric to a trimeric state. The authors test the capability of oligomerization in the 2H system (Fig. 1). Why are the authors using a construct that lacks the C2 domain, and not a full-length construct for the crosslinking assay in Fig. 1E (alpha1L construct). What is not understandable is why the authors use either this nearly full-length construct and compare it with a construct containing just the HECT domain lacking alpha1 helix (both in fusion with MBP), to conclude that the alpha 1 helix is interfering with the oligomerization. One cannot draw this conclusion, the correct one would be that the region comprising the alpha1 helix and the WW domains are interfering with the oligomerization. The authors would have to make a near full-length construct in which they delete the alpha1 helix. It follows that both experiments of 1E and 1F are no very useful and do not provide the relevant information.
2. Instead of crosslinking, can the authors show the monomeric and oligomeric states on a non-denaturing, native gel? That would be more convincing.
3. The authors test their model by 2H analysis, using either RSP5 K432,K438,K411R mutant, or a catalytically inactive mutant. The triple K mutant reduces the interaction to some extent. What was the effect with the single mutants, especially K432R, as, according to the model, ubiquitylation of K432 seems to be relevant? Surprisingly, the inactive RSP5C777K mutant, has a much stronger effect than the triple K mutant. Wouldn't that mean that there must be other ubiquitylation sites that are as important as K432 for the interaction? The authors do not address this issue at all, but they should and come up with some explanations. I would not talk about in vivo in the context of the 2H system, this is still a very artificial system.

4. Fig. 2F. How many times has this been repeated? Quantification and statistics should be provided (same for Fig. 4E, and 5E).
5. When testing NEDD4 on FGFR1, the authors mutate K523, 525R, although they previously talked only about K525. What happens if only K525 is mutated?
6. Page 3 last paragraph, a hypothesis is tested.....the phrase, "As expected, while the....." is not appropriate, as it gives the impression that the authors knew the result already in advanced. "As hypothesized,....." would be better.

Referee #2:

The manuscript by Attali et al. describes an investigation into the function of the Nedd4 family of HECT E3 ubiquitin ligases. Using a series of biophysical, biochemical and functional approaches, the authors propose a model where E3 function is negatively controlled through the oligomerization of the E3. Specifically, the authors identify a helical unit at the N-terminus of the HECT catalytic domain that contains a lysine residue that becomes ubiquitylated in cis. In the author's model, this ubiquitylation event drives the association of the ubiquitin with a ubiquitin binding domain on the HECT that displaces the N-terminal helix, thus enabling oligomerization and inactivation of the E3.

Determining the mechanism of action of E3 ligases and how these enzymes are regulated in the cell are of great interest. The authors' proposal for Nedd4 ligase regulation is novel, and if correct, represents a significant step forward. However, my main concern is with the in vitro ubiquitylation assays. Specifically, the activity shown for Rsp5 HECT is very weak, product formation is measured over extremely long time scales that are unlikely to be physiologically relevant, and the comparison of activity is purely qualitative. The upshot is that the differences in the activities of the various HECT constructs are very subtle and still leaves significant doubt, at least in the mind of this review, as to how oligomerization affects HECT activity.

The authors should address this by using a more convincing activity assay. For instance, the in vitro Rsp5 activity assay developed by Kamadurai and Schulman (Elife, 2013) has far more robust activity than the assay presented here. I realize that since the Rsp5 protein used in that study contains the third WW domain, my proposal would entail working out a method to ubiquitylate Lys 432, and I acknowledge that this is going to be challenging work. However, I think that it is justified since the authors' current approach of fusing ubiquitin to the N-terminus of the HECT domain has obvious limitations and drawbacks. In its current form, I cannot support this paper in EMBO.

Specific points

1. The authors co-express the ubiquitylation cascade enzymes with Rsp5 in E.coli and map the K432 ubiquitylation site by MS. Do the authors see modification of this lysine residue with ubiquitin from Rsp5 protein derived from yeast cells?
2. Does expression of the Rsp5 catalytic cysteine mutant disrupt ubiquitylation of K432 in yeast? As a side-note, why did the authors mutate the cysteine to a lysine residue? Others have shown that this may result in the permanent modification of the lysine residue with ubiquitin which could also affect oligomerization.
3. What was the rational behind the triple lys to arginine mutant (K432, K438, and K411) in Figure 2E? The authors mention that K438 was identified as a ubiquitylation site in the literature, but what about K411? The authors should include results for the single K432R mutant since this is most relevant to the in vitro ubiquitylation assays.
4. The modeling results in Figure 3 are not convincing and should be supported by mutations to ionic residues at both sites in ubiquitin and the HECT domain. Is the I537D mutation complemented by a second I44K mutation in the same HECT and result in restoration of oligomerization? This result would go a long way towards substantiating their model.
5. How many lysine residues are there in the Rpn10 substrate? This is important because it is unclear whether the authors are observing multi-mono or poly-ubiquitylation of substrate. Indeed on p 7, 2nd paragraph, the authors imply that the substrate is poly-ubiquitylated. Does this mean that Rpn10 is only modified at one lysine?
6. How would all of this work in the cell? What is the role of the Ubp2 de-ubiquitylating enzyme (that is known to form a stable complex with Rsp5 in yeast)? I support the authors' contention that these questions are beyond the scope of this manuscript, and I also acknowledge that the authors

touch on the subject in the Discussion section. However, their model does suggest that Rps5 would rapidly inactivate itself in cells, and a more thorough discussion of the literature in light of this would be helpful.

Referee #3:

Nedd4 ligases belong to the HECT family of E3s and regulate a large variety of cellular processes. Some Nedd4 family members are inhibited by intramolecular interactions, which in turn are regulated by post-translational modifications such as phosphorylation and ubiquitylation.

In this manuscript the authors describe a novel mechanism of Nedd4 regulation, which relies on a ubiquitylation-dependent self-association, which inactivates catalytic activity. This mechanism has been identified in the yeast Nedd4 protein Rsp5 and is shown by the authors to be conserved in mammalian cells. This is a very interesting study that adds another level of regulation to E3 ubiquitin ligase activity.

The manuscript is well written and overall the data presented are of high technical quality. My main concern with the data presented is that almost all experiments have been carried out using MBP-fusion proteins, which is far from ideal when investigating intramolecular interactions and self-association. As described below, the authors need to provide clear evidence that the large MBP tag has no influence on the behaviour of the proteins under investigation.

Major points:

1) The experiments shown in Figure 2B using an MBP-Ub-HECT should be repeated without a MBP tag. This tag is very large and may influence the experiment. I do not understand why the authors chose to carry out these experiments with a fusion protein when the tag can be easily removed as shown in Figure 1 F.
The same applies for the experiments shown in Figures 4 and 5. The authors should repeat the experiments with the tag removed or at least need to provide additional data that clearly show that the MBP tag has absolutely no influence on self-association.

2) Figure 2F: As the authors show in Figures 2D and S3, Ub- α 1 is in a monomer-trimer-hexamer equilibrium. This implies that even after collecting the monomer and oligomeric fractions on SEC, they should re-equilibrate and hence contain the same 1:3:6 ratio. Why do they show different levels of activity? Does this mean that this equilibrium is very slow - slower than collecting the fractions and carrying out the experiment?
Please test the monomeric fraction after 1, 2,4 hours on SEC to check if the equilibrium has been re-established.

3) The authors should provide SDS gels across the SEC fractions to show that the void peak does not contain the mutant proteins.

4) I do not fully understand the MST experiment shown in Figure 4D - in this experiments the authors are looking at exchange of labelled oligomers with unlabelled ones. What model was used to fit the data, given that a monomer-trimer-hexamer equilibrium was analysed and hence what does the Kd refer to?

Minor points:

1) Reference Ronchi et al. is incomplete

2) Figure 1 C: A close up of the clash might be helpful in this figure.

3) How can the authors exclude that the WW domains that are present in the α1L construct do not influence self-association?

4) The authors should add a panel to Figure 2 to show the structure of Rsp5 and indicate the position of K432 to allow the reader to judge for themselves how well a fusion of ubiquitin to the N-terminus of a1 may mimick ubiquitylation (or refer to Figure 3B).

6) Why does the majority of the G747E mutant elute in the void? (Fig 5B). This does not agree with the ratio of monomer-trimer-hexamer determined by SE (Table S1). Are there contaminants in the mixture? The authors should provide an SDS gel of the different peak fractions.

7) In Figure 6C the authors show that after 30 min there is no difference between the activity of apo HECT and Ub-HECT anymore. What do the authors think the reason for this is?

1st Revision - authors' response 25 September 2016

We are very pleased to submit a revised version of the manuscript entitled: *'Ubiquitylation-dependent oligomerization regulates activity of Nedd4 ligases'*
We very much appreciate and thank the referees' suggestions and your consideration and valuable comments. As you will see in the revised manuscript we addressed these comments and believe they all significantly improved the manuscript.

In the rebuttal letter, you will see that we address all the comments point-by-point.

Most importantly, with the aim of answering the most critical ensemble of concerns raised, we setup an *in vitro* system with the full-length un-tagged Rsp5, for self- and PPXY-containing substrate ubiquitylation, using a fluorescein labeled ubiquitin. In this system we show that an Rsp5 3K->R mutant of the critical lysine residues that are responsible for the oligomerization and inactivation of the enzyme, presents a significant increased activity both in self- and PPXY-substrate ubiquitylation. These results strongly corroborate our previous data with human NEDD4 in cells, and summarize the identified novel allosteric mechanism for inactivation of the NEDD4 family members.

We hope that you will find the revised manuscript suitable for publication in The EMBO Journal.

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see from the reports, the referees all express interest in the findings reported in your manuscript although they also raise a number of concerns - mainly related to protein constructs used, further mutagenesis, and the size of the regulatory effect - that you will have to address experimentally before they can support publication of your study in The EMBO Journal.

Given the referees' overall positive recommendations, I would like to invite you to submit a revised version of the manuscript, in which you address the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance or rejection of your manuscript will therefore depend on the completeness of your responses in this revised version.

For the revised manuscript I would particularly ask you to focus your efforts on the following points:

-> Both ref #1 and #3 raise concerns about the choice of protein construct used for the analysis (truncation and presence of MBP tag) and it will therefore be important for you to extend these experiments using full-length and untagged protein.

We thank the referees for this comment. As requested, we now demonstrate oligomerization-dependent regulation of catalytic activity of Rsp5 using full-length and untagged protein. As shown in the revised manuscript (Revised Figure 2C) we purified a full-length untagged WT-Rsp5 or an unrestrained-Rsp5 mutant (3K->R) and performed an *in-vitro* ubiquitylation assay with the PPXY-containing substrate Rvs167. In these experiments we also used a fluorescein labeled Ub, which

provided a robust signal that could be easily observed without further processing. Our data clearly supports the suggested mechanism as the unrestrained mutant enzyme presented a significantly higher activity both in terms of self and substrate ubiquitylation.

-> In addition, please include additional mutagenesis work as requested by refs #1 and #2.

We thank the referees for this important point as additional mutants allowed us to dissect the contribution of specific Lys residues to the restrains mechanism of the ligase.
Interestingly, we found that in human Nedd4 only K525 plays a role. This residue is conserved in all eukaryotes. Rsp5-K438 plays the same role, as it is the conserved residue to K525 of the human Nedd4. However, in variation to Nedd4, ubiquitylation of two other lysine residues in the vicinity in Rsp5 can also promote oligomerization of the enzyme.

-> You will see that referee #2 is the most critical of the three and that this person would need to see the findings repeated using a more robust Rps5 activity assay. The referee outlines one potential strategy for doing this but we would also welcome your suggestions for an alternative way to approach this question. As you will see from the report, the referee finds further experimental data for this point to be a prerequisite for supporting publication in The EMBO Journal.

We obtained a fluorescein-Ub, re-purified the proteins of the ubiquitylation cascades and repeated the *in-vitro* Rsp5 ubiquitylation assays as requested. This is a very powerful technique not only because it reads the signal more directly and in a more sensitive manner, but also because it circumvents the complications derived from the Western-blot analysis. As you will see in the revised manuscript we repeated the *in vitro* ubiquitylation assays and demonstrated in a highly robust manner that self-ubiquitylation of Rsp5 at the identified residues inactivates the enzyme.

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. 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.

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

RESPONSE TO REFEREES

Referee #1:

In this interesting manuscript, it is shown that NEDD4 ligases are able to oligomerize, specifically trimerize, a status in which these ligases are proposed to be inactive. The transition from a monomeric, active state, into the trimerization state is proposed to be controlled by ubiquitylation of lysines present in the alpha1 helix that is localized at the N-terminal end of the HECT domain. The evidence for such a mechanism is tested by yeast two-hybrid system, size exclusion chromatography, crosslinking experiments and sedimentation velocity experiments. Computer based modelling is also applied, as well as a number of other sophisticated techniques. The physiological relevance is tested in the yeast on the proteasome subunit RPN10, and in mammalian cells on NEDD4 dependent ubiquitylation of FGFR1, and on Iks channels.

Comments:
1. Based on previous crystallization studies it is proposed that the alpha1 helix present in the NEDD4 family members interferes with the transition from monomeric to a trimeric state. The

authors test the capability of oligomerization in the 2H system (Fig. 1). Why are the authors using a construct that lacks the C2 domain, and not a full-length construct for the crosslinking assay in Fig. 1E (alpha1L construct). What is not understandable is why the authors use either this nearly full-length construct and compare it with a construct containing just the HECT domain lacking alpha1 helix (both in fusion with MBP), to conclude that the alpha 1 helix is interfering with the oligomerization. One cannot draw this conclusion, the correct one would be that the region comprising the alpha1 helix and the WW domains are interfering with the oligomerization. The authors would have to make a near full-length construct in which they delete the alpha1 helix. It follows that both experiments of
1E and 1F are no very useful and do not provide the relevant information.

We agree that the direct conclusion presented in the original manuscript cannot be drawn from the data as originally presented.
We found that the C2 domain interacts (probably in a non-specific manner) with the bacterial membrane. Thus, an α1L construct (termed ΔC2 in the revised manuscript) facilitates the purification, and was used as a control of the nearly full-length protein for the cross-linking and the AUC experiments.
Recently, we developed a purification protocol that circumvents this limitation and purified a full-length Rsp5 fused to a removable MBP moiety.
In the revised manuscript we reordered the results along the line of our conclusion, in a manner that makes more sense:
First we show the structural model of the trimer based on the structure of E6AP. The model provides two hypotheses: *i.* the Δα1 dictates the oligomerization states; *ii*. The oligomerization order is of a trimer.
We then performed SEC analysis with full-length untagged, α1 or Δα1 Rsp5 derivatives to assess the first hypothesis with Rsp5. SEC profile of α1-containing HECT and of Δα1 clearly indicates that α1 removal shifts equilibrium towards self-assembly.
Then we used the nearly full-length (ΔC2) vs. Δα1 to assess the second hypothesis (with use of cross-linking and AUC); note that the AUC was done with untagged proteins.
Reordering the results this way makes the article more coherent and logical. We therefore very much appreciate the reviewer comment that significantly improved this section of the manuscript.
*Please note that our hypothesis on the effect of α1 is also reinforced at a later stage by the sedimentation equilibrium experiment presented in figure 2.

2. Instead of crosslinking, can the authors show the monomeric and oligomeric states on a non-denaturing, native gel? That would be more convincing.
This is a very interesting idea, and we therefore gave it a try. We used both commercial (Invitrogen NativePAGE) and home made NB-Gels but unfortunately in most cases the experiments did not yield presentable images for publication, but a smeared distribution of the complexes in the gel. In the case of the G747E experiment, where the complex is probably less dynamic, a presentable gel was obtained, and therefore presented (Revised Fig. 5C). As we showed the oligomerization in bioinformatics, genetics, biochemical and biophysical approaches we decided to focus on more critical comments.

3. The authors test their model by 2H analysis, using either RSP5 K432,K438,K411R mutant, or a catalytically inactive mutant. The triple K mutant reduces the interaction to some extent. What was the effect with the single mutants, especially K432R, as, according to the model, ubiquitylation of K432 seems to be relevant? Surprisingly, the inactive RSP5C777K mutant, has a much stronger effect than the triple K mutant. Wouldn't that mean that there must be other ubiquitylation sites that are as important as K432 for the interaction? The authors do not address this issue at all, but they should and come up with some explanations. I would not talk about in vivo in the context of the 2H system, this is still a very artificial system.

In the revised manuscript we dissected the contribution of individual lysine residues to the oligomerization of both NEDD4 and Rsp5. In the case of NEDD4 we found that the most conserved lysine, K525, plays a pivotal role in ubiquitylation-dependent oligomerization and K523R did not show any effect (EV Fig. 6). Surprisingly in Rsp5, single mutations at all three lysine residues reduced the oligomerization (as read by the beta-gal units; Fig 2B). Moreover, the triple and the catalytic Cys mutants showed apparently lower beta-gal units, suggesting that cumulative ubiquitylation may totally shuts-off the enzyme activity. It also indicates of course that

ubiquitylation of each lysine is sufficient to significantly reduce the enzyme activity.
We thank the reviewer for this comment as our new experiments provide higher resolution and deeper understanding of the regulation process, and thus significantly improve the manuscript.
As the referee suggested, we changed the terminology for Y2H from *in vivo* to yeast cells.

4. Fig. 2F. How many times has this been repeated? Quantification and statistics should be provided (same for Fig. 4E, and 5E).
In the previous version we repeated the experiment shown in Fig 2F three times but at different time scales so we could not use these for statistics. However, for the revised manuscript, due to comments of the other reviewers we have changed the experimental setup and used fluorescein-labeled Ub instead. With this new approach we repeated all the *in vitro* experiments with Rsp5 (Including the new assay with the untagged full-length WT and 3K->R mutant) between three to five times, quantified them and provide SD as shown in the revised manuscript.

5. When testing NEDD4 on FGFR1, the authors mutate K523, 525R, although they previously talked only about K525. What happens if only K525 is mutated?
As suggested, we tested the individual contributions of each of the NEDD4 lysine residues (EV Fig 6). As seen in the revised manuscript we found that only K525 plays a role in the ubiquitylation dependent restrain mechanism.

6. Page 3 last paragraph, a hypothesis is tested.....the phrase, "As expected, while the....." is not appropriate, as it gives the impression that the authors knew the result already in advanced. "As hypothesized,....." would be better.
We corrected the text accordingly.

Referee #2:

The manuscript by Attali et al. describes an investigation into the function of the Nedd4 family of HECT E3 ubiquitin ligases. Using a series of biophysical, biochemical and functional approaches, the authors propose a model where E3 function is negatively controlled through the oligomerization of the E3. Specifically, the authors identify a helical unit at the N-terminus of the HECT catalytic domain that contains a lysine residue that becomes ubiquitylated in cis. In the author's model, this ubiquitylation event drives the association of the ubiquitin with a ubiquitin binding domain on the HECT that displaces the N-terminal helix, thus enabling oligomerization and inactivation of the E3.

Determining the mechanism of action of E3 ligases and how these enzymes are regulated in the cell are of great interest. The authors' proposal for Nedd4 ligase regulation is novel, and if correct, represents a significant step forward. However, my main concern is with the in vitro ubiquitylation assays. Specifically, the activity shown for Rsp5 HECT is very weak, product formation is measured over extremely long time scales that are unlikely to be physiologically relevant, and the comparison of activity is purely qualitative. The upshot is that the differences in the activities of the various HECT constructs are very subtle and still leaves significant doubt, at least in the mind of this review, as to how oligomerization affects HECT activity.

The authors should address this by using a more convincing activity assay. For instance, the in vitro Rsp5 activity assay developed by Kamadurai and Schulman (Elife, 2013) has far more robust activity than the assay presented here. I realize that since the Rsp5 protein used in that study contains the third WW domain, my proposal would entail working out a method to ubiquitylate Lys 432, and I acknowledge that this is going to be challenging work. However, I think that it is justified since the authors' current approach of fusing ubiquitin to the N-terminus of the HECT domain has obvious limitations and drawbacks. In its current form, I cannot support this paper in EMBO.

We appreciate the reviewer ‘s concern about the fusion-constructs and the sensitivity of the detection for this ubiquitylation method used in the original manuscript. As you will see in the revised manuscript we addressed both concerns by using a full-length untagged self-ubiquitylated WT Rsp5 or 3K>R mutant that cannot undergo ubiquitylation at the critical sites. We also used a fluorescein-labeled-Ub as suggested. Together, our new experiments fully address these main

concerns. Specifically, we demonstrated that 'real' ubiquitylation of the untagged full-length Rsp5 at the identified critical lysine residues inactivates the enzyme. We used a highly robust in vitro ubiquitylation assay with fluorescein-Ub and a classical PPXY containing substrate (Rvs167).

Specific points

1. The authors co-express the ubiquitylation cascade enzymes with Rsp5 in E.coli and map the K432 ubiquitylation site by MS. Do the authors see modification of this lysine residue with ubiquitin from Rsp5 protein derived from yeast cells?
This was previously done by others (Swaney et al. Nat Methods. 2013) in general surveys of the yeast ubiquitome. In this yeast proteomic studies K411 and K432 were identified with the GG-peptide.

2. Does expression of the Rsp5 catalytic cysteine mutant disrupt ubiquitylation of K432 in yeast?
We did not test it directly. Our Y2H indirectly implies this.
As a side-note, why did the authors mutate the cysteine to a lysine residue? Others have shown that this may result in the permanent modification of the lysine residue with ubiquitin which could also affect oligomerization.
The simple answer is that we used this mutant because we had it available. We previously generated it because we wanted to have a permanent conjugation for crystallography purpose (in the past). Since this mutant can accept Ub, but cannot transfer it to other lysine residues it should not form oligomers. In fact, we showed by Y2H (Fig. 2B) that C777K mutant forms only monomeric protein, corroborating our structural model, since even if Ub is conjugated to K777, this cannot open the α1-helix and cannot form linear oligomer.

3. What was the rational behind the triple lys to arginine mutant (K432, K438, and K411) in Figure 2E?
In our MS analysis using the E.coli system we identified K411 and K432 as self-ubiquitylation sites. These two sites were also detected in proteomic studies in vivo in yeast (prior to our study). Since K438 is the most conserved lysine on α1 we chose to mutate this lysine as well.

The authors mention that K438 was identified as a ubiquitylation site in the literature, but what about K411? The authors should include results for the single K432R mutant since this is most relevant to the in vitro ubiquitylation assays.
It is K432 that we identified as ubiquitylation site in the literature and not K438 as mentioned above. In the revised manuscript we addressed this issue in the new Y2H assay presented in Fig. 2B.
We understand that one of our sentences ("However K438, which is was previously detected as target for ubiquitylation, is conserved across the whole Nedd4 family") may have been misleading and we therefore modified it. The meaning was that K438-homologous lysine in higher eukaryotes were shown to undergo ubiquitylation. In yeast ubiquitylation was detected on K432 and K411.

4. The modeling results in Figure 3 are not convincing and should be supported by mutations to ionic residues at both sites in ubiquitin and the HECT domain. Is the I537D mutation complemented by a second I44K mutation in the same HECT and result in restoration of oligomerization? This result would go a long way towards substantiating their model.
The presented model is based on crystal structure of Rsp5 with non-covalent Ub bound to the UBD. We did not change the crystallographic model at this region at all. Huibregtse and co-workers (EMBO Reports 2011) carefully assessed the binding mode presented in this model. Moreover, at the same time the structure of NEDD4-Ub non-covalent complex was also determined and carefully assessed by Polo and co-workers (EMBO Reports 2011). Both structures have essentially identical binding mode thus the request of the referee to repeat this analysis seems to require experiment that are redundant with these previously published studies. Moreover, the specific requested mutations (Ub-I44K and Rsp5-I537D) have low probability to restore binding as they are both located at the center of hydrophobic patches and thus such mutation are predicted to disrupt the entire patch. In such cases the presumably new electrostatic (or salt-bridge) bond cannot compensate for the destruction of the hydrophobic interaction. Such idea may work in case where native electrostatic interaction already exists and exchanging the positive and the negative residues restores the interaction. Actually, we recently demonstrated such exchange based on our crystal structure of a novel UBD, ENTH domain (*Nature Methods,* to be online on Oct 3rd 2016)

5. How many lysine residues are there in the Rpn10 substrate?
This is important because it is unclear whether the authors are observing multi-mono or poly-ubiquitylation of substrate. Indeed on p 7, 2nd paragraph, the authors imply that the substrate is poly-ubiquitylated. Does this mean that Rpn10 is only modified at one lysine?
There are 9 lysine residues in Rpn10, where K84 was identified as the major ubiquitylation site. Moreover, Rpn10 was shown to undergoes mono-ubiquitylation in vivo in yeast. As clearly seen in the revised manuscript Rpn10 undergoes mono-ubiquitylation when we used the fluorescein-labeled-K48C-Ub (Figs 4E-F and 6B-C).

6. How would all of this work in the cell? What is the role of the Ubp2 de-ubiquitylating enzyme (that is known to form a stable complex with Rsp5 in yeast)? I support the authors' contention that these questions are beyond the scope of this manuscript, and I also acknowledge that the authors touch on the subject in the Discussion section. However, their model does suggest that Rps5 would rapidly inactivate itself in cells, and a more thorough discussion of the literature in light of this would be helpful.
We thank the referee for this comment and we expanded the discussion on these topics in the revised discussion.

Referee #3:

Nedd4 ligases belong to the HECT family of E3s and regulate a large variety of cellular processes. Some Nedd4 family members are inhibited by intramolecular interactions, which in turn are regulated by post-translational modifications such as phosphorylation and ubiquitylation.

In this manuscript the authors describe a novel mechanism of Nedd4 regulation, which relies on a ubiquitylation-dependent self-association, which inactivates catalytic activity. This mechanism has been identified in the yeast Nedd4 protein Rsp5 and is shown by the authors to be conserved in mammalian cells. This is a very interesting study that adds another level of regulation to E3 ubiquitin ligase activity.

The manuscript is well written and overall the data presented are of high technical quality. My main concern with the data presented is that almost all experiments have been carried out using MBP-fusion proteins, which is far from ideal when investigating intramolecular interactions and self-association. As described below, the authors need to provide clear evidence that the large MBP tag has no influence on the behaviour of the proteins under investigation.

The reviewer comment on the use of MBP tagged protein is indeed highly important as we had a few experiments with untagged Rsp5. We originally assumed that the presented work with untagged NEDD4 would be sufficient to convince that MBP does not affect the mechanism we proposed. However, retrospectively, we are much more satisfied with the revised manuscript where we used untagged full-length (native) Rsp5 to address this comment (see Revised Fig. 2).

Major points:

1) The experiments shown in Figure 2B using an MBP-Ub-HECT should be repeated without a MBP tag. This tag is very large and may influence the experiment. I do not understand why the authors chose to carry out these experiments with a fusion protein when the tag can be easily removed as shown in Figure 1 F. The same applies for the experiments shown in Figures 4 and 5. The authors should repeat the experiments with the tag removed or at least need to provide additional data that clearly show that the MBP tag has absolutely no influence on self-association.

We thank the reviewer and the editor for these excellent comments.
To show that MBP does not influence our model, we undertook a very challenging approach and developed an *in-vitro* assay with full-length untagged "more authentic" proteins undergoing genuine ubiquitylation. Specifically, we purified a full-length WT Rsp5 and a 3K>R mutant and removed the MBP for *in vitro* activity assays. Moreover, using fluorescein-labeled Ub we performed a highly

robust ubiquitylation assays with the WT and the mutant proteins. Our results are in perfect agreement with the suggested model, showing that the identified lysine residues undergo rapid ubiquitylation leading to restraining of enzymatic activity (both in self-ubiquitylation and in PPXY or UBD-containing substrate ubiquitylation).

In figure 5 we also removed MBP from WT and G747E mutant. The results with untagged proteins perfectly agree with the results from the original manuscript both in terms of oligomerization and activity.

Further support to the idea that MBP does not influence oligomerization can be found in the self-assembly shown in the Y2H system, upon ubiquitylation on α1-lysine residues in the absence of MBP and our in vitro and in vivo experiments with NEDD4 presented in the original manuscript.

2) Figure 2F: As the authors show in Figures 2D and S3, Ub- α 1 is in a monomer-trimer-hexamer equilibrium. This implies that even after collecting the monomer and oligomeric fractions on SEC, they should re-equilibrate and hence contain the same 1:3:6 ratio. Why do they show different levels of activity? Does this mean that this equilibrium is very slow - slower than collecting the fractions and carrying out the experiment?
Please test the monomeric fraction after 1, 2,4 hours on SEC to check if the equilibrium has been re-established.

The proteins used for ultracentrifugation were collected from the oligomeric peak in SEC as it consists of a population in equilibrium rather than a single oligomeric state. Indeed, exchange between different oligomeric states in this sample is shown by MST, in which the signal depends on association dynamics between labeled and unlabeled proteins (in this case labeled Rsp5 with unlabeled Rsp5). The MST clearly shows how self-association depends on Rsp5 concentration. It seems that the monomeric peak consists of proteins incapable of oligomerization, following alterations/damage incurred along the purification process. Interestingly, we did not encounter this phenomenon during Nedd4 purification.

3) The authors should provide SDS gels across the SEC fractions to show that the void peak does not contain the mutant proteins.
In the revised manuscript we used newly prepared proteins following MBP cleavage. Specifically the G747E mutant as well as the other derivatives do not show significant voids, and we therefore did not add SDS-PAGE analysis.

4) I do not fully understand the MST experiment shown in Figure 4D - in this experiments the authors are looking at exchange of labelled oligomers with unlabelled ones. What model was used to fit the data, given that a monomer-trimer-hexamer equilibrium was analysed and hence what does the Kd refer to?
This is an excellent point. There is no good model applied for oligomers (higher than dimer) in the MST data analysis (or in the new analysis software by Chad Brautigam and co-workers Analytical Biochemistry 2015). Analysis is therefore based on dimerization rather than a trimerization. The Kd value therefore depends on the actual association of the whole complex but can only be referred to as apparent affinity for a 1:1 model. It can therefore not be interpreted as constant for trimerization, but provides a good sense of exchange between oligomeric states in solution. To remove doubt we clarified this point in the revised text.

Minor points:

1) Reference Ronchi et al. is incomplete
The reference has been corrected.

2) Figure 1 C: A close up of the clash might be helpful in this figure.
This image was added as EV Figure 1

3) How can the authors exclude that the WW domains that are present in the α1L construct do not

influence self-association?
As explained to referee #1 this is good point. We cannot draw such conclusion from experiments with α1L (termed ΔC2 in the revised manuscript) and we therefore re-arranged our results and conclusion in a more logical order (please see our reply to comment #1 of referee #1).
In short, the new SEC results from the revised Figure 1 with FL-RSp5, α1-containing HECT and Δα1-HECT indicate that it is the presence or absence of α1 that affect the oligomeric state of the protein.
These results are strengthened by AUC sedimentation equilibrium experiment shown in Figure 2E.

4) The authors should add a panel to Figure 2 to show the structure of Rsp5 and indicate the position of K432 to allow the reader to judge for themselves how well a fusion of ubiquitin to the N-terminus of a1 may mimick ubiquitylation (or refer to Figure 3B).

The reference to Figure 3 has been added.

6) Why does the majority of the G747E mutant elute in the void? (Fig 5B). This does not agree with the ratio of monomer-trimer-hexamer determined by SE (Table S1). Are there contaminants in the mixture? The authors should provide an SDS gel of the different peak fractions.
We are not sure about the reason for the large void peak, which often depends on how protein stability is maintained along the purification process (often depending on the protocol used). Due to the requests to remove the MBP tag we repeated the SEC and other experiments of Figure 5 with untagged protein. Since there is no void peak in the revised version, we found no point in running previous samples (Fig. 5B).

7) In Figure 6C the authors show that after 30 min there is no difference between the activity of apo HECT and Ub-HECT anymore. What do the authors think the reason for this is?
The apo HECT undergoes ubiquitylation on the α1, which results in inactivation.
Our assays with full-length Rsp5 WT vs. 3K->R support this idea (Fig. 2C-2E).

2nd Editorial Decision 18 October 2016

Thank you again for submitting a revised version of your manuscript to The EMBO Journal. It has now been seen by all three original referees and their comments are shown below. As you will, all three of them find that the criticisms have been sufficiently addressed and they therefore recommend the manuscript for publication. However, before we can officially accept your manuscript there are a few editorial issues concerning text and figures that I would ask you to address in a final revised version of your study:

-> Please clarify the three minor points raised by ref #3 with text revisions.

-> For all figures displaying statistics please make sure that the nature of the error bars (SD or SEM), the test used, and the number and type of replicas (biological vs technical) is indicated in the figure legend.

-> We generally encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, and please feel free to contact me with any questions for the formatting points listed above. I look forward to receiving your final revision.

REFEREE REPORTS

Referee #1:

The authors have addressed all my original concerns in a satisfactorily manner.

Referee #2:

The manuscript by Attali et al. describes an investigation into the function of the Nedd4 family of HECT E3 ubiquitin ligases. Using a series of biophysical, biochemical and functional approaches, the authors propose an innovative model where E3 function is negatively controlled through the oligomerization of the E3. Specifically, the authors identify a helical unit at the N-terminus of the HECT catalytic domain that contains lysine residues that becomes ubiquitylated in cis. This ubiquitylation event drives the association of the ubiquitin with a ubiquitin binding domain on the HECT that displaces the N-terminal helix, thus enabling oligomerization and inactivation of the E3. This hypothesis is then tested in a functional setting using mammalian cells grown in tissue culture.

The authors addressed my main concern regarding the very weak Rsp5 activity shown in the original manuscript by setting up an in vitro reconstituted ubiquitylation system that contained full-length Rsp5 protein as well as protein substrate. While the results do show a statistically significant change in Rvs167 substrate ubiquitylation comparing wild-type and triple mutant Rsp5, the effect is rather modest at 2- to 3-fold (Figure 2C,D). Furthermore, the Rsp5 activity is still weak and the kinetics are slow compared to activity assays developed by others such as Kamadurai and Schulman.

While these weaknesses are still considerable in the mind of this reviewer, they are without doubt surmounted by the many strong experiments shown throughout the work. The authors did an admirable job in attempting to address the concerns of all three reviewers, and thus I am happy to recommend publication of this manuscript in EMBO.

Referee #3:

The inclusion of new experiments, especially those with untagged versions of Rsp5 have improved this manuscript significantly and I am now happy to recommend publication.

There are just some minor points the authors might want to consider:

- Page 6 new paragraph "Fusing MBP instead of Ub directly...." - according to the figure legend both of these constructs are tagged with MBP. Please clarify.

Generally it is important to ensure that each time a MBP-tagged protein is used this is said explicitly. Sometimes this is not absolutely clear.

- Figures 2 I&J: the signal for ubiquitylated alpha1 at 30min looks much stronger than for Ub-alpha1mono on the gel in 2I but the relative activity in 2J is the other way around. Is this a mistake?

2nd Revision - authors' response 25 November 2016

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The authors have addressed all my original concerns in a satisfactorily manner.

We thank the referee for the review process that significantly improved the manuscript.

Referee #2:

The manuscript by Attali et al. describes an investigation into the function of the Nedd4 family of

HECT E3 ubiquitin ligases. Using a series of biophysical, biochemical and functional approaches, the authors propose an innovative model where E3 function is negatively controlled through the oligomerization of the E3. Specifically, the authors identify a helical unit at the N-terminus of the HECT catalytic domain that contains lysine residues that becomes ubiquitylated in cis. This ubiquitylation event drives the association of the ubiquitin with a ubiquitin binding domain on the HECT that displaces the N-terminal helix, thus enabling oligomerization and inactivation of the E3. This hypothesis is then tested in a functional setting using mammalian cells grown in tissue culture.

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We do not have the equipment that Schulman has to stop the reaction after fraction of a second. However, this does not indicate that our assay developed in a slower manner.
We can not exclude the possibility that a similar signal would obtained at such short time.

While these weaknesses are still considerable in the mind of this reviewer, they are without doubt surmounted by the many strong experiments shown throughout the work. The authors did an admirable job in attempting to address the concerns of all three reviewers, and thus I am happy to recommend publication of this manuscript in EMBO.
We thank the referee for recognizing.

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The inclusion of new experiments, especially those with untagged versions of Rsp5 have improved this manuscript significantly and I am now happy to recommend publication.

There are just some minor points the authors might want to consider:

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This was clarified

Generally it is important to ensure that each time a MBP-tagged protein is used this is said explicitly. Sometimes this is not absolutely clear.
Done

- Figures 2 I&J: the signal for ubiquitylated alpha1 at 30min looks much stronger than for Ub-alpha1mono on the gel in 2I but the relative activity in 2J is the other way around. Is this a mistake?
There is no mistake. The referee was probably confused with the bands of Rsp5 self-ubiquitylation. To clarify and facilitate the reading /viewing we added a subtitle "Rpn10-Ub above the bar-plot. We did the same in the other bar-plots.

EMBO PRESS

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Gali Prag
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Manuscript Number: EMBOJ-2016-94314R

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

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

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the

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E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
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F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under '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	N/A
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
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22. 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.	N/A

G- Dual use research of concern

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