EMBO reports

Manuscript EMBOR-2015-40298

# Phosphorylation of ubiquitin at Ser65 affects its polymerization, targets and proteome-wide turnover

Danielle L. Swaney, Ricard A. Rodríguez-Mias and Judit Villén

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**Review timeline:**

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|---|---|
| Submission date: | 27 February 2015 |
| Editorial Decision: | 19 March 2015 |
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| Editorial Decision: | 01 June 2015 |
| Revision received: | 15 June 2015 |
| Accepted: | 15 June 2015 |

## 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: Nonia Pariente

1st Editorial Decision 19 March 2015

Thank you for your submission to EMBO reports. We have now received reports from the two referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, although the referees find the topic of interest, they both raise several issues which they believe should be addressed for publication.

As the reports are below, I will not detail them here. I think all issues raised are pertinent and should be addressed. Pease note that it is journal policy that all proteomics datasets are deposited in publicly available databases, as referee 2 indicates.

As you know, timing is of essence in this case, so I would like to ask you to reply to these issues with a revised version within the next four weeks if possible, six weeks maximum.

Please note that it is our policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review. If any of the concerns raised poses a serious problem, please let me know.

Please contact me if I can be of any assistance.

REFEREE REPORTS:

Referee #1:

Swaney et al explore functions of ubiquitin (Ub) phosphorylation in yeast. Ubiquitin phosphorylation on Ser65 by PINK1 has been recently suggested to activate E3 ligase parkin on mitochondria or inhibit both Ub chain assembly by E3 enzymes and disassembly by DUBs. In this work, numerous phospho-sites on ubiquitin are identified in yeast and also suggested to be present in mammals. The authors focus on S65 phosphorylation and show that this Ub modification is increased upon oxidative stress accompanied by general accumulation of diverse Ub chains. Consistent with Wauer et al, S65 phosphomimetics induces upregulation of Ub chains attributed to inhibited by S65E DUB activity. Finally, authors identify substrates of phospho-Ub that include histones and SNARE proteins.

This work proposes novel regulatory mechanisms of Ub phosphorylation in vivo. Despite that a few findings have already been reported, the study is rather novel with many interesting parts that could further promote this highly dynamic and exciting area of research. However, certain points need to be addressed better, including effects of S63E on Ub chain assembly and disassembly. The question is also if PINK1 overexpression can generate pS65-modified Ub and mimic oxidative stress phenotypes. There are comments below that could further improve this work.

Major comments:

1. Data on Figure 3b and c are not convincing. Quantify or generate better pictures making sure that bands on WBs are well separated.

2. To strengthen this work, authors could also analyze effects of S65E on interaction with ubiquitin-binding domains.

3. Could data on Figure 4a be quantified or assayed for viability in another more quantitative way?

4. It would be interesting to examine autophagy stimulation/inhibition as in Figure 4a.

5. Does proteasome or DUB inhibition increase pS65 Ub modification?

6. It would be important to validate several hits, histone or SNARE proteins as targets of S65E or pS65 modification.

Minor comments:

1. Page 2, 13th line, there are other UBLs that could be modified by other PTMs, for example LC3.

2. Page 3, 26th line, rephrase or specify what you mean by 'in order to investigate the role of ubiquitin ligases.'

3. Figure 2, please, explain why HECT domain of Rsp5 neutralizes the effect of S65E.

4. Figure 3, why is S65A more sensitive to DUB disassembly?

5. Page 4, 6th line, add appropriate reference here.

6. Page 4, 10th line, remove extra 'of'.

7. Page 4, 5-7th lines from the bottom, this is speculation and may not be needed at this point but could be used for discussion.

8. Reference 24 is not complete in your reference list.

Referee #2:

In this manuscript Swaney et al. used yeast strains expressing ubiquitin, or ubiquitin S65A, or S65E. They used in-vitro ubiquitylation assays to show that when incubated with two different E2 enzymes the S65E mutant fails to synthesize ubiquitin chains. However, it can synthesize poly-ubiquitin chain if incubated with E2 and E3 enzymes (Ubc4 and RSP5). As published recently, the authors show that ubiquitin S65E mutant is resistant to certain DUBs. They tested the sensitivity of these yeast strains for various stress-causing agents and found that S65E mutant expressing yeast is sensitive to canavanine and H2O2. They used quantitative mass spectrometry to compare ubiquitylation levels in cells, and show that ubiquitylation is globally increased in S65E cells compared to WT. They also analyzed protein degradation and turnover and suggest that S65E mutation affects both of these processes.
Recently, several studies have reported a role of ubiquitin S65 in mammalian cells. This study used yeast as a model to study function of this phosphorylation site. While some of their observations are not entirely new, their data supports observations published from mammalian cells. Their approach has strength to study function of S65 in cells exclusively expressing WT or mutant ubiquitin, but a major drawback is that mutants lack the dynamics of phosphorylation. While the data from mutants are informative, the effects of mutants likely overestimate the phenotype of S65 phosphorylation. The authors should candidly discuss these limitations. Some of their analyses, for example, analysis of ubiquitin mutants on protein degradation and turnover are very superficial; the results are poorly described and discussed. Also, the manuscript has several issues that need to be addressed.
Specific comments:
1. The figure 1 contains no new information and should be moved to supplement.
2. What is the status of other ubiquitin phosphorylation sites after H2O2 and canavanine.
3. While the authors state that USP5, the functional homolog of the yeast Ubp14, is primary responsible for chain disassembly, the data shown in Fig 3B show little effect of this DUB on ubiquitin chain disassembly of ubiquitin WT and there is no clear difference between deubiquitylation of ubiquitin WT and S65E mutant chains. Can authors explain this apparent discrepancy?
4. The authors state "We generated poly-ubiquitin free chains.....", but in the Fig 3B it seems to the opposite. From their migration pattern, the chains shown in this figure appear to be of poly-ubiquitin?
5. The authors discuss about the stoichiometry of ubiquitin S65 phosphorylation, but provide no data. The authors should accurately measure the stoichiometry of this site (i.e. by using AQUA-based approach) in unperturbed cells as well as in cells treated with H2O2 and canavanine. This is particularly important for understanding the functional roles of this phosphorylation site. Because the mutants used are somewhat artificial in the sense that they lack the entire dynamics of phosphorylation as modification.
6. The authors should clearly state in the manuscript text (in results, discussion) that they have analyzed di-Gly sites, which are mostly derived from ubiquitin, but they did not specifically quantify ubiquitylation sites. The authors should cite the original references that described the di-Gly approach (Wagner et al., MCP, 2011, and Kim at al. Mol Cell, 2011), instead of self-citing their own work published much later.
7. Supplementary tables for di-Gly modification sites should include detailed information about the quantified peptides, such as sequences of modified peptides, their search scores etc. Also, the authors need to elaborate on what parameters were used for searching MS spectra. All raw data need to be deposited in a public repository, it is not sufficient to provide a web link to authors´ laboratory.
8. For all proteomics experiments the authors should clearly state how many biological replicates were performed for each set type of analysis, and include these data in supplement so that the readers can independently assess the quantitative reproducibility of the data.

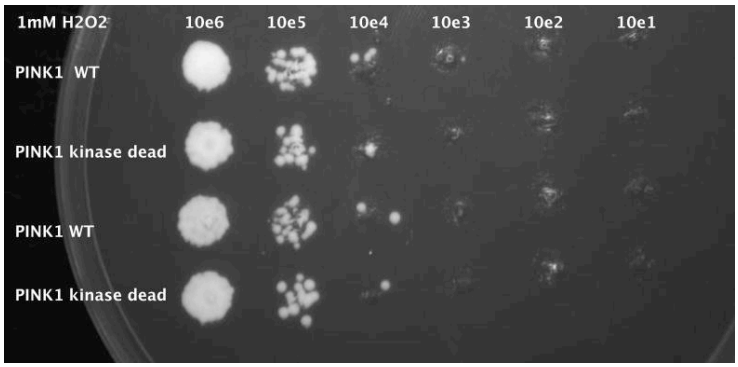
EMBOR-2015-40298 RESPONSE TO REVIEWERS

Referee #1:

Swaney et al explore functions of ubiquitin (Ub) phosphorylation in yeast. Ubiquitin phosphorylation on Ser65 by PINK1 has been recently suggested to activate E3 ligase parkin on mitochondria or inhibit both Ub chain assembly by E3 enzymes and disassembly by DUBs. In this work, numerous phospho-sites on ubiquitin are identified in yeast and also suggested to be present in mammals. The authors focus on S65 phosphorylation and show that this Ub modification is increased upon oxidative stress accompanied by general accumulation of diverse Ub chains. Consistent with Wauer et al, S65 phosphomimetics induces upregulation of Ub chains attributed to inhibited by S65E DUB activity. Finally, authors identify substrates of phospho-Ub that include histones and SNARE proteins.

This work proposes novel regulatory mechanisms of Ub phosphorylation in vivo. Despite that a few findings have already been reported, the study is rather novel with many interesting parts that could further promote this highly dynamic and exciting area of research. However, certain points need to be addressed better, including effects of S65E on Ub chain assembly and disassembly. The question is also if PINK1 overexpression can generate pS65-modified Ub and mimic oxidative stress phenotypes. There are comments below that could further improve this work.

***Response: We greatly appreciate the reviewer's feedback on our manuscript. We have performed yeast viability experiments to test if PINK1 over-expression can mimic the oxidative stress phenotype by transforming yeast with the PINK1 plasmids described in (Koyano et al. Nature 2014). The resulting strains were spotted onto C-Ura +2% raffinose +2% galactose plates containing a range of H2O2 concentrations (1mM, 2mM, 3mM, 5mM). Under these conditions cell growth was only observed at 1mM H2O2. As shown below, we find that expression of wild-type PINK1 and kinase-dead PINK1 render yeast equally viable. Thus we conclude that PINK1 activity does not mimic the oxidative stress phenotype of S65E mutants in yeast. We cannot draw further conclusions of this result without knowing whether endogenous ubiquitin is phosphorylated by introducing PINK1 in yeast and its stoichiometry, an experiment that we could not perform due to time constraints. For this reason, we opted to leave this Figure out of the manuscript.***




Major comments:

1. Data on Figure 3b and c are not convincing. Quantify or generate better pictures making sure that bands on WBs are well separated.

***Response: Unfortunately our attempts to resolve or quantify the bands have failed. We have decided to move this experiment to Expanded View, and show it now duplicate. In addition, we have performed a dose response experiment in which the same dimers are exposed to different concentrations of Usp5. Here again, we observe that the S65E homodimer is more stable than WT to DUB disassembly (new Fig 3B). This data is supported by quantification as shown in Figure 3C. Because both reviewers had issues with Figure 3B, we decided to remove it.***

2. To strengthen this work, authors could also analyze effects of S65E on interaction with ubiquitin-binding domains.
***Response: This is a great suggestion that has allowed us to expand the scope of the paper. Rather than focusing on a particular UBD-containing protein, we decided to take an unbiased approach. We used recombinant His-tagged ubiquitin (WT, S65E and S65A) to pulldown ubiquitin interacting proteins. We then used reductive dimethylation chemistry to incorporate isotopic labels, and performed relative quantification (WT vs S65E and WT vs S65A) by mass spectrometry. We identified a number of proteins involved in the ubiquitylation/deubiquitylation process, and several proteins containing ubiquitin-binding domains, some of which showed differential binding to S65E ubiquitin. A new section has been added to "Results" explaining this experiment and our findings, quantified UBD-containing proteins are displayed in Table 1, and the full dataset is provided as "Expanded View Dataset E1".***

3. Could data on Figure 4a be quantified or assayed for viability in another more quantitative way?
***Response: Yeast spotting assays are one of the most widely used methods of identifying differences in yeast strain viability. In cases where differences in viability are small, we agree that a more quantitative assay would be beneficial. However, for the conditions assayed and highlighted, the viability differences are sufficiently dramatic, such that little additional information would be gained by changing to a more quantitative assay.***

4. It would be interesting to examine autophagy stimulation/inhibition as in Figure 4a.
***Response: While we agree that exploring the relationship between autophagy and S65 mutants could be interesting, we feel it is outside of the scope of this manuscript.***

5. Does proteasome or DUB inhibition increase pS65 Ub modification?
***Response: Again, this is a great suggestion, but looking into this possible feedback mechanism will require more than just measuring pS65 Ub modification. This is a direction that we will be pursuing in our future work.***

6. It would be important to validate several hits, histone or SNARE proteins as targets of S65E or pS65 modification.
***Response: We would like to highlight that the nature of our experimental design incorporates already numerous controls to support our findings. We apologize for not explaining this sufficiently in the manuscript, and have added text to better explain this. First, we look for proteins that show preferential association with S65E vs WT ubiquitin, but also, that do not show preferential association with S65A ubiquitin. Second, for most proteins, we also have the relative quantitation of these proteins in the His-tag flow-through (Extended Dataset file 2), and find that for nearly every case the abundance of these proteins is indifferent to the ubiquitin mutant***

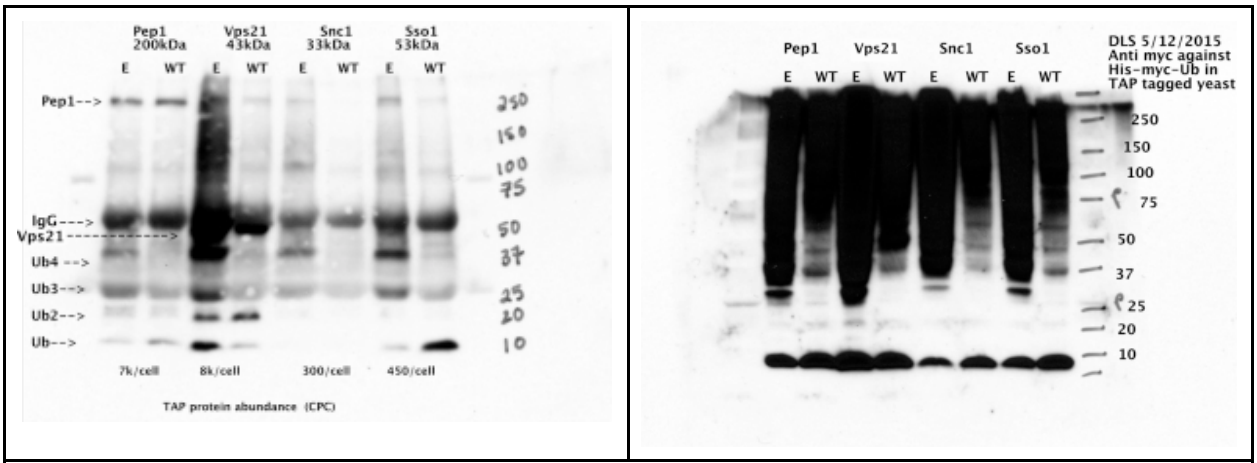
 

***present in the cell. This measurement indicates that the increase in abundance of SNARE, histone associated proteins, etc., is specific to S65E ubiquitin conjugates, rather than an alternative scenario, where the presence of S65E ubiquitin in the cell causes a global increase in these proteins.***

***Furthermore, for some protein hits, such as histone 2B, our experiment provides additional, site specific, quantitative information in support of a preference for ubiquitylation by S65E. In particular for H2B, we observe a 9.4-fold preference for K50 ubiquitylation by S65E vs WT ubiquitin, 2.4 fold preference for K112, and a 1.9-fold preference for K124. We have now included these additional details in the text.***

***Finally, we have attempted to validate several of SNARE proteins using orthogonal methods (co-IP) as described below, but obtained inconclusive results, such as in our validation experiment we cannot distinguish covalent from non-covalent association of S65E to our hits. Specifically, we selected yeast strains for all TAP-tagged SNARE proteins available in the yeast TAP-tagged collection (Vps21, Pep1, Snc1 and Sso1), introduced His-myc-tagged ubiquitin (WT or S65E), performed affinity purification of SNARE proteins and detected tagged ubiquitin by Western blot against the anti-myc antibody. For Vps21 we observe an increase in S65E ubiquitin compared to WT. In addition, we observe interaction of all these proteins with free ubiquitin and unanchored ubiquitin chains, that can be direct or maybe via another interacting protein. This interactions are preferentially towards S65E ubiquitin, but may be an effect of higher abundance of ubiquitin on the S65E His-myc-Ubiquitin strains (see WB for control of protein extracts used as IP input).***

***Therefore, while we observe differential association of SNARE proteins, our validation does not rule out non covalent binding. In order to be certain about a covalent association top-down mass spectrometry analysis would be required, which is not a routine analysis for proteins of this size, and definitely something we cannot accomplish within the limited time of the revision. Note that because in the original proteomics experiments purifications were performed under denaturing conditions, all associations observed there are likely to be covalent.***




Western-blot with anti-myc antibody to visualize His-myc-Ubiquitin. Left: Eluate from affinity purification. Right: Affinity purification input. Yeast strains with TAP-tagged SNARE proteins were transfected with S65E mutant ubiquitin (E) or wild-type ubiquitin (WT).

Minor comments:

1. Page 2, 13th line, there are other UBLs that could be modified by other PTMs, for example LC3.
***Response: We agree with the reviewer that this point could be better emphasized. We have modified the text to be inclusive of UBLs as follows: "Ubiquitin and other ubiquitin-like (UBL) proteins can themselves be modified by other PTMs, adding another layer of complexity that cannot occur on many other modifications".***

2. Page 3, 26th line, rephrase or specify what you mean by 'in order to investigate the role of ubiquitin ligases.'
***Response: The text has been modified to read: "Next, we conducted ubiquitylation reactions in the presence of an E3 ubiquitin ligase. Specifically, we used the HECT domain of the E3 Rsp5 and the E2 Ubc4, and Rsp5 autoubiquitination was monitored over time (Fig 2D)."***

3. Figure 2, please, explain why HECT domain of Rsp5 neutralizes the effect of S65E.
***Response: With S65E we observe an impairment in discharge from the E2. Because E3s assist with the discharge of ubiquitin from E2, it is not surprising the defect is diminished. We have addressed the issue in the text, and reworded to say "mitigates" instead of "neuralizes".***

4. Figure 3, why is S65A more sensitive to DUB disassembly?
***Response: We have eliminated this figure from the manuscript for being confusing.***

5. Page 4, 6th line, add appropriate reference here.
***Response: The appropriate reference has been added.***

6. Page 4, 10th line, remove extra 'of'.
***Response: This edit has been made.***

7. Page 4, 5-7th lines from the bottom, this is speculation and may not be needed at this point but could be used for discussion.
***Response: This text has been moved to the discussion as requested.***

8. Reference 24 is not complete in your reference list.
***Response: This reference has been completed.***

Referee #2:

In this manuscript Swaney et al. used yeast strains expressing ubiquitin, or ubiquitin S65A, or S65E. They used in-vitro ubiquitylation assays to show that when incubated with two different E2 enzymes the S65E mutant fails to synthesize ubiquitin chains. However, it can synthesize poly-ubiquitin chain if incubated with E2 and E3 enzymes (Ubc4 and RSP5). As published recently, the authors show that ubiquitin S65E mutant is resistant to certain DUBs. They tested the sensitivity of these yeast strains for various stress-causing agents and found that S65E mutant expressing yeast is sensitive to canavanine and H2O2. They used quantitative mass spectrometry to compare ubiquitylation levels in cells, and show that ubiquitylation is globally increased in S65E cells compared to WT. They also analyzed protein degradation and turnover and suggest that S65E mutation affects both of these processes.

Recently, several studies have reported a role of ubiquitin S65 in mammalian cells. This study used yeast as a model to study function of this phosphorylation site. While some of their observations are not entirely new, their data supports observations published from mammalian cells. Their approach has strength to study function of S65 in cells exclusively expressing WT or mutant ubiquitin, but a major drawback is that mutants lack the dynamics of phosphorylation. While the data from mutants are informative, the effects of mutants likely overestimate the phenotype of S65 phosphorylation. The authors should candidly discuss these limitations. Some of their analyses, for example, analysis of ubiquitin mutants on protein degradation and turnover are very superficial; the results are poorly described and discussed. Also, the manuscript has several issues that need to be addressed.

***Response: We greatly appreciate the reviewers constructive criticisms on our manuscript. In response to this feedback we have expanded our discussion of the limitations of using a phosphomimetic mutants with regards to residue mimicry, phosphorylation stoichiometry, and phosphorylation dynamics.***
***We have also expanded the results section related to protein turnover and degradation to discuss some of the individual and categorical differences in turnover found within the dataset. Specifically, we looked at proteins with the largest differences between S65E and S65A/WT (new Fig E8). We found Upf2 and Upf3 to have much slower turnover in S65E. These proteins are part of a 3 protein complex (Upf1 (NAM7), Upf2 (NMD2), and Upf3) responsible for nonsense mediated mRNA decay and ubiquitin mediated degradation of the truncated polypeptide.***
***We also analyzed turnover rates in the context of cellular compartments, and show that in some compartments WT ubiquitin behaves more like S65A than in others (new Fig E7). This suggests that different compartments recognize and utilize S65A differently, however why these differences occur and what the biological significance of these differences are is not yet clear.***

Specific comments:
1. The figure 1 contains no new information and should be moved to supplement.
***Response: We agree that Figure 1 contains no new results, and we will be OK with moving this to Supplement. However, we felt as ubiquitin phosphorylation emerges as a new topic in ubiquitin biology, it would be informative to visually recapitulate ubiquitin phosphorylation sites in one Figure (current Fig 1A). This information is not easily accessible to readers, since it is buried in supplementary datasets of proteomics papers. We would like to leave it at the discretion of the editor to decide.***

2. What is the status of other ubiquitin phosphorylation sites after H2O2 and canavanine.
***Response: To address this question we have performed additional SILAC experiments. We find that most phosphorylation sites on ubiquitin show minimal changes upon H2O2 treatment. We have added quantitative data displaying a moderate increase in abundance for ubiquitin S57 phosphorylation following H2O2 exposure (Figure 4B). Experiments analyzing ubiquitin phosphorylation upon canavanine show a 3-fold increase in ubiquitin abundance, while S65 phosphorylation remains constant, resulting in a decrease of S65 phosphorylation stoichiometry (new Fig E4). On the other hand, S57 phosphorylation stoichiometry remains invariable.***

3. While the authors state that USP5, the functional homolog of the yeast Ubp14, is primary responsible for chain disassembly, the data shown in Fig 3B show little effect of this DUB on ubiquitin chain disassembly of ubiquitin WT and there is no clear difference between

deubiquitylation of ubiquitin WT and S65E mutant chains. Can authors explain this apparent discrepancy?
***Response: Because both reviewers raised issues with Figure 3B, we opted for removing it, and instead focus on the competition assay with the dimers.***

4. The authors state "We generated poly-ubiquitin free chains.....", but in the Fig 3B it seems to the opposite. From their migration pattern, the chains shown in this figure appear to be of poly-ubiquitin?
***Response: We apologize for poor wording. We were referring to unanchored poly-ubiquitin chains. This text has been removed together with Figure 3B.***

5. The authors discuss about the stoichiometry of ubiquitin S65 phosphorylation, but provide no data. The authors should accurately measure the stoichiometry of this site (i.e. by using AQUA-based approach) in unperturbed cells as well as in cells treated with H2O2 and canavanine. This is particularly important for understanding the functional roles of this phosphorylation site. Because the mutants used are somewhat artificial in the sense that they lack the entire dynamics of phosphorylation as modification.
***Response: We have conducted AQUA mass spectrometry experiments and quantified basal S65 phosphorylation levels of ubiquitin at <0.5% (Fig E1). We agree with the reviewer comment about the limitations of using mutants, and we have added these limitations to the discussion. On the positive side, however, mutants provide a controlled manner to study phosphorylation in vivo, and also allow detecting extreme phenotypes.***

6. The authors should clearly state in the manuscript text (in results, discussion) that they have analyzed di-Gly sites, which are mostly derived from ubiquitin, but they did not specifically quantify ubiquitylation sites. The authors should cite the original references that described the di-Gly approach (Wagner et al., MCP, 2011, and Kim at al. Mol Cell, 2011), instead of self-citing their own work published much later.
***Response: We apologize for these omissions. We have added text to the results to state that di-Gly sites were measured and that these are suggestive of ubiquitylation, and also added the Wagner and Kim references to this statement. Additionally, we had originally cited our own work to direct readers to our methods of enrichment for ubiquitin-phosphorylation interactions, utilized in this work as well, but agree with the reviewer that it is also important to cite the original di-Gly approach references in the methods.***

7. Supplementary tables for di-Gly modification sites should include detailed information about the quantified peptides, such as sequences of modified peptides, their search scores etc. Also, the authors need to elaborate on what parameters were used for searching MS spectra. All raw data need to be deposited in a public repository, it is not sufficient to provide a web link to authors´ laboratory.
***Response: Additional requested information for results files has been added, and all MS searching criteria have also been specified. Both the RAW data files and the results files have also been uploaded to the PRIDE repository.***

8. For all proteomics experiments the authors should clearly state how many biological replicates were performed for each set type of analysis, and include these data in supplement so that the readers can independently assess the quantitative reproducibility of the data.
***Response: The number of biological replicates has been added to the descriptive paragraph at the top of each Extended Data file. We are also providing the data for***

***each independent replicate so the reader can assess data quality for proteomics experiments.***

2nd Editorial Decision 01 June 2015

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the reports below, both referees are now positive about its publication in EMBO reports. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

We can only accommodate up to 5 expanded view figures. These are presented in an expandable format inline in the main text so that readers who are interested can access them directly as they read the article. They are also provided for download in a separate typeset PDF to accompany the Article PDF. These should be those of particular value to specialist readers, but which are not required to follow the main thread of the paper (and not additional controls or reagent optimization).

Additional figures and tables should be labeled Appendix figure/table X, and will appear as one combined PDF available as a separate download.

The data sets that you have provided will be linked as source data with the appropriate tables and figures.

After all remaining corrections have been attended to, you will receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Given that these are all minor changes, please submit the final version of the study within a week.

REFEREE REPORTS:

Referee #1:

The authors have significantly improved the manuscript by additional data. No further changes are needed.

Referee #2:

This reviewer feels that the authors have adequately addressed the concerns raised and the manuscript can be recommended for publication in EMBO reports.

2nd Revision - authors' response 15 June 2015

I just uploaded the final version in the system. Looking forward to further instructions.
Thanks for making the editorial process so smooth.

3rd Editorial Decision 15 June 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. I will ask our production team to fast-track online publication as much as possible.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication.