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An E2 dimer simultaneously engages donor and acceptor ubiquitins to form Lys48-linked ubiquitin chains

WeiXiao Liu, Yonglaing Shang, Yan Zeng, Chao Liu, Yanchang Li, Linhui Zhai, Pan Wang, Jizhong Lu, Ping Xu, Yihong Ye, Wei Li

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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 02 May 2013

Thank you again for submitting your manuscript on ubiquitin chain formation by Ube2g2 dimers for consideration by The EMBO Journal. We have now received the reports of three expert referees, which you will find copied below. As you will see, all referees consider the basic mechanistic concept worked out here potentially interesting and in principle suited for reporting in our journal. However, they also raise a number of major concerns that would need to be adequately addressed and clarified before the study would be publishable. Of particular importance in this respect are the concerns of referees 1 and 3 regarding the dimerization data, which would need to be substantially strengthened by additional, decisive biophysical experiments as suggested by both of these referees. Furthermore, these reviewers also raise important issues concerning support by quantitative data and excluding the possibility of general folding defects obscuring the analysis of mutant E2 proteins.

Should you be able to satisfactorily improve these aspects of the study, as well as to adequately address the various other specific points, then we should be able to consider a revised manuscript further for publication. I realize that this may require a substantial amount of additional time and effort, and would therefore also understand if you preferred to rather seek rapid publication without major changes elsewhere; however it is clear that only such more definitive data will make the study a sufficiently compelling candidate for publication in a broad general journal such as The EMBO Journal.

If you do decide to revise the manuscript for The EMBO Journal, please be reminded that it is our policy to allow only a single round of major revision, and that it is therefore essential to carefully respond to all points being raised and to convince the referees with additional data and clarifications at this stage. 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.

Thank you for the opportunity to consider this work publication, and please do not hesitate to get back to me should you require further clarification regarding this decision or your revision.

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REFEREE REPORTS:

Referee #1:

In this manuscript the mechanism of K48 ubiquitin chain formation by E2G2 is analyzed and a model is proposed where two thio-ester linked E2 enzymes dimerize to promote ubiquitin chain formation. The arguments are nicely built up, starting from a set of mutations that affect either donor or acceptor ubiquitin function that can complement each other on separate E2s, followed by some evidence for dimerization. Based on this assumption a Haddock model is made with restraints that would allow the K48 to come close to the active site. This model then suggests a charged interaction which is then validated by a charge swap mutant. Interestingly, the K48 specificity can then be lost by the mutants.
Mechanisms for ubiquitin chain formation are a major puzzle, and the one proposed here is plausible and provides a highly interesting variation on the Ubc13/Mms2 model of chain formation, since both E2s now make a thioester. That aspect fits beautifully with the fact that E2G2 holds on to chains on its active site cysteine. Overall the arguments look reasonable and the charge swap validation of the model is very nice. Nevertheless, a large number of the intermediate results are currently not in publishable state and need further validation, in particular there are two major issues that need to be adressed.
Moreover, the quantitative data are not very good yet, and at least require error bars.

1)The dimerization data are not convincing, and there is no data with ubiquitin.
a.Since the model is based on dimerization of the thio-esters it would be great to get some data on this: it is definitely worth trying to make the active site cys to lys mutant to see if the Ron Hay trick of making a stable E2-ub link can be emulated here. If that works, many different options exist to follow dimerization, but gel filtration might be the easiest.
b.The SPR data clearly have some non-specific binding component: hence it is necessary to present the raw data of both the WT and the mutant side-by-side (and preferably the full binding curves, if that starts at -10, just give it from -10) and refrain from trying to fit those data with a curve, just give the results.
c.The cross-linking data are very confusing: how could a single cys crosslink give a set of bands? Clearly the interpretation is in direct contradiction to the actual gels. If the experiments suggested above work it may be better to leave this out. Gel filtration of the E2 dimer might anyway give sufficient shift to prove the dimerization
2)The charge swap mutants are very suggestive, but it would be very interesting to now test them in the context of the donor/acceptor mutations, to really nail the point that the model is correct.

Other points
-The loss of k48 modification in the K48R/E51R mutant is convincing. However, from the S6 figure it is clear that the shift is not exclusively to K63. Hence this aspect needs to be toned down. Alternatively, further evidence for this K63 exclusivity needs to be provided.
-It is necessary to establish that the proposed model is compatible with E3 binding as determined in crystal and NMR complexes.
-I don't understand the phrase 'catalyze cysteine 89' in the second line of page 8, please rephrase.
-As far as I can tell Fig 1 B and D depict the same information: please keep them together.

Referee #2 :
Liu et al. present a generally solid study with some interesting conclusions about a poorly understood, perhaps even a little controversial, mechanism of ubiquitin chain formation. In this mechanism, the chain is attached to the substrate by en bloc transfer from an E2 ubiquitin conjugating enzyme (Ube2g2) active site (catalyzed by the gp78 E3 ligase). The data demonstrate formation of a Ube2g2 dimer by mutagenesis, modeling, and chemical crosslinking, and that this dimer is important for Ube2g2 in vivo function in ERAD and in vitro in forming K48-ubiquitin chains on the Ube2g2 active site cysteine. In addition, an interaction between a specific Arg residue in the donor E2 and acidic residues in the acceptor ubiquitin is shown to be needed for K48 linkage specificity and also seems to contribute to the in vivo ERAD function of Ube2g2. Mass spectrometric determination of linkage specificity changes and nice genetic suppression tests were used to support this last set of results.

Overall, this is a well-executed analysis with only a few minor rough spots, in my opinion. This will be of interest to ubiquitin aficionados, including me, although I wonder a little about the general interest of the story given that the ability of this E2 (Ube2g2) and E3 (gp78) to perform en bloc transfer has been published in several previous papers (Li et al., Nature 2007; Li et al., PNAS, 2009). Moreover, the ability of specific E2s to make specific ubiquitin chain linkages is understood in other examples, particularly Ubc13/Mms2. I like the new mechanistic details in the present study, however, which are different from what has been seen in other systems.

Minor comments:

1. The biochemical data are generally of high quality but some of the in vivo validation is not so compelling. For example, the blots in Figs. 3B, C are overexposed and messy-looking. The inhibition of TCRalpha degradation (Fig. 3D) is pretty small, probably explained mostly by an initial lag in degradation. However, the experiment is well-controlled. Similarly, the effect of the R109E mutation (Fig. 5E) is on the edge of believable, with only the 3-h time point showing any real difference from WT.

2. The protein interaction measurements were done on a ForteBio Octet RED96 platform (p. 8). I had not been familiar with this machine, and in any case a more detailed description of how these measurements were made is needed in the methods section. In general, the methods section is a little sparse.

Referee #3:

Critique of An E2 dimer simultaneously engages donor and acceptor ubiquitins to form Lys48-linked ubiquitin chains
By Weixiao Liu, Yongliang Shang, Chao Liu, Yanchang Li, Linhui Zhai, Pan Wang, Ping Xu, Yihong Ye, Wei Li1
Summary
Ubiquitination plays a role in response to cellular stress in the endoplasmic reticulum by aiding in degradation of retrotranslocated products. The primary ubiquitin conjugating enzyme involved in this process is Ube2g2. Ube2g2 is unique among studied E2 enzymes in that it forms ubiquitin chains on the catalytic cysteine of the E2 and apparently transfers the chain en bloc to the substrate lysine. Liu and coworkers examined the biochemical mechanism of ubiquitin chain specificity. They found that Ube2g2 forms dimers and propose a few residues that are involved in mediating this interaction. They further go on to make a structural model of the dimer and support their model of biochemical and cellular assays. Ultimately, Liu and coworkers propose a model for ubiquitin chain specificity and show they can change the chain specificity of Ube2g2.
The findings are potentially novel and interesting to the ubiquitin field and this reviewer appreciates the amount of work in the manuscript in performing in vitro and in vivo experiments. However, I am not convinced there are not alternative explanations or models from those presented here. Specifically, are the mutations functional mutations or are they structural? Addressing this issue and the others noted below along with significant editing of the text and figures is needed before

publication.
Major concerns
1.) An alternative explanation that fits your data is simply that the proteins containing sequence changes are locally or globally disordered. For the Ube2g2 cysteine mutants, M3, M4, and R109E along with the two ubiquitin mutants some confirmation that the proteins are not functional because of structural defects is needed. This data can come from circular dichroism, multi-angle light scattering, NMR, crystallography, etc. Just please provide additional data that the proteins are folded and the defects observed are due to functional changes.

2.) The presentation of the model is difficult to follow in figures S3, S4 and 5. For example, Figure S3C seems to be critical for your later conclusions but I cannot see how you came to your conclusions based on this figure because all the potential models overlap and the labeling is not clear. One way to simplify the figure would be to show the alignment of the Ube2g2 model and Ubc1 model as one panel, then Ube2g2 aligned with UbcH5, and continue this scheme side-by-side. I think this will help convince the reader of your decision to use Ubc1 for placement of the ubiquitin. Similar editing and simplification of Figures S4 and 5 will aid the reader in understanding the data and conclusions.

3.) I would like to see more clarity in the defining restraints for modeling. When the authors say "The carboxyl-terminal carbon atom of Gly76 of the donor ubiquitin should be close to Cys89 in the donor Ube2g2", what is close? Van der Waals distance or 6 ≈ or a longer distance? The restraints can alter the top scoring models as defining the distances too narrowly makes any other configuration impossible. This goes back to the point 1, where additional experiments to show that the protein isn't disordered by the mutations would support narrow distance restraints in the model.

4.) I urge caution in using the current Ub~E2 structures in the PDB for modeling ubiquitin position as many of these structures have the placement of the donor ubiquitin altered by crystallographic contacts (2GMI from Eddins, et al. 2006 is a well-known example). Are the proposed configurations of the donor ubiquitin and incoming lysine in the acceptor ubiquitin in the model appropriate for the chemistry of ubiquitin transfer? Based on Figure S4, the configuration does not look like it is chemically appropriate, which then leads me to question if the model is correct. However, because the difficulty in reading that figure as noted in point 2, it could be simply poor orientation of the model.

5.) There are numerous spelling, grammatical, and labeling errors throughout the manuscript (including several misspellings of ubiquitin!). Please edit the text and figures to correct these issues.
Minor Concerns
1.) An additional and simple experiment supporting your model would be to see the effect of increasing the solution salt concentration (NaCl, MgCl2, etc.) on dimerization/cysteine crosslinking and ubiquitin chain formation activity. This would provide non-mutagenic evidence that your structural model is plausible.
2.) Figure S1A would be helpful to see in the main figures as it clarifies your experimental setup greatly.
3.) The fit of the SPR data in Figure 2H is questionable because of the point at 100 uM. Please provide error bars or a confidence interval on your data so the reader can evaluate the quality of your data. Also, please provide statistics on the SPR data in Figure 4C.

1st Revision - authors' response 30 July 2013

We thank the reviewers for their constructive suggestions. We are glad to learn that the consensus is that our study is interesting and potentially suitable for the EMBO journal. During the last three months, we have performed many experiments in order to address these comments. In a quick summary, the revised manuscript contains new biochemical data, further supporting the notion that ubiquitin charging promotes Ube2g2 dimerization. We also measure the secondary structures of our E2 variants with circular dichroism (CD) to exclude the possibility that the mutations may induce folding defects, which affect the interpretation of the data. We also re-measure the binding affinity using Biolayer Interferometry. The new data are consistent with our previous SPR results. We also

provide statistical analyses on protein-protein interaction kinetics and include detailed methods describing how the Biolayer Interferometry, CD spectroscopy, Ube2g2 C89K-Ub dimer synthesis and the protein purification experiments were performed. Please see the point-by-point response below for details. We hope that the modified manuscript addresses your concerns satisfactorily.

Referee #1
In this manuscript the mechanism of K48 ubiquitin chain formation by E2G2 is analyzed and a model is proposed where two thioester linked E2 enzymes dimerize to promote ubiquitin chain formation. The arguments are nicely built up, starting from a set of mutations that affect either donor or acceptor ubiquitin function that can complement each other on separate E2s, followed by some evidence for dimerization. Based on this assumption a Haddock model is made with restraints that would allow the K48 to come close to the active site. This model then suggests a charged interaction which is then validated by a charge swap mutant. Interestingly, the K48 specificity can then be lost by the mutants.
Mechanisms for ubiquitin chain formation are a major puzzle, and the one proposed here is plausible and provides a highly interesting variation on the Ubc13/Mms2 model of chain formation, since both E2s now make a thioester. That aspect fits beautifully with the fact that E2G2 holds on to chains on its active site cysteine. Overall the arguments look reasonable and the charge swap validation of the model is very nice. Nevertheless, a large number of the intermediate results are currently not in publishable state and need further validation, in particular there are two major issues that need to be addressed. Moreover, the quantitative data are not very good yet, and at least require error bars.

R: We thank this reviewer for his positive view on the biological significance of our study. We followed the suggestions and performed a large number of experiments. We believe that the new results have significantly strengthened our model. Specifically, we have synthesized and purified Ube2g2 C89K-linked ubiquitin complex, and found that ubiquitin charged to the active site indeed facilitates its dimerization as determined by gel filtration experiments. We have re-done all the Ube2g2 binding affinity using Biolayer Interferometry and provided statistics analyses on the data. We further characterized Ube2g2 R109E and ubiquitin E51R mutants, we found that Ube2g2 R109E mutant is defective in its donor function, and E51R ubiquitin is defective in accepting ubiquitin from the donor E2, but still can be transferred to another ubiquitin charged on the acceptor Ube2g2 which strongly support our model. The detailed responses are described below.

1) The dimerization data are not convincing, and there is no data with ubiquitin.
a. Since the model is based on dimerization of the thioesters it would be great to get some data on this: it is definitely worth trying to make the active site cys to lys mutant to see if the Ron Hay trick of making a stable E2-ub link can be emulated here. If that works, many different options exist to follow dimerization, but gel filtration might be the easiest.

R: Thanks for this valuable suggestion. We created a Ube2g2 C89K mutant, and charging this E2 mutant with ubiquitin under a high pH condition. Although the charging efficiency was not very high, we managed to purify enough Ube2g2 C89K-ubiquitin complex for biochemical characterization. Under these conditions, size exclusion chromatography shows that a fraction of this heterodimer migrates at a position corresponding to a 60KD protein, suggestive of a dimer. Under the same experimental condition, uncharged Ube2g2 C89K migrates exclusively as a monomer (~20kD). The fact that only a fraction of Ube2g2-Ub complex is detected as a dimer is consistent with the interaction data, showing that the E2-E2 interactions are highly dynamic. These new results (shown in Figure 5) suggest that ubiquitin charging promotes Ube2g2 dimerization.

b. The SPR data clearly have some non-specific binding component: hence it is necessary to present the raw data of both the WT and the mutant side-by-side (and preferably the full binding curves, if that starts at -10, just give it from -10) and refrain from trying to fit those data with a curve, just give the results.

R: We re-analyzed Ube2g2 dimerization using Biolayer Interferometry on a Fortebio Octet ^{RED}96 instrument, which generates binding data comparable to that by SPR. This assay uses less protein and is much faster. The new data are consistent with the ones obtained previously. We now present the raw data with statistical analyses in Figure S3 and Table S2, respectively. The reviewer is absolutely right that many biophysical assays for protein-protein interactions have limitations. For

biosensor based assays, they often require immobilization of the bait protein on a sensor CHIP, which may result in non-specific binding. However, we believe that the E2 self-interaction reported here is specific because we can reduce this interaction by only introducing one single point mutation, which does not affect the folding of this E2. Moreover, the biosensor assay is only one of the several methods that we use to demonstrate that Ube2g2 can form a dimer. The data are consistent with other methods used in this study including crosslinking and gel filtration.

c. The cross-linking data are very confusing: how could a single cys crosslink give a set of bands? Clearly the interpretation is in direct contradiction to the actual gels. If the experiments suggested above work it may be better to leave this out. Gel filtration of the E2 dimer might anyway give sufficient shift to prove the dimerization

R: We apologize for not making it clear. Most of the crosslinking experiments were done with single cysteine mutation (only bearing one C to A substitution). Nonetheless, mutating either C48 or C89 is sufficient to abolish both E2 dimer and oligomer. This clearly suggests that the high molecular weight E2 oligomers are formed between these two cysteine residues. We agree with the reviewer that the crosslinking result obtained with the single cysteine mutant is a little bit surprising. In addition to dimer, a small amount of what appears to be tetramer can be seen by western blotting. It is unclear to us how this oligomer species was formed. Given that the gel filtration experiment further demonstrates that the E2 can form a dimer, particularly when it is charged with ubiquitin, we follow this reviewer's suggestion and take out this panel (Previous Figure 2G).

2)The charge swap mutants are very suggestive, but it would be very interesting to now test them in the context of the donor/acceptor mutations, to really nail the point that the model is correct.

R: (1) Following this reviewer's suggestion, the Ube2g2 R109E and ubiquitin E51R mutants were further characterized. We found that Ube2g2 R109E mutant is defective in its donor function (Figure 6B), and E51R ubiquitin is defective in accepting ubiquitin from the donor Ube2g2 but still can transfer ubiquitin to another ubiquitin on an acceptor Ube2g2 (Figure 7A), these results further strengthen our model.
(2) In addition, we tested the charging efficiency, donor and acceptor functions and their assembly polyubiquitin chain assembly on E2 ability of M3 and M4 mutants. We found the charging efficiency is comparable to wild type Ube2g2 (Figure 4F). However, both M3 and M4 failed to serve either as a ubiquitin donor or acceptor E2 (Figure 4H). These results are a bit surprising, but they are not contradictory to our model. Because the surface on the donor E2 corresponding to the dimerization surface on the acceptor E2 is close to the E3 binding site, and maybe involved in E2-E3 communication. Thus, it is expected that mutations on this surface with disrupt both acceptor and donor E2 function. Conversely, the donor E2 dimerization surface in acceptor E2 may have other functions in ubiquitin transfer on the acceptor side, which would explain why mutations on this surface affect both the donor and acceptor E2 functions. These functional analyses also suggest that although M3 and M4 mutants when mixed together can partially restore the E2 dimer interaction, a mutant E2 dimer consisting of one M3 and one M4 mutant should not be active. This prediction is now directly tested using an in vitro ubiquitination assay (Figure 4G).
Other points
-The loss of k48 modification in the K48R/E51R mutant is convincing. However, from the S6 figure it is clear that the shift is not exclusively to K63. Hence this aspect needs to be toned down. Alternatively, further evidence for this K63 exclusivity needs to be provided.

R: We totally agree with this reviewer's judgment "the shift is not exclusively to K63" in the previous Figure 6S. We now remove this panel to save room for other data and tone done our conclusion on page 12. The text reads as "mass spectrometry analysis showed that chains made by UbE51R were predominantly linked by Lys63". Instead of using "exclusively", we use the word "predominantly".

-It is necessary to establish that the proposed model is compatible with E3 binding as determined in crystal and NMR complexes.
R: To address this concern, we superimposed the G2BR domain of gp78 on the donor E2 in our model. We made these models based on the known G2BR-Ube2g2 structure solved by either crystallography or NMR. Neither model shows any structural conflict between G2BR and the

E2~Ub dimer (Figure S8). In addition, the putative RING binding site in the donor E2, which involves H1 and the two small loops are clearly exposed in our model. For these reasons, we believe that our model is compatible with E3 binding.

-I don't understand the phrase 'catalyze cysteine 89' in the second line of page 8, please rephrase.

R: We change it to "catalytic cysteine 89", thanks!

-As far as I can tell Fig 1 B and D depict the same information: please keep them together.

R: They were put together in the Figure 1 C and D.

Referee #2 (Remarks to the Author):

Liu et al. present a generally solid study with some interesting conclusions about a poorly understood, perhaps even a little controversial, mechanism of ubiquitin chain formation. In this mechanism, the chain is attached to the substrate by en bloc transfer from an E2 ubiquitin conjugating enzyme (Ube2g2) active site (catalyzed by the gp78 E3 ligase). The data demonstrate formation of a Ube2g2 dimer by mutagenesis, modeling, and chemical crosslinking, and that this dimer is important for Ube2g2 in vivo function in ERAD and in vitro in forming K48-ubiquitin chains on the Ube2g2 active site cysteine. In addition, an interaction between a specific Arg residue in the donor E2 and acidic residues in the acceptor ubiquitin is shown to be needed for K48 linkage specificity and also seems to contribute to the in vivo ERAD function of Ube2g2. Mass spectrometric determination of linkage specificity changes and nice genetic suppression tests were used to support this last set of results.

Overall, this is a well-executed analysis with only a few minor rough spots, in my opinion. This will be of interest to ubiquitin aficionados, including me, although I wonder a little about the general interest of the story given that the ability of this E2 (Ube2g2) and E3 (gp78) to perform en bloc transfer has been published in several previous papers (Li et al., Nature 2007; Li et al., PNAS, 2009). Moreover, the ability of specific E2s to make specific ubiquitin chain linkages is understood in other examples, particularly Ubc13/Mms2. I like the new mechanistic details in the present study, however, which are different from what has been seen in other systems.

R: We are thankful that this reviewer feels that our study is a well-executed analysis that will be of interest to researchers in the ubiquitin field. We agree that this story is an extension of our previous study, but the study addresses several key mechanistic questions that have not been answered by previous studies. Specifically, the study illustrates how Ube2g2 dimerizes to promote chain assembly on its active site and how an E2 dimer communicates with both donor and acceptor ubiquitin to catalyze Lys48-linked ubiquitin chain formation. The ion pairing mechanism between Ube2g2 and ubiquitin required for synthesizing Lys48-linked ubiquitin chain is distinct from the other reported E2 systems. We therefore believe that our study represents a major step forward in the field.

Minor comments:
1. The biochemical data are generally of high quality but some of the in vivo validation is not so compelling. For example, the blots in Figs. 3B, C are overexposed and messy-looking. The inhibition of TCRalpha degradation (Fig. 3D) is pretty small, probably explained mostly by an initial lag in degradation. However, the experiment is well-controlled. Similarly, the effect of the R109E mutation (Fig. 5E) is on the edge of believable, with only the 3-h time point showing any real difference from WT.

R: The cell based experiments are a little bit tricky to do because cell lysates often contain a large amount of DNA, which causes the messy appearance of the Western blots. However, as the reviewer point out, we carefully controlled the experiment and also repeated each experiments several times. The immunoblotting were all performed using fluorescence labeled secondary antibodies and the results are quantified by an Odyssey scanner. We now provide statistics analyses for some of the time points for these chase experiments, which show that the differences are statistically significant. I hope that these analyses will ease the reviewer's concern on the in vivo data.

2. The protein interaction measurements were done on a ForteBio Octet RED96 platform (p. 8). I had not been familiar with this machine, and in any case a more detailed description of how these measurements were made is needed in the methods section. In general, the methods section is a little sparse.

R: We now provide detailed methods about Biolayer Interferometry, CD spectroscopy, Ube2g2 C89K-Ub dimer synthesis and purification in the methods section. We hope this will help the reviewer and readers follow our experiments.

Referee #3 (Remarks to the Author):
Critique of An E2 dimer simultaneously engages donor and acceptor ubiquitins to form Lys48-linked ubiquitin chains
By Weixiao Liu, Yongliang Shang, Chao Liu, Yanchang Li, Linhui Zhai, Pan Wang, Ping Xu, Yihong Ye, Wei Li1
Summary
Ubiquitination plays a role in response to cellular stress in the endoplasmic reticulum by aiding in degradation of retrotranslocated products. The primary ubiquitin conjugating enzyme involved in this process is Ube2g2. Ube2g2 is unique among studied E2 enzymes in that it forms ubiquitin chains on the catalytic cysteine of the E2 and apparently transfers the chain en bloc to the substrate lysine. Liu and coworkers examined the biochemical mechanism of ubiquitin chain specificity. They found that Ube2g2 forms dimers and propose a few residues that are involved in mediating this interaction. They further go on to make a structural model of the dimer and support their model of biochemical and cellular assays. Ultimately, Liu and coworkers propose a model for ubiquitin chain specificity and show they can change the chain specificity of Ube2g2.
The findings are potentially novel and interesting to the ubiquitin field and this reviewer appreciates the amount of work in the manuscript in performing in vitro and in vivo experiments. However, I am not convinced there are not alternative explanations or models from those presented here. Specifically, are the mutations functional mutations or are they structural? Addressing this issue and the others noted below along with significant editing of the text and figures is needed before publication.

Major concerns
1.) An alternative explanation that fits your data is simply that the proteins containing sequence changes are locally or globally disordered. For the Ube2g2 cysteine mutants, M3, M4, and R109E along with the two ubiquitin mutants some confirmation that the proteins are not functional because of structural defects is needed. This data can come from circular dichroism, multi-angle light scattering, NMR, crystallography, etc. Just please provide additional data that the proteins are folded and the defects observed are due to functional changes.

R: We are thankful that the reviewer feels that our findings are potentially novel and interesting. The major concern of this reviewer is whether the mutations we introduced are functional or structural mutations. To address this question, we determined the secondary structures of the key Ube2g2 mutants used in this study including C48A, M3, M4 and R109E and that of the E51R and D58R ubiquitin mutants by circular dichroism (CD), as suggested by the referee. The results show that the mutations do not cause any significant folding defects in either E2 or ubiquitin (Figure S4, Figure 4D-E, Figure S10 B). Only the CD spectra of the D58R ubiquitin mutant show a subtle difference when compared to the wild type counterpart. Since D58R ubiquitin mutant is used as a control, we believe that this small folding variation should not affect our conclusion.

2.) The presentation of the model is difficult to follow in figures S3, S4 and 5. For example, Figure S3C seems to be critical for your later conclusions but I cannot see how you came to your conclusions based on this figure because all the potential models overlap and the labeling is not clear. One way to simplify the figure would be to show the alignment of the Ube2g2 model and Ubc1 model as one panel, then Ube2g2 aligned with UbcH5, and continue this scheme side-by-side. I think this will help convince the reader of your decision to use Ubc1 for placement of the ubiquitin. Similar editing and simplification of Figures S4 and 5 will aid the reader in understanding the data and conclusions.

R: We thank the referee for these helpful suggestions. To improve the presentation, we now split the

previous Figure S3 into two figures, Figure S5 and Figure S6. In the Figure S6, our Ube2g2 model is presented side-by-side with the six reported E2~ubiquitin structures respectively, which were shown as Figure S6A-F.

3.) I would like to see more clarity in the defining restraints for modeling. When the authors say "The carboxyl-terminal carbon atom of Gly76 of the donor ubiquitin should be close to Cys89 in the donor Ube2g2", what is close? Van der Waals distance or 6 Å or a longer distance? The restraints can alter the top scoring models as defining the distances too narrowly makes any other configuration impossible. This goes back to the point 1, where additional experiments to show that the protein isn't disordered by the mutations would support narrow distance restraints in the model.

R: We now specify the distance constraint in the text. The text reads as "The carboxyl-terminal carbon atom of Gly76 of the donor ubiquitin should be close to Cys89 (2 Å) in the donor Ube2g2" in our new manuscript.

4.) I urge caution in using the current Ub~E2 structures in the PDB for modeling ubiquitin position as many of these structures have the placement of the donor ubiquitin altered by crystallographic contacts (2GMI from Eddins, et al. 2006 is a well-known example). Are the proposed configurations of the donor ubiquitin and incoming lysine in the acceptor ubiquitin in the model appropriate for the chemistry of ubiquitin transfer? Based on Figure S4, the configuration does not look like it is chemically appropriate, which then leads me to question if the model is correct. However, because the difficulty in reading that figure as noted in point 2, it could be simply poor orientation of the model.

R: Thanks for the suggestion. As in point 2, we have considered all six available Ub~E2 structures in the PDB, and found the Ubc1~ubiquitin complex (1FXT) produced no major structural conflict with the model, after refined with HARDOCK. Another E2~Ub complex (PDB code 4AP4) also produces a similar structure (Figure S6F), but it contains some minor structural conflicts. The fact that two E2~Ub structures crystallized under different conditions lead to a similar model suggests that the position of the ubiquitin in our model is unlikely a result of crystal packing. In addition, the side chain of Lys48 from acceptor ubiquitin aligns perfectly within the channel and points right toward the catalytic cys of the donor E2. The C-temimus of the donor ubiquitin and incoming lysine in the acceptor ubiquitin is about 6.0 Å apart from each other. The flexibility of the ubiquitin C terminus may further shorten this distance during the transfer of ubiquitin from the donor E2 to the acceptor (Figure S7B). Since a single figure is difficult to conveyall the information, we provide the PDB file of our model.

5.) There are numerous spelling, grammatical, and labeling errors throughout the manuscript (including several misspellings of ubiquitin!). Please edit the text and figures to correct these issues.

R: We have tried out best to correct as many spelling, grammatical mistakes and the labeling errors.

Minor Concerns
1.) An additional and simple experiment supporting your model would be to see the effect of increasing the solution salt concentration (NaCl, MgCl2, etc.) on dimerization/cysteine crosslinking and ubiquitin chain formation activity. This would provide non-mutagenic evidence that your structural model is plausible.

R: We thank the reviewer for this great suggestion. We have tested the effect of salt on Ube2g2 dimerization by gel filtration. Consistent with our model, we found that dimerization of the Ube2g2-Ub hetero-complex is significantly reduced with salt treatment (Figure 5F). This finding further supports the proposed electrostatic interactions between two Ube2g2 molecules. In addition, when the E3-independent di-ubiquitin formation experiment was performed in the presence of salt, we found that the formation of Ube2g2-linked di-ubiquitin is highly sensitive to salt concentration (Figure 5G). This experiment indicates that the established electrostatic interactions between two Ube2g2 molecules are functional.

2.) Figure S1A would be helpful to see in the main figures as it clarifies your experimental setup greatly.

R: We move Figure S1A to the main text.

3.) The fit of the SPR data in Figure 2H is questionable because of the point at 100 uM. Please provide error bars or a confidence interval on your data so the reader can evaluate the quality of your data. Also, please provide statistics on the SPR data in Figure 4C.

R: We repeated all the interaction experiments using a new experimental set-up that is assessable to us. This so called Biolayer interferometry technology is similar to Biacore in measuring protein-protein interaction. We measure the dimerization kinetics for wild type Ube2g2 and several mutant variants. The raw data are presented in Figure S3, Table S2 and statistic analyses were provided in Figure 2C and Figure 4C.

2nd Editorial Decision 26 August 2013

Thank you for submitting your revised manuscript for our consideration, and please excuse the delay associated with its re-review during the summer vacation period. Two of the original referees have now evaluated it once more, and while both of them find the study considerably improved, one of them still retains major reservations. These concerns refer mainly to the biochemical/biophysical protein-protein interaction analyses and their quantification, and therefore unfortunately affect a key aspect of the study. I am afraid that in our view, the revised manuscript is at this point still not acceptable for publication in The EMBO Journal.

As mentioned before, it is our policy to allow a single round of major revision only. However, since two referees are at this point supportive, and the well-taken remaining problems raised by referee 1 should be in principle addressable (albeit only through a significant amount of additional experimental efforts), I would be inclined to offer you one more opportunity to address the open issues with biophysical analyses, data quantitation and protein interaction specificities through an exceptional second round of revision. I will not reiterate the referee's comments here, as they appear to be very clearly explained in their report. However, I have to stress that all these issues will have to be clarified to the referee's satisfaction, and it also clear that the requested follow-up experiments may ultimately confound the original conclusions of the study. I therefore hope you understand that I am not in a position to make any strong commitments on whether we may ultimately be able to accept this manuscript for publication in The EMBO Journal. Nevertheless, should you be confident that you may be able to convince the referee by substantially improving the criticized analyses and strengthening the original conclusions, then please resubmit a revised manuscript, together with a detailed point-by-point response letter, via the hyperlink provided below.

I am therefore returning the manuscript to you once again at this stage, hoping you will be able to obtain the decisive data required for making this study a strong candidate for an EMBO Journal article during an additional round of major revision. Please note that this will however have to be the final revision round for this manuscript at our journal. Should you have any question in this regard, please do not hesitate to contact me.

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REFEREE REPORTS:

Referee #1:

This manuscript has improved considerably, particularly with the gelfiltration of the E2-K-Ub, suggesting that dimerization occurs. Nevertheless, there are still serious concerns with the data in this manuscript
1) The analysis of the protein interaction studies is not yet acceptable. Similar to the SPR data the new Octet biolayer interferometry data clearly reveal the presence of a non-specific component. As discussed in the previous review, this does not have to be a problem if the data are only analyzed

qualitatively, by showing the traces of WT and mutants side by side. If the concentrations used and the amount of protein immobilized are equivalent (now not possible to evaluate, since these numbers are not given anywhere), these can be viewed and discussed. However, for quantitative analysis of such data it is necessary to have a physical meaningful model and acceptable residuals. The individual analysis of kinetic analysis of four different concentrations with single-site binding kinetics, with each giving different answers just glosses over the fact that other things are going on and is not acceptible. In such a case, an equilibrium analysis is better than a kinetic analysis, but 4 concentrations are not sufficient and hence this will require a more thorough analysis.
In these experiments the M3 mutant seems to have a serious problem of non-specific precipitation onto the sensor and should be removed entirely. The RU is much higher and goes up with the loading, with no release whatsoever. The calculation of kinetic data is meaningless and this mutant is not suitable for analysis with this method. That is also true in the M3-M4 case.
2) In the gelfiltration experiment the protein concentration is not indicated. It could be of interest to try to do concentration series and show that the equilibrium shifts into the larger peak. It is necessary to give the protein concentrations and the column used in materials and methods.
3) The cross-linking data were problematic since the single cysteine mutant had unexplained extra bands. Now this data point is removed, but how likely is it that the underlying problem is also present in the other cross-linking experiments? The presented quantification is only on the cross-linked products, which are a mix of different bands on a western blot. Surely it would be better to quantify the E2 band itself, which does not vary as much and is less sensitive to subtle westernblot changes.

Referee #3 :

Summary

Ubiquitination plays a role in response to cellular stress in the endoplasmic reticulum by aiding in degradation of retrotranslocated products. The primary ubiquitin conjugating enzyme involved in this process is Ube2g2. Ube2g2 is unique among studied E2 enzymes in that it forms ubiquitin chains on the catalytic cysteine of the E2 and apparently transfers the chain en bloc to the substrate lysine. Liu and coworkers examined the biochemical mechanism of ubiquitin chain specificity. They found that Ube2g2 forms dimers and propose a few residues that are involved in mediating this interaction. They further go on to make a structural model of the dimer and support their model of biochemical and cellular assays. Ultimately, Liu and coworkers propose a model for ubiquitin chain specificity and show they can change the chain specificity of Ube2g2. These findings offer fresh insight into E2 reaction specificity.

The revised version of the manuscript is greatly improved and this reviewer appreciates the time the authors took in doing the science and considering reviewer suggestions. I am satisfied with the manuscript and believe the authors did a good job of addressing my concerns and the concerns of the other reviewers.

2nd Revision - authors' response 25 October 2013

Referee #1 :
This manuscript has improved considerably, particularly with the gel filtration of the E2-K-Ub, suggesting that dimerization occurs. Nevertheless, there are still serious concerns with the data in this manuscript
1) The analysis of the protein interaction studies is not yet acceptable. Similar to the SPR data the new Octet biolayer interferometry data clearly reveal the presence of a non-specific component. As discussed in the previous review, this does not have to be a problem if the data are only analyzed qualitatively, by showing the traces of WT and mutants side by side. If the concentrations used and the amount of protein immobilized are equivalent (now not possible to evaluate, since these numbers are not given anywhere), these can be viewed and discussed. However, for quantitative analysis of such data it is necessary to have a physical meaningful model and acceptable residuals. The individual analysis of kinetic analysis of four different concentrations with single-site binding

kinetics, with each giving different answers just glosses over the fact that other things are going on and is not acceptible. In such a case, an equilibrium analysis is better than a kinetic analysis, but 4 concentrations are not sufficient and hence this will require a more thorough analysis. In these experiments the M3 mutant seems to have a serious problem of non-specific precipitation onto the sensor and should be removed entirely. The RU is much higher and goes up with the loading, with no release whatsoever. The calculation of kinetic data is meaningless and this mutant is not suitable for analysis with this method. That is also true in the M3-M4 case.

R: We repeated the biolayer interferometry experiments with various concentrations of WT and the C48A mutant proteins, and carefully compared the interaction response side by side from 1.25μM to 80μM. We then did steady state analysis which is similar to the equilibrium analysis of SPR to get the KD of these two proteins. Our results showed that the dimerization affinity of the C48A mutant decreased significantly compared to wild type Ube2g2 (Figure 2F, Supplementary Figure S3B, C).

We agree with the reviewer that the chip or sensor-based protein interaction studies are potentially confounded by background non-specific interactions that occur between the chip/sensor and certain mutant E2 proteins that carry multiple mutations (e.g. M3 and M4). To overcome this problem, we used another biophysical method to measure the E2-E2 interaction. We measured directly the rupture force among WT Ube2g2 and its variants pairs by atomic force microscopy (AFM). The rupture force between two C48A mutants was 17.63±0.56pN compared to 24.08±1.19pN for wild type Ube2g2 (Figure 2H). This result provides additional evidence that the self-association between two C48A Ube2g2 mutants is weaker than that for wild type Ube2g2.

Because AFM measures the rupture force between two single molecular, thus it can overcome the non-specific precipitation problem of the M3 and M4 mutants. We therefore also measured the rupture force of M3-M3, M4-M4 and M4-M3. Consistent with the crosslinking results, the rupture forces required to dissociation M3 and M4 homo-dimers were significantly lower than that required for breaking wild type Ube2g2 dimer. By contrast, the rupture force between M4-M3 was comparable to that of wild type Ube2g2 (Figure 4C and Supplementary Figure S3D-G). We removed the Biolayer data for the M3 and M4 mutant to give room to these new results.

2) In the gel filtration experiment the protein concentration is not indicated. It could be of interest to try to do concentration series and show that the equilibrium shifts into the larger peak. It is necessary to give the protein concentrations and the column used in materials and methods.
R: The concentration and amount of proteins used in the gel filtration experiment are now provided either in the figure or in the figure legend (Figure 5).

We also tried the suggest titration experiment. With increasing concentration of Ube2g2~Ub, the percentage of dimer increased significantly (Figure 5 F, G), suggesting the dimer and monomer of Ube2g2~Ub are in an equilibrium state.

The column used was Superdex 75 10/300 GL, and the information is now provided in the 'materials and methods' part now.

3) The cross-linking data were problematic since the single cysteine mutant had unexplained extra bands. Now this data point is removed, but how likely is it that the underlying problem is also present in the other cross-linking experiments? The presented quantification is only on the cross-linked products, which are a mix of different bands on a western blot. Surely it would be better to quantify the E2 band itself, which does not vary as much and is less sensitive to subtle western blot changes.
R: As shown in the Ube2g2 structure, C89 and C48 are on the opposite sides of the molecule. Therefore, the only way to crosslink these two residues is to have two E2s interact with each other in a front-to-back orientation (see the model below). It is conceivable that in the model, multiple Ube2g2 molecules will be crosslinked to form larger oligomers via these two cysteine residues. This explains why the crosslinking products are multiple bands, rather than a single E2 dimer species. We do not understand why the single cysteine mutants (each carrying two mutations) we generated still had some background crosslinking. This might be related to the non-specific E2 interactions discussed above, which appears to form when too many mutations were introduced in this E2. However, the data obtained with mutants carrying a single amino acid substitution are clear and convincing. Mutating either C48 or C89 significantly affects the crosslinking products, whereas the C75A mutation had no significant effect (Figures 2C, D, E).

As one can see from Figure 5, only a small amount of crosslinked E2 oligomers (no more than 10 %) was formed under our experimental conditions. As a result, the maxiumum reduction in the E2 band after crosslinking is only less than 10%, which under the current Western condition is

almost invisible because the signal of the E2 band is close to saturation. On the other hand, the signals for the crosslinking products are in the linear dynamic range and the change in the Western signal has a larger dynamic range.

Acceptance letter 4 November 2013

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by the original referee 1, and I am happy to inform you that there are no further objections towards publication in The EMBO Journal!

