# EMBO *reports*

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# Molecular Insights into RBR E3 Ligase Ubiquitin Transfer Mechanisms

Katja Dove, Benjamin Stieglitz, Emily Duncan, Katrin Rittinger and Rachel Klevit

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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.)

**The EMBO Journal**

1st Editorial Decision                                                                                        09 February 2016

Thank you again for submitting your manuscript on RBR E3 ubiquitin transfer mechanisms for our consideration. I have now obtained the reports from two expert referees, which are copied below for your information.

As you will see, both referees acknowledge the importance the topic, but also have some significant reservations as to whether your present results offer sufficiently decisive and direct new insights into how transthiolation is favored over non-specific ubiquitination. In addition to several experimental and technical issues related to this main concern, they also feel that the study is currently not well presented, and also not adequately set out and discussed in the context of various earlier studies relevant to the present findings.

Given the overall interest of the subject, as well as the potential complementarity of your analyses to the very recently reported structural data on HOIP RBR/E2-Ub interaction, I would nevertheless remain open to considering a single, expedited round of revision for The EMBO Journal. For such a revision to be eventually successful, it will however be important to satisfactorily respond to all key points of the referees, and in particular:
- please reorganize and carefully edit the study, making sure to introduce all relevant earlier papers in the introduction, and to discuss your findings better in the context of the body of published work.

While the recent HOIP structure would not yet need to be referenced in the introduction, it would clearly have to be taken account in the discussion as well, and possibly also at some points in the results (see below).

- please also reorganize the presentation and organization of the data, following the comments and suggestions of the referees. Please also remove the two footnotes, integrating their contents into the main text at the appropriate positions.

- several technical concerns need to be experimentally addressed: referee 1's issue with Figure 1 controls; referee 2's concern about assays and quantifications used in Figures 1 & 5; referee 2's request regarding the extended R1-E2 interaction surface, and question regarding HHARI R2-catalyzed ubiquitination.

- for referee 2's question regarding the analyses of non-covalent ubiquitin binding sites, which have been studied in previous papers and are also a main thrust of the recent HOIP structural study: while any direct data you may have to answer this would clearly be helpful, I feel it may not be absolutely essential to follow this up in detail with mutagenesis, but maybe to use the reported mutational and (new) structural data to discuss these issues in context.

- finally, may I suggest slight rewording of the title into "Molecular insights into RBR E3 ligase ubiquitin transfer mechanisms" to make it more generally accessible?

To ensure timely publication of this work, we hope you would ideally be able to get such a revised manuscript, together with a detailed point-by-point response for the referees, back to us within two months. As usual at The EMBO Journal, competing work published elsewhere during this period would have no negative impact on our final assessment of your own study. In any case, should you have any specific questions/comments regarding the referee reports or your revision work, please do not hesitate to get in touch with me ahead of time for further discussions.

Thank you again for the opportunity to consider this work for The EMBO Journal, and I look forward to hearing from you.


REFEREE REPORTS

Referee #1:

Dove and colleagues characterize the interactions between ubiquitin-conjugated E2 enzymes and RBR E3 ligases using activity assays and NMR spectroscopy. The paper builds heavily on previous studies from the Klevit group that show the some E2~Ub conjugates adopt a closed conformation that favors ubiquitin transfer to lysine residues of substrates (aminolysis). Here, the authors show that E2~Ub binding to RBR-family E3 ligases favors the open conformation and hence transfer to the E3 catalytic cysteine (transthiolation). There is a lot of interest in RBR ligases and the structure of an E2~Ub and RBR ligase complex would be very significant. However, most of the evidence in the current manuscript is indirect and there isn't any insight into the actual mechanism of transthiolation. A key element that is missing is a mechanistic explanation of why the closed conformation is more reactive toward lysine residues. Is the E2~Ub thioester more exposed when Ub is held out of the way of incoming lysine residues?

The manuscript also suffers from poor presentation. The results are spread over 14 main and supplemental figures and there are a number of small errors and inconsistent terminology that make reading it difficult. As far as I could tell, the authors have mislabeled the mutation T341N as T431N in half of the occurrences. Much of the supplemental material should be included in the main figures while Figure 4 should be removed.

While this manuscript was under review, the structure of a complex of the HOIP RBR in complex with E2~Ub was published (Lechtenberg et al, Nature, 2016). That structure shows that the E2~Ub conjugate is indeed in an extended (open) conformation with key interactions with the hydrophobic patch of Ub mediated by a helix that precedes RING2. The linchpin RING1 residue that Dove et al suggest plays a role in activating classical RING ligases is far from the action in the RBR structure.

Thus the finding that the conformation of E2~Ub bound to RING1 domains biases ubiquitin transfer to intermolecular lysine or intramolecular cysteine residues is likely to be true but the significance of the evidence presented here is debatable. The difference between classical RING and RBR E3

ligases is more profound than a bias in the conformation of the E2~Ub upon RING1 binding.

Points for improvement:

Abstract, pg 2, I'd suggest "it exposes the hydrophobic" rather than "it reveals the hydrophobic". The following sentence "Mutations in either..." either has an extra word or is missing a word. I'd suggest changing the sentence to conclude "being critical for Ub transfer by RBR ligases."

pg 3, top, "Introduction" not "Intro"

Throughout the paper, the abbreviations of R1 and R2 for RING1 and RING2 are inconsistently applied. The text and figures will be clearer with the names written out as RING1 and RING2.

pg 3, bottom, "Only a few years ago, a third class" should be "Only a few years ago, the third class" since the three classes have already been introduced in the first paragraph.

pg 5, change "the final step in Ub transfer by RING-type E3s" to "the final step in Ub transfer". It is the final step for all E3s, not just RING-type E3s.

pg 5, "canonical RING/Ubox". U-box ligases should be mentioned in the introduction.

pg 7, "absence of an E3 UbcH7-O-Ub" is missing a comma.

pg 9, simplify the sentence "Finally, we wondered whether HHARI R1 can disrupt closed conformations of other E2~Ub species that detectably populate E3-independent closed conformations in the absence of E3 such as Ubc13~Ub (Pruneda et al, 2011)."

pg 10, first paragraph. I didn't understand the importance of transthiolation as an isoenergetic reaction given that both aminolysis and transthiolation are spontaneous and do not require energy. The excess of free lysine in the experiments of S5b,c should drive the reaction forward despite the difference in energy between the isopeptide bond and thioester. (My guess is the isopeptide bond is lower energy anyhow.)

pg 13, remove "extremely" from "extremely high concentration"

pg 14, misformatted reference "Stieglitz, 2013 #56"

pg 21, the residue numbers for the GST-Parkin(R0-RBR) construct should be given. That fragment is (presumably) mistakenly referred to as GST-Parkin(RBR) in the legend to Figure 1a. "PO4" should be used instead of "Pi". "SDS-page" should be fully capitalized.

pg 22, unbalanced parenthesis in the Fig 3 legend.

pg 23, "unknown position". The sentence should be reworded to include possibility that the peak simply broadens and is undetectable.

Figure 1 needs a control experiment with a classical RING E3 (such as FLAG-BRCA1) that shows a difference between WT and UbcH5 L104Q. MW markers need to be indicated on all the gels and in the different bands identified in panel 1c (also in Figures 5, S5, and S6.) The figure legend should specify that the Parkin W403A mutant was used.

Figure 2, a linear horizontal scale for the graph in 2a would be preferable since it would allow the unambiguous determination of the residue numbers. Most importantly, the full plot of CSP for UbcH7-O-Ub(I44A) should be presented for comparison. The formula for the CSP should be given in the Materials and Methods section. In the legend, the meaning of the phrase "CSPs greater than 1 stdv" better explained. What was the standard deviation? The sentence, "A black arrow.... cross-over helix", is unnecessary and should be removed from the legend.

Figure 3, the supplemental plot in Fig S3a should be included in the main figure. The reversion of two chemical shifts upon RING1 binding feels very anecdotal. For example, why does the chemical

shift of residue 48 not shift upon RING1 binding? In Fig S3b-d, are the differences simply related to affinity?

Figure 4, I didn't see the point of this experiment. Showing that the U-box is active without the RING2 catalytic cysteine doesn't add anything to the manuscript.

Figure 5, the difference between I44A Ub in the HHARI and Parkin assays is interesting.

Figure 7, a simple schematic model showing the open and closed conformations of E2~Ub bound to RING1 domains would help and might be preferable to structures shown. In panel b, the vertical, horizontal, checkered circles would be clearer as a,b,c etc. Panels c and d are not useful.

Given the large number of different E3 and E2 enzymes compared, it is important that the figures are clearly labeled. The reader shouldn't have to know that arih1 and HHARI are equivalent terms (e.g. in Figs S1c and S7).

Figure S1a, using different colors for the Ub and UbcH7 peaks would make the figure clearer.

Figure S5. Why is the lysine reactivity in the "No E3" controls different in panels b and c? The mixed use of the asterisk in the two panels is confusing. (It is not necessary in panel c.) The decrease in reactivity in the presence of the HHARI RING1 is not convincing. Are the replicates in Fig S5d completely independent experiments and gels? Why does the "No E3" lane in panel b show 100% free UbcH5 but only 30% in the plot in panel d? The first sentence in the legend is missing the word "(red)".


Referee #2:

General opinion:
The manuscript from Dove et al describes biophysical and biochemical insights into the mechanism of ubiquitin transfer in the RBR class of E3 ligases. Work over the last few years has uncovered that RING type ligases promote formation of closed E2~ub (charged E2) states, with substantial evidence that these states are most favorable for discharge of ubiquitin onto lysine residues. For HECT ligases there does not appear to be a requirement for a closed E2~ub state. In 2011, the Klevit lab made the landmark breakthrough discovery that RBR-type ligases were genuinely catalytic, harboring a catalytic cysteine required for activity. Since then, many labs have been interested in understanding the RBR-ligase mechanism. In this manuscript Dove et al make the observation that the transfer of ubiquitin from E2 to catalytic cysteine in RBR ligases is achieved by disfavoring closed E2~Ub states. They also observe non-covalent ubiquitin binding by a BR linker within the RBR unit to be a prerequisite for activity. On the whole, I find this advance to be somewhat incremental and not fully examined. The authors' previous study (Pruneda et al., 2012, Mol Cell., figure 3) shows that non RING E3s (HECTs & RBRs) do not require closed E2~Ub states to catalyze ubiquitination events. Furthermore, it is not clear if non-covalent ubiquitin binding site close to R2 of the RBR unit is required for the isoenergetic transthiolation step (E2~Ub to RBR~Ub) or aminolysis step (RBR~Ub to target ubiquitination) or both.

Major issues:

I find the manuscript to be quite specialized with respect to E2~ub/E3 interactions however, data pertaining to subsequent crucial events such as the ubiquitin loading of RBR and substrate/target ubiquitination are somewhat incomplete. In general the manuscript is difficult to follow, many different constructs of proteins are used, tagged, truncated, yet often referred to just by the name of the protein. In conjunction, the figures are difficult to follow, for example, figure 1: It would be useful to have sequence limits for each protein under study, as a cartoon. Items that are necessary for following the manuscript are in supplementary figures, and the quality of data in some of the main figures is not always great.

In figure1 the authors use a Coomassie assay for HOIP, but western blots for HHARI, Parkin and TRIAD1. Western blots are not reliably quantitative, and to draw the conclusion that L104Q mutation has no effect on the activity of GST-Parkin RBR in particular is difficult to support from

the data presented. There are many other assays (Coomassie staining in Sauve 2015, EMBO J, fluorescence in Stieglitz 2012, EMBO Rep, Kumar 2015, EMBO J) that could generate quantifiable data that would allow the authors to address the question of whether this mutation has an effect on activity. A modified assay setup would also allow a comparison of RBR activity between UbcH5, UbcH5 L104Q and UbcH7. In particular with HHARI that has a higher affinity to UbcH7 than UbcH5. Also the setup used in Figure 5c (HOIP H887A ~Ub charging) is an elegant setup and should be adapted for all RBRs and in particular for figure 5a assay.

I find the use of the RBR module, with tags, a little troublesome. Particularly in the case of the most-studied RBR, Parkin, there is a RING0/RING1 interface, as well as a RING0/RING2 interface, thus the RING0 domain sits in between the two R domains, therefore how meaningful is a GST-RBR construct? A similar argument holds for the HHARI RBR module. In the case of HOIP, we do not yet know where any other domains from either HOIP, or HOIL-1L/Sharpin, therefore the use of the minimal unit is understandable, but it is more difficult to support in the case of those whose domain architecture is known.

The authors propose the R1 within RBRs have an extended interaction surface on E2s that overlaps (and hence outcompetes) the Ub binding surface seen in closed E2~Ub conformations. Thus in contrast to canonical RINGs, there would be no difference in R1 affinity to charged versus uncharged E2s. This needs to be tested for multiple R1s. Recent work (Kumar (2015) EMBO J) also suggest the Parkin R1 can support non-covalent ubiquitin interaction.

The authors also observe that UbcH7~Ub and Ubc13~Ub adopt closed conformation in absence of an E3. An earlier study (Matsuda et al. 2006, J Biol Chem) using a tagged BR only construct (i.e lacking R1) is capable of catalyzing ubiquitination with multiple E2s including the two studied here. Will the HHARI R2 construct used in Figure6 catalyze ubiquitination? If so, will the addition of HHARI R1 (in trans) influence this reaction?

The presence of a non-covalent ubiquitin binding site close to R2 of the RBR unit has been previously proposed/demonstrated (Chaugule et al., 2011, EMBO J., Zheng & Hunter, 2013, Cell Res., Rankin et. al., 2014, Biochemistry). Rankin et. al. (figure 9) also denote a UIM region which is distinct from that proposed in this study. The true nature of the ubiquitin binding domain could easily be tested using peptides that span just the BR linker (HHARI 325 to 342, Parkin 392 to 415). Finally, the residues Thr341/E352 in HHARI and T415/E426 in Parkin proposed by the authors to be involved in ubiquitin interaction have been earlier proposed (Figure 3, Spratt et al., 2013, Nat Commun.) to participate as catalytic residues during R2~Ub loading and/or off loading. The authors should either attempt to experimentally determine roles for these residues or discuss alternatives.

The authors also suggest major rearrangements to occur in RBRs that allow R2 to be recruited to the R1/E2~Ub module. In particular, the straightening of a kinked R1 helix is required based on the recent Phosphoubiquitin bound Parkin truncated structure (Wauer et al. 2015 EMBO J). This is very speculative and should be toned down. Also Figure 7b suggest the extended E2~Ub conformation could also be stabilized by the UBA domain of HHARI. This domain has been reported to bind NEDD8 and not Ubiquitin (Kelsall et al., 2013, EMBO J). Thus the model as stands is not supported by current data.

The referencing is patchy. For example, HHARI and Parkin structures are referred to as full length but the citations from Wauer (2013) and Riley (2013) are not full length Parkin structures, while other Parkin structures with all domains present are not referred to : Kumar (2015) EMBO J; Sauve (2015) EMBO J. Also the discovery that RBRs bind to ubiquitin non-covalently has been observed previously in Chaugule (2011) EMBO J, indeed figure 5a is almost identical to figures 7a/b in the above mentioned paper, including mutation of the I44 hydrophobic patch. Zheng and Hunter (2013) CELL RES also reports that binding to ubiquitin is in the case of Parkin critical for activity. This finding should be discussed in the context of the existing literature. Further, despite multiple references to inhibited states of RBRs, the original paper defining the inhibition is not cited: Chaugule (2011) EMBO J. Finally, although the Lechtenberg/Riedl paper describing the RBR of HOIP bound to E2~ub was not yet published when this manuscript was submitted, the findings in that paper will need to be discussed and put into context. It may help to put the footnote describing the findings from Smit et al (2012) into perspective.

More generally there are several statements without correct (or any) citations, e.g. p10 RBRs have been shown to be active with a variety of E2s. No citation. HHARI seems to work best with UbcH7, for years Parkin was only shown to function with UbcH7 or UbcH8, there are papers with these 'multiple E2' experiments in them, and they should be included.

The discussion also lacks proper citations. Linkers are referred to on p17 without reference to the primary literature, full-length parkin structures are attributed to Wauer and Komander and Riley et al., despite both these structures lacking 17kDa of the protein.
p18 'details of release of auto inhibition are specific for each RBR' has no citation, latter half of that sentence refers to removal of inhibitory domains without referring to Sauve 2015/Chaugule 2011/Kumar 2015 all of which show inhibition of parkin RBR by a ubiquitin-like domain.

Supplementary figure 1 should be part of figure 1. The authors are establishing the notion of open vs closed conformation when adding HHARI RING in isolation, the CSPs are modest, but present. These are high quality data.

S2 should be part of figure 2.
S3b-e make THE critical point that is the crux of the paper, high quality data that demonstrate that R1s behave differently to RINGs, that disfavoring of the closed state being specific to the RING1 proteins, and should not be in supplementary data.

What about activators? How do they fit in? For example phosphoubiquitin? Does an activator position the RING1 as per the proposed model? Or does an activator create ubiquitin binding domains? Some discussion of current understanding of RBR mechanisms would be helpful.

Minor points:
Figure S4A is introduced in the text before S3B-E.

I like the hybrid experiment, using the U-box, it's a clever and informative experiment.
Fig 2 legend says INSERT, do the authors mean inset?

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**1st Revision - authors' response**　　　　　　　　　　　　　　　　　　　　　29 March 2016

I am pleased to submit a revised manuscript for consideration by EMBO J. titled "Molecular Insights into RBR E3 Ligase Ubiquitin Transfer Mechanisms." We have carefully considered and responded to all the reviewers'' comments and suggestions and believe that the resulting manuscript is both easier to read and stronger. The contents of figures have been reorganized to follow the reviewers' suggestions and additional controls have been included where requested. Citations and mentions of previous papers with a bearing on our story have been added. We have also taken the opportunity to include the new HOIP-E2~Ub structure paper in our Discussion section as it provides complementary insights to ours. At the same time, we have tried to highlight the new and additional insights that our studies provide as well as a brief mention of ways in which our results are distinct from the crystal structure.

We include point-by-point responses to the highly detailed set of comments provided by the two reviewers. We hope they will find us to be responsive and that they are satisfied with the revisions made.

We look forward to hearing concerning our contribution in the near future.

Referee #1:

*Dove and colleagues characterize the interactions between ubiquitin-conjugated E2 enzymes and RBR E3 ligases using activity assays and NMR spectroscopy. The paper builds heavily on previous studies from the Klevit group that show the some E2~Ub conjugates adopt a closed conformation that favors ubiquitin transfer to lysine residues of substrates (aminolysis). Here, the authors show that E2~Ub binding to RBR-family E3 ligases favors the open conformation and hence transfer to the E3 catalytic cysteine (transthiolation). There is a lot of interest in RBR ligases and the structure*

*of an E2~Ub and RBR ligase complex would be very significant. However, most of the evidence in the current manuscript is indirect and there isn't any insight into the actual mechanism of transthiolation. A key element that is missing is a mechanistic explanation of why the closed conformation is more reactive toward lysine residues. Is the E2~Ub thioester more exposed when Ub is held out of the way of incoming lysine residues?*

**Response:** We agree that a mechanistic explanation of why closed conformations are more reactive toward lysine residues is an important question. But the focus of this study involves transfer to cysteine not lysine, so the question is outside the scope of the current study.

*The manuscript also suffers from poor presentation. The results are spread over 14 main and supplemental figures and there are a number of small errors and inconsistent terminology that make reading it difficult. As far as I could tell, the authors have mislabeled the mutation T341N as T431N in half of the occurrences. Much of the supplemental material should be included in the main figures while Figure 4 should be removed.*

**Response:** We simplified the terminology and made it consistent throughout the manuscript. A new Table (1) lists the different constructs used in our study. We do not feel we can decrease the total number of figures and still effectively present our results, but we have simplified some of the figures by removing some data panels. We moved Supplemental Figures S1 and S3B-D to the main text to enhance readability. Figure 4 presents a key point of the manuscript which is that the induction of **OPEN** E2~UB is functionally relevant in the context of RBR E3s, as closed states would likely lead to off-target ubiquitination effects. Hence, RING1 domains do not **lack** a function (compared to canonical RING domains), but rather their function differs purposefully. Please note that Reviewer #2 felt that Figure 4 was a particularly strong aspect of the manuscript.

*While this manuscript was under review, the structure of a complex of the HOIP RBR in complex with E2~Ub was published (Lechtenberg et al, Nature, 2016). That structure shows that the E2~Ub conjugate is indeed in an extended (open) conformation with key interactions with the hydrophobic patch of Ub mediated by a helix that precedes RING2. The linchpin RING1 residue that Dove et al suggest plays a role in activating classical RING ligases is far from the action in the RBR structure. Thus the finding that the conformation of E2~Ub bound to RING1 domains biases ubiquitin transfer to intermolecular lysine or intramolecular cysteine residues is likely to be true but the significance of the evidence presented here is debatable. The difference between classical RING and RBR E3 ligases is more profound than a bias in the conformation of the E2~Ub upon RING1 binding.*

**Response:** While the differences between canonical RING domains and RBR E3 ligases extend beyond differences between RING1 of RBR E3 and canonical RINGs, the differences we uncovered between these two structurally related domains are both significant and surprising. Indeed we find that they function in the *opposite* direction to canonical RINGs. RING1 domains actively favor open E2~Ub conformations to ultimately enforce Ub transfer through the RING2 active site Cys by a) not activating the E2~Ub for aminolysis and b) expose the hydrophobic patch of Ub that binds to and recruits RING2. Thus, the favoring of open versus closed E2~Ub conformations have large functional consequences.

Points for improvement:

*Abstract, pg 2, I'd suggest "it exposes the hydrophobic" rather than "it reveals the hydrophobic". The following sentence "Mutations in either..." either has an extra word or is missing a word. I'd suggest changing the sentence to conclude "being critical for Ub transfer by RBR ligases."*

**Response:** Changes made.

*pg 3, top, "Introduction" not "Intro"*

**Response:** Changes made.

*Throughout the paper, the abbreviations of R1 and R2 for RING1 and RING2 are inconsistently applied. The text and figures will be clearer with the names written out as RING1 and RING2.*

**Response:** Changes made.

*pg 3, bottom, "Only a few years ago, a third class" should be "Only a few years ago, the third class" since the three classes have already been introduced in the first paragraph.*

**Response:** Changes made.

*pg 5, change "the final step in Ub transfer by RING-type E3s" to "the final step in Ub transfer". It is the final step for all E3s, not just RING-type E3s.*

**Response:** Here the final step in transfer by RING-type E3s refers to the transfer of Ub from the E2~Ub to the substrate while the final step for HECT-and RBR-type E3 is the transfer from the E3~Ub onto a substrate. To clarify this point we added "activated for aminolysis reactions that define the final step…".

*pg 5, "canonical RING/Ubox". U-box ligases should be mentioned in the introduction.*

**Response:** Ubox E3 ligases are now introduced in the first paragraph.

*pg 7, "absence of an E3 UbcH7-O-Ub" is missing a comma.*

**Response:** Comma added.

*pg 9, simplify the sentence "Finally, we wondered whether HHARI R1 can disrupt closed conformations of other E2~Ub species that detectably populate E3-independent closed conformations in the absence of E3 such as Ubc13~Ub (Pruneda et al, 2011)."*

**Response:** We split this sentence into two to simply it: *"Finally, we wondered whether HHARI RING1 can disrupt closed conformations of other E2~Ub species. Ubc13~Ub detectably populates closed conformations in the absence of E3 (Pruneda et al, 2011)."*

*pg 10, first paragraph. I didn't understand the importance of transthiolation as an isoenergetic reaction given that both aminolysis and transthiolation are spontaneous and do not require energy. The excess of free lysine in the experiments of S5b,c should drive the reaction forward despite the difference in energy between the isopeptide bond and thioester. (My guess is the isopeptide bond is lower energy anyhow.)*

**Response:** We removed Fig. S5b and the sentence regarding the isoenergetic reaction.

*pg 13, remove "extremely" from "extremely high concentration"*

**Response:** Changes made.

*pg 14, misformatted reference "Stieglitz, 2013 #56"*

**Response:** Changes made.

*pg 21, the residue numbers for the GST-Parkin(R0-RBR) construct should be given. That fragment is (presumably) mistakenly referred to as GST-Parkin(RBR) in the legend to Figure 1a. "PO4" should be used instead of "Pi". "SDS-page" should be fully capitalized.*

**Response:** All studies for this manuscript were done using PARKIN-RBR (without RING0). Everything is now labelled correctly. "PO4" was changed to "Pi" and SDS PAGE was fully capitalized.

*pg 22, unbalanced parenthesis in the Fig 3 legend.*

**Response:** Changes made.

*pg 23, "unknown position". The sentence should be reworded to include possibility that the peak simply broadens and is undetectable.*

**Response:** It is true that broadening is a possibility. We added "either broadens significantly or shifts…" to the sentence to allow for that possibility.

*Figure 1 needs a control experiment with a classical RING E3 (such as FLAG-BRCA1) that shows a difference between WT and UbcH5 L104Q. MW markers need to be indicated on all the gels and in the different bands identified in panel 1c (also in Figures 5, S5, and S6.) The figure legend should specify that the Parkin W403A mutant was used.*

**Response:** Fig. 1 now includes experiments with Flag-BRCA1/BARD1 (a canonical RING dimer) along with assays with HHARI and Parkin using the same stocks of UbcH5 wt and mutant). The new assays in Figure 1 were performed with WT parkin. MW markers were added to all gels in the manuscript.

*Figure 2, a linear horizontal scale for the graph in 2a would be preferable since it would allow the unambiguous determination of the residue numbers. Most importantly, the full plot of CSP for UbcH7-O-Ub(I44A) should be presented for comparison. The formula for the CSP should be given in the Materials and Methods section. In the legend, the meaning of the phrase "CSPs greater than 1 stdv" better explained. What was the standard deviation? The sentence, "A black arrow.... cross-over helix", is unnecessary and should be removed from the legend.*

**Response:** Due to limited space we were unable to generate a linear horizontal scale. We have changed the labelling from every 10th residue to every 4th residue to make it easier to read. The formula used to calculate CSPs is added to the Materials and Methods section. We added the values (mean + 1stdv) that were used to determine cut-offs for chemical shifts and intensity loss. The sentence "A black arrow…" was removed.

*Figure 3, the supplemental plot in Fig S3a should be included in the main figure. The reversion of two chemical shifts upon RING1 binding feels very anecdotal. For example, why does the chemical shift of residue 48 not shift upon RING1 binding?*

**Response:** We expanded the histogram for better readability, but due to its size we believe that it does not fit well into a main figure. In the main figures, we present two clear examples of chemical shift perturbations that support the main points that UbcH7~Ub populates closed states independent of an E3 (Fig. 2) and RING1 binding disrupts those closed states (Fig. 3). Ub residue Q49 exemplifies a chemical shift being affected in "reverse" order upon RING1 binding and we note in the text that peaks do not completely shift back to their exact original (free Ub) position, but rather a position that is close to free Ub. We state that the subtle differences reflect changes of the chemical environment which are likely caused by the presence of RING1. Fig. 3b presents data from the point-of-view of UbcH7 that further confirm our conclusion that RING1 disrupts closed UbcH7~Ub states. Importantly, the peak corresponding to the methyl group ($-CH_3$) of Ala 110 disappears (broadens or shifts) when UbcH7 is conjugated to Ub and reappears at almost the same position as free $-CH_3$ (Ala110 – blue peak) when RING1 is added to UbcH7~Ub (red peak). Full spectral overlays are provided in Figure S5 for the reader as well.

*In Fig S3b-d, are the differences simply related to affinity?*

RING1 domains have higher affinity for UbcH7~Ub than canonical RING-type domains. We therefore added 3-fold excess of the Ubox domain of E4BU to ensure generation of sufficient E3-bound UbcH7~Ub complex (Fig. S6). Likewise, the affinity of RING1 to Ubc13~Ub is quite modest so the complex is far from saturated under attainable conditions (Fig. S7), but a perturbation of the closed state towards the open ("free") Ub state is readily observable.

*Figure 4, I didn't see the point of this experiment. Showing that the U-box is active without the RING2 catalytic cysteine doesn't add anything to the manuscript.*

We believe that Figure 4 represent a key point of the manuscript which is that the induction of **OPEN** E2~Ub is functionally relevant in the context of RBR E3s. In this experiment the Ubox domain is used as a tool to simulate a condition where a RING1 might induce a closed state, so we could ask whether the promotion of open E2~Ub is relevant in the context of the RBR. This experiment shows that *IF* RING1 domains were to behave like canonical RINGs (as simulated with the Ubox hybrid experiment) this would lead to off-target ubiquitination events that do not proceed through the RING2 active site. Hence, RING1 domains do not lack a function (compared to canonical RING domains), but rather their function differs purposefully.

*Figure 5, the difference between I44A Ub in the HHARI and Parkin assays is interesting.*

**Response:** We agree that the different effects of I44A and other Ub mutations tested among RBR E3s is quite interesting. We point out in the discussion that there is little conservation of the proposed Ub binding surface on RING2. Analysis of various Ub binding motifs that specifically bind to the hydrophobic patch of Ub shows that Ub binding motifs differ greatly. Therefore, differences on the binding surface on each RING2 (and therefore slightly different Ub:RING2 binding modes) may account for the differences seen among the various Ub mutants we tested.

*Figure 7, a simple schematic model showing the open and closed conformations of E2~Ub bound to RING1 domains would help and might be preferable to structures shown. In panel b, the vertical, horizontal, checkered circles would be clearer as a,b,c etc. Panels c and d are not useful.*

**Response:** We have followed the suggestion and include a new, simplified schematic. We have also moved panels c and d into the supplement, as we think they contribute smaller points to the discussion section. We hope that the new version of the figure will help the reader understand the proposed mechanism better.

*Given the large number of different E3 and E2 enzymes compared, it is important that the figures are clearly labeled. The reader shouldn't have to know that arih1 and HHARI are equivalent terms (e.g. in Figs S1c and S7).*

**Response:** We have changed the names accordingly.

*Figure S1a, using different colors for the Ub and UbcH7 peaks would make the figure clearer.*

**Response:** Unfortunately, it is not possible to color peaks of Ub vs. UbcH7 separately because the spectrum was collected as single data set of the E2~Ub. As all peaks were collected in the SAME experiment and they will always have the same color unless we digitally alter the image which we are unwilling to do.

*Figure S5. Why is the lysine reactivity in the "No E3" controls different in panels b and c? The mixed use of the asterisk in the two panels is confusing. (It is not necessary in panel c.) The decrease in reactivity in the presence of the HHARI RING1 is not convincing. Are the replicates in Fig S5d completely independent experiments and gels? Why does the "No E3" lane in panel b show 100% free UbcH5 but only 30% in the plot in panel d? The first sentence in the legend is missing the word "(red)".*

**Response:** Although, we believe that the Lys reactivity assays added a nice nuance to the story, we decided to remove those panels as those don't significantly change the outcome of the manuscript and seemed to cause confusion.

Referee #2:

General opinion:
*The manuscript from Dove et al describes biophysical and biochemical insights into the mechanism of ubiquitin transfer in the RBR class of E3 ligases. Work over the last few years has uncovered that RING type ligases promote formation of closed E2~ub (charged E2) states, with substantial evidence that these states are most favorable for discharge of ubiquitin onto lysine residues. For HECT ligases there does not appear to be a requirement for a closed E2~ub state. In 2011, the Klevit lab made the landmark breakthrough discovery that RBR-type ligases were genuinely catalytic, harboring a catalytic cysteine required for activity. Since then, many labs have been interested in understanding the RBR-ligase mechanism. In this manuscript Dove et al make the observation that the transfer of ubiquitin from E2 to catalytic cysteine in RBR ligases is achieved by disfavoring closed E2~Ub states. They also observe non-covalent ubiquitin binding by a BR linker within the RBR unit to be a prerequisite for activity. On the whole, I find this advance to be somewhat incremental and not fully examined. The authors' previous study (Pruneda et al., 2012, Mol Cell., figure 3) shows that non RING E3s (HECTs & RBRs) do not require closed E2~Ub states to catalyze ubiquitination events. Furthermore, it is not clear if non-covalent ubiquitin binding site*

*close to R2 of the RBR unit is required for the isoenergetic transthiolation step (E2~Ub to RBR~Ub) or aminolysis step (RBR~Ub to target ubiquitination) or both.*

**Response:** While previous studies have shown data that **imply** that RBR E3s do not require closed conformations of E2~Ub, our study goes far beyond what had been shown previously:

1) We show that not only do RBR E3s not **require** closed E2~Ub, they specifically **promote** OPEN E2~Ub.
2) We show that keeping the E2~Ub in open conformations is functionally relevant to promote transfer of Ub onto the active cite CYS of RING2 (and hence prevent off-target ubiquitination).
3) We identify a Ub binding function and map its surface on RING2. This binding is functionally critical as mutations in either Ub or RING2 that decrease binding also decrease ubiquitination activity by several RBR E3s.
4) In addition, we show that Ub mutations (V70A for HHARI) that decrease binding to RING2 also show decrease generation of the E3~Ub demonstrating that Ub binding is crucial for the Ub transfer to the E3 active site.

Major issues:

*I find the manuscript to be quite specialized with respect to E2~ub/E3 interactions however, data pertaining to subsequent crucial events such as the ubiquitin loading of RBR and substrate/target ubiquitination are somewhat incomplete. In general the manuscript is difficult to follow, many different constructs of proteins are used, tagged, truncated, yet often referred to just by the name of the protein. In conjunction, the figures are difficult to follow, for example, figure 1: It would be useful to have sequence limits for each protein under study, as a cartoon. Items that are necessary for following the manuscript are in supplementary figures, and the quality of data in some of the main figures is not always great.*

**Response:** We have taken pains to simplify and clarify the manuscript to make it easier to follow. We now include a Table (1) that provides information regarding all constructs used. Following the reviewer's suggestion, we moved Figures S1 and S3b-d into the main text. We hope the revised manuscript is easier to follow and thank the reviewer for these suggestions.

*In figure1 the authors use a Coomassie assay for HOIP, but western blots for HHARI, Parkin and TRIAD1. Western blots are not reliably quantitative, and to draw the conclusion that L104Q mutation has no effect on the activity of GST-Parkin RBR in particular is difficult to support from the data presented. There are many other assays (Coomassie staining in Sauve 2015, EMBO J, fluorescence in Stieglitz 2012, EMBO Rep, Kumar, 2015, EMBO J) that could generate quantifiable data that would allow the authors to address the question of whether this mutation has an effect on activity. A modified assay setup would also allow a comparison of RBR activity between UbcH5, UbcH5 L104Q and UbcH7. In particular with HHARI that has a higher affinity to UbcH7 than UbcH5.*

**Response:** We added a control experiment using BRCA/BARD1 to Figure 1 to show that auto-ubiquitination activity is substantially reduced with UbcH5-L104Q in contrast to what is observed for RBR E3s. We agree that Western blot analysis does not support a quantitative interpretation. In our estimation, the compared activity of RBR E3s and BRCA1/BARD1with UbcH5-L104Q (run in a single experiment using the same stocks of enzymes for each reaction) is so robustly different that a quantitative analysis (if possible) would not significantly contribution to this study.

*Also the setup used in Figure 5c (HOIP H887A ~Ub charging) is an elegant setup and should be adapted for all RBRs and in particular for figure 5a assay.*

**Response:** We have now included a similar assay using H359A-HHARI--a mutation for which an E3~Ub thioester has been trapped before (Duda et al, 2013 Structure)—and observe decreased formation of HHARI~Ub with V70A-Ub. To our knowledge, no one (including us) has been able to trap and detect the E3~Ub for Parkin.

*I find the use of the RBR module, with tags, a little troublesome. Particularly in the case of the most-studied RBR, Parkin, there is a RING0/RING1 interface, as well as a RING0/RING2 interface, thus the RING0 domain sits in between the two R domains, therefore how meaningful is a GST-RBR*

*construct? A similar argument holds for the HHARI RBR module. In the case of HOIP, we do not yet know where any other domains from either HOIP, or HOIL-1L/Sharpin, therefore the use of the minimal unit is understandable, but it is more difficult to support in the case of those whose domain architecture is known.*

**Response:** All Parkin constructs used in this study lack the RING0 domain, therefore this is considered an "activated" construct, similar to the HHARI-ΔARI (Duda et al, 2013 *Structure*), Also, Wauer et al (2013 *EMBO J*) have shown that GST-Parkin (that includes RING0) is as reactive with a suicide probe as activating mutants such as W403A, indicating that a GST-tag does activate the enzyme. We use activated RBR constructs as a tool to address questions regarding the transfer of Ub from E2~Ub onto RING2. Many other studies, including Spratt et al (2013 *Nat Commun*) have used even more truncated constructs (such as RING2) to answer specific questions.

*The authors propose the R1 within RBRs have an extended interaction surface on E2s that overlaps (and hence outcompetes) the Ub binding surface seen in closed E2~Ub conformations. Thus in contrast to canonical RINGs, there would be no difference in R1 affinity to charged versus uncharged E2s. This needs to be tested for multiple R1s. Recent work (Kumar (2015) EMBO J) also suggest the Parkin R1 can support non-covalent ubiquitin interaction.*

**Response:** We performed ITC measurements with HHARI and RNF144 RING1 and either free UbcH7 or UbcH7~Ub. For either RING1 domain, the difference between binding the E2~Ub vs. free E2 was two-fold at most. While interesting, we don't believe that the binding data contributes significantly to this study and decided to not include it here. (N.B. Differences in $K_d$ for unconjugated versus conjugated E2s binding to canonical RINGs span a wide spectrum from no detectable difference to as much as 60-fold. We expect a similar idiosyncratic situation among the RING1 domains.)

*The authors also observe that UbcH7~Ub and Ubc13~Ub adopt closed conformation in absence of an E3. An earlier study (Matsuda et al. 2006, J Biol Chem) using a tagged BR only construct (i.e lacking R1) is capable of catalyzing ubiquitination with multiple E2s including the two studied here. Will the HHARI R2 construct used in Figure6 catalyze ubiquitination? If so, will the addition of HHARI R1 (in trans) influence this reaction?*

**Response:** HHARI RING2 has been shown to have activity on its own (Capili et al, 2004 *J Mol Biol*) and we observe ubiquitination activity with a RING2 construct (res 326-395) similar to the one used in our NMR experiments shown in Figure 6 (res 325-396). We, too, had wondered about the possibility of an effect of having RING1 in *trans* but did not observe a measurable difference. We repeated these experiments after reading the reviewers' comments, but due to the RING2-only constructs' low activity, extremely high concentrations are required for observable *in vitro* ubiquitination activity, which makes performing the *in trans* experiment under conditions where a difference can be detected technically challenging.

*The presence of a non-covalent ubiquitin binding site close to R2 of the RBR unit has been previously proposed/demonstrated (Chaugule et al., 2011, EMBO J., Zheng & Hunter, 2013, Cell Res., Rankin et. al., 2014, Biochemistry). Rankin et. al. (figure 9) also denote a UIM region which is distinct from that proposed in this study. The true nature of the ubiquitin binding domain could easily be tested using peptides that span just the BR linker (HHARI 325 to 342, Parkin 392 to 415). Finally, the residues Thr341/E352 in HHARI and T415/E426 in Parkin proposed by the authors to be involved in ubiquitin interaction have been earlier proposed (Figure 3, Spratt et al., 2013, Nat Commun.) to participate as catalytic residues during R2~Ub loading and/or off loading. The authors should either attempt to experimentally determine roles for these residues or discuss alternatives.*

**Response:** In the course of our study to define the first step of ubiquitin transfer mechanisms utilized by RBR E3s, we identified a Ub binding site on HHARI RING2 using NMR. In contrast to peptide binding studies where the native secondary structure of the contained sequence may be destabilized, NMR provides the advantage of studying protein-protein interactions of proteins in solution and their structured context as well as providing residue-level information. We believe that little additional information would be gained from the suggested peptide binding experiments. We note that we failed to discuss an earlier study proposing that T415 is involved in a hydrogen-bonding network around the active site of Parkin and have now included this work in our revised discussion.

*The authors also suggest major rearrangements to occur in RBRs that allow R2 to be recruited to the R1/E2~Ub module. In particular, the straightening of a kinked R1 helix is required based on the recent Phosphoubiquitin bound Parkin truncated structure (Wauer et al. 2015 EMBO J). This is very speculative and should be toned down. Also Figure 7b suggest the extended E2~Ub conformation could also be stabilized by the UBA domain of HHARI. This domain has been reported to bind NEDD8 and not Ubiquitin (Kelsall et al., 2013, EMBO J). Thus the model as stands is not supported by current data.*

**Response:** We agree that some of our ideas regarding major domain rearrangements are speculative and have removed the more detailed structural suggestions (such as the straightening of the kinked helix). We have generated a new schematic for Fig. 7b to clearly summarize our mechanistic model.

*The referencing is patchy. For example, HHARI and Parkin structures are referred to as full length but the citations from Wauer (2013) and Riley (2013) are not full length Parkin structures, while other Parkin structures with all domains present are not referred to : Kumar (2015) EMBO J; Sauve (2015) EMBO J. Also the discovery that RBRs bind to ubiquitin non-covalently has been observed previously in Chaugule (2011) EMBO J, indeed figure 5a is almost identical to figures 7a/b in the above mentioned paper, including mutation of the I44 hydrophobic patch. Zheng and Hunter (2013) CELL RES also reports that binding to ubiquitin is in the case of Parkin critical for activity. This finding should be discussed in the context of the existing literature. Further, despite multiple references to inhibited states of RBRs, the original paper defining the inhibition is not cited: Chaugule (2011) EMBO J. Finally, although the Lechtenberg/Riedl paper describing the RBR of HOIP bound to E2~ub was*
*not yet published when this manuscript was submitted, the findings in that paper will need to be discussed and put into context. It may help to put the footnote describing the findings from Smit et al (2012) into perspective.*

*More generally there are several statements without correct (or any) citations, e.g. p10 RBRs have been shown to be active with a variety of E2s. No citation. HHARI seems to work best with UbcH7, for years Parkin was only shown to function with UbcH7 or UbcH8, there are papers with these 'multiple E2' experiments in them, and they should be included.*

**Response:** We added appropriate citations where they were missing. We agree that HHARI works best with UbcH7, and that many biochemical studies with Parkin are done with UbcH7 as well. There are, however, several cell-based studies that propose that Parkin functions with other E2s (Fiesel et. al, 2014 *J Cell Sci*; Haddad et al, 2013 *Mol Cell*). In addition, HHARI and HOIP have been shown to work with UbcH5 (Wenzel at al, 2011 *Nature*; Stieglitz et al, 2013 *Nature*). Finally, while this manuscript was under review a structure of the HOIP-RBR bound to UbcH5~Ub was published (Lechtenberg et al, 2016 *Nature*). We therefore believe that understanding how RBR E3 ligases work with E2s other than UbcH7 is relevant and highly informative.

*The discussion also lacks proper citations. Linkers are referred to on p17 without reference to the primary literature, full-length parkin structures are attributed to Wauer and Komander and Riley et al., despite both these structures lacking 17kDa of the protein.*
*p18 'details of release of auto inhibition are specific for each RBR' has no citation, latter half of that sentence refers to removal of inhibitory domains without referring to Sauve 2015/Chaugule 2011/Kumar 2015 all of which show inhibition of parkin RBR by a ubiquitin-like domain.*

**Response:** We have added missing citations or removed/changed the wording.

*Supplementary figure 1 should be part of figure 1. The authors are establishing the notion of open vs closed conformation when adding HHARI RING in isolation, the CSPs are modest, but present. These are high quality data.*

**Response:** We agree and have added the former Fig. S1 to Fig. 1 (panels b-d).

*S2 should be part of figure 2.*

**Response:** Figure 2 presents the E3-independent formation of UbcH7~Ub closed states, while Fig. S2 addresses the effects of RING1 binding to unconjugated UbcH7 and UbcH5. We believe that Fig. S2 logically works better in conjunction with Fig. 3 which focus on effects caused by RING1 binding to UbcH7~Ub.

*S3b-e make THE critical point that is the crux of the paper, high quality data that demonstrate that R1s behave differently to RINGs, that disfavoring of the closed state being specific to the RING1 proteins, and should not be in supplementary data.*

**Response:** Thank you. We agree and moved former panels S3b-d into the main text.

*What about activators? How do they fit in? For example phosphoubiquitin? Does an activator position the RING1 as per the proposed model? Or does an activator create ubiquitin binding domains? Some discussion of current understanding of RBR mechanisms would be helpful.*

**Response:** We agree that the question of how activators fit into the mechanism is interesting but believe such a discussion is beyond of the scope of our manuscript. HHARI is not activated by phospho-Ub (our unpublished data) and it is not yet clear which other RBR E3s are activated by phospho-Ub binding. Our manuscript focuses specifically on the transfer of Ub from the RING1-bound E2~Ub onto the active site of RING2 and how and if activators might affect this step is a speculative discussion for most RBRs at this point.

*Minor points:*
*Figure S4A is introduced in the text before S3B-E.*

**Response:** All figures are introduced in order now.

*I like the hybrid experiment, using the U-box, it's a clever and informative experiment.*

**Response:** Thank you!

*Fig 2 legend says INSERT, do the authors mean inset?*

**Response:** Yes, we changed it to inset.

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2nd Editorial Decision                                                                      28 April 2016

Thank you for submitting your revised manuscript for our consideration, and please excuse the delay in getting back to you with a decision on it. I am afraid that the reevaluation by the referees was not straightforward and prompted several additional consultations, as I will try to explain in the following.

As you will see from the comments of the two original reviewers below, neither of them is convinced that many of their major initial concerns have been addressed in a sufficiently head-on and satisfactory manner in this revision. Although they do appreciate that your study demonstrates some important points (such as the RBR E3 promotion and requirement of an open E2~Ub conformation), they still feel that the manuscript on the whole provides an insufficient overall advance to warrant EMBO Journal publication. They also retain a number of the original presentational concerns. I discussed the reports, as well as our initial decision, in detail with my colleagues. Unfortunately, these unanimous critical opinions of both referees even after major revision left us with little choice but to conclude that we cannot offer publication of this revised manuscript in The EMBO Journal.

As I emphasized in my first decision letter, we were (as per our policies) open to considering a single round of major revision for this study. Clearly, satisfactorily addressing the persisting experimental concerns of the referees would amount to another major round of revision, and given your responses in the current version it is also unclear how feasible such further consolidation and follow-up work would be within a limited time-frame. Moreover, in light of the already published Lechtenberg et al paper, I feel that further delaying publication would compromise the timeliness of your key new results and therefore not be in this study's best interest.

In this situation, I have therefore taken the liberty to discuss your manuscript and its review history with my colleagues at our sister journal, EMBO reports, to explore the possibility of rapid publication of the current version in their pages. This required some additional time, but I am now

pleased to say that following consultations with a trusted editorial advisor (who is familiar both with the field and with EMBO reports and its scope), my colleague Dr. Achim Breiling agreed in principle to publishing this work without further rounds of review, following only minor additional textual/presentation changes and adaptations (which would be discussed in follow-up correspondence after eventual manuscript transfer).

Should you be interested in this option, please simply utilize the hyperlink at the end of this email to channel the manuscript to EMBO reports.

Once again, I apologize for the drawn-out evaluation, and I am sorry that the outcome of the re-review did not allow me to be more positive regarding publication in The EMBO Journal, but very much hope that you will consider the possibility of publishing this work in EMBO reports, which over the past few years has certainly published a number of significant papers in the ubiquitin field.

REFEREE REPORTS

Referee #1:

The authors have addressed many of the technical and presentation concerns raised in the first round of reviews. However, the paper remains an incremental contribution to the literature. Critically, it lacks mechanistic insight. The most important element missing is an explanation of why the closed E2~Ub state favours aminolysis. This has not been addressed in sufficient detail. On the positive side, the approach of the paper is original, well executed, and will be of interest to specialists in the field.

A second concern is the fact that the linchpin residue does not contact the E2~Ub in the new HOIP/E2~Ub structure. This makes the experiments shown in Fig. 1a much less interesting. Moreover, I would argue that Ser, Thr, and Asp are hydrogen-bonding residues, contrary to the authors' claim (see 2nd paragraph page 7, as well as Fig. 1d, residues highlighted in yellow).

Minor points: The sentence "Linkers between IBR and RING2 domains are either not observed or are unstructured in existing crystal structures of HHARI and Parkin", (p.17, 7th line from bottom), is not quite true. The REP in Parkin is observed and structured. However, it is true that the other parts of the linker are unstructured.

The preparation of some figures still needs to be improved. The text sizes should be more consistent and there is an extraneous line that runs through the top of Figure 3c.

Referee #2:

Review of resubmitted manuscript from Dove et al. describing insights into RING1 binding to charged-E2s.

Summary:
The authors response to this review begins with a reassertion of the 4 points they wished to communicate in the manuscript. I accept points 1 and 2 and endorse it as the key take-home message of the paper. However, points 3 and 4 remain under-examined in this revision. The family of RBRs (12-13 members) is relatively small in comparison to the 'classical' RING. In order to appreciate the significance of the proposed RBR RING2 interactions with the hydrophobic surface of Ubiquitin requires some carefully designed biochemical experiments. While this is not trivial, I feel it is still possible. There are apparent differences observed in productive HHARI and Parkin interactions with the hydrophobic surface mutants of Ubiquitin (Figure 5a, I44A defected with Parkin and not HHARI, while Q49E shows activity with HHARI but not Parkin). However these need to presented in a better way and/or quantified in order to truly appreciate its significance. Indeed, the concept of significance itself requires quantification and statistical analysis, the word 'significant' is used throughout the manuscript and the authors' response, without any quantification of activity assays. It is also not clear whether the proposed Ub and IBR-R2 linker interaction is exclusively required for R2~Ub formation (HHARI R2~Ub V70A intermediate is formed albeit at a reduced level) or for the

aminolysis step (R2~Ub to substrate, in this case auto-ubiquitination) or both. The impact of IBR-R2 linker residues (HHARI T341, E352 and Parkin T415, E352) need to be further examined given its role in a hydrogen-bonding network around the R2 catalytic site. In summary, this reviewer accepts that the authors demonstrate features of a productive E2~Ub interaction with R1 of RBRs however, the authors do not address this reviewers concerns regarding events that occur during R2~Ub formation and its generic nature among the RBR family. The title of the manuscript refers to RBR family mechanisms, so this is an important point! Otherwise the manuscript needs to be called 'HHARI RING1 promotes open E2~Ub conformation (and disfavours closed conformation)....!'

Specific points:
A quantifiable readout is possible and required for figure1 given the liberal use of 'activated' RBR constructs (and varied time points). In fact, simple E2~Ub discharge assays (commassie stain gels or use of labelled Ub allR) would allow for a better comparison of RBR activity. In general the manuscript would benefit from better biochemical assays that clearly demonstrate the molecular events of ubiquitin transfers in the RBR family.

In regards to R2~Ub formation (Figure 5), I agree that a Parkin R2~Ub thioester has not as yet been demonstrated, however several examples of oxyester states exist both in cells ((Zheng and Hunter, 2013, Cell Res, Lazarou et al, 2013, J Cell Biol.) and in vitro (Spratt et al, 2013, Nat Commun.) (Zheng and Hunter, 2013, Cell Res, Lazarou et al, 2013, J Cell Biol.).

The reported affinity between an activated HHARI (HHARI deltaAri) and UbcH7/Ube2L3 is around 190nM (Duda et al. 2013, Structure), while there are been no reports of a similar RNF144 interaction. An activated HHARI would include the proposed IBR-R2 linker based Ub binding site and as well any cryptic surfaces. It is difficult to assess from the authors comments what affinity range is observed with the isolated HHARI RING1 domain. A 'two-fold' difference in the nano molar range seems potentially important given the weak nature of Ub interactions. Furthermore, the same journal has published a report on the Parkin R1 (Kumar et al, 2015, EMBO J.) to support ubiquitin interaction. Given the broader implications of the manuscript in regards to RBR biology, the authors need to include such binding data in the manuscript (or at least in response to reviewer concerns), present their findings in the context of the prevailing literature and discuss the same. Furthermore, the authors' prevailing argument in the manuscript is that what holds for HHARI holds for all RBRs, namely that the R1s of RBRs are all functioning the same way and distinct from (all?) canonical RINGs. Then they say they expect the same range of differences in (~12) R1s binding to charged E2s as is seen in (~550) RINGs. These two comments seem quite inconsistent to me. What were the data for the binding? Why do the authors think it not significant? What does significant mean in this context?

Still the referencing is not accurate. Again, inhibition of RBRs is not referenced, neither is the fact that I44A ubiquitin not supporting RBR activity has been seen before (Chaugule, 2011, Zheng and Hunter 2013). On page 13 the wrong Spratt et al paper is cited - the paper cited in line 2 is Spratt et al Nature Comms, 2013, describing Parkin R2; the paper containing the NMR spectrum assignments of HHARI RING2 is Spratt, Mercier and Shaw, 2013, PLoS ONE. The reference to Kumar at the end the first paragraph on p17 is wrong. The paper describing the H-bonding network is Spratt et al., 2013 Nature Comms. There may be other inappropriate citations elsewhere. It is important to be accurate when discussing the primary literature, and it was a key point in my original review.

In regards to the suggested peptide experiment, I don't really follow the the argument that peptides are by their very nature destabilised, and therefore the suggested experiment will not (in the authors' opinion) add to the manuscript. However, in their discussion on page 18, the authors state the following:
'We propose that the linker (IBR-R2 linker) is extended and/or disordered in auto-inhibited states.... Upon release of the inhibitory domain(s), the linker could undergo a coil-to-helix transition to complete the Ub-binding site on RING2, enabling it to be recruited to the conjugates Ub moiety at RING1.'
This is the additional information that can be gained with a peptide binding experiment and is crucial to understanding Ub transfer mechanisms of RBR ligases. I feel the authors have missed an opportunity to carefully address functions of extended or disordered regions in Ub enzymatic mechanisms. Recent work in the SUMO field has shown how similar disordered regions (SUMO interaction motifs (SIMs)) can activate the E2~SUMO intermediate (Cappadocia et al., 2015, Nat

Struct Mol Biol., Eisenhardt et al., 2015, Nat Struct Mol Biol.).

Reviewer 1's concerns also appear not to have been addressed. Reviewer 1 also noticed that the defect using I44A ubiquitin seems different between HHARI and Parkin. If both RING1s are binding the hydrophobic patch of ubiquitin, an I44A ubiquitin mutant would be expected to have a similar effect. Indeed in the case of HHARI, I44A and V70A have quite different impacts. This is surprising given the 5Å distance between these two residues on ubiquitin and the frequency with which the two residues form part of the same binding surface. This experiment needs repeating, quantifying and statistical analyses prior to being able to draw the conclusion that the hydrophobic patch of ubiquitin interacts with RBRs.

Reviewer 1 was also concerned about the lysine reactivity assay that has now been removed. We agree with the authors that this experiment doesn't add to the paper, but the reviewer's concerns about the inconsistency between the gel data and that in the plot are reasonable and remain unaddressed.

Finally, obviously it is an editorial decision whether to accept the manuscript for publication, I can only provide my opinion of the data and their interpretations. Most of my original concerns remain, and possibly those of reviewer 1.

**EMBO reports**

| | |
|---|---|
| 1st Editorial Decision | 03 May 2016 |

Thank you for the transfer of your manuscript to EMBO reports. In light of the input on your manuscript and the original referee reports that we have received from an expert advisor of our journal (which you will find at the end of this e-mail), we are happy to offer publication of your study in our journal. However, some remaining issues need to be addressed:

- As you will see from the advisor's comments, our advisor suggests the inclusion and discussion of original papers on LUBAC, as well as the rewording of certain conclusions related to aminolysis being favored by closed E2~Ub states, in response to a concern raised by referee 1.

- Referee 1 raised another minor discussion point and a few remaining issues with figure presentation, which I would ask you to incorporate.

- In response to referee 2's remaining concerns, please once more carefully check and adjust the referencing of primary articles as discussed in the report.

Furthermore, please consider whether any of the other referee comments would warrant additional clarifications or discussions in the manuscript text (e.g. the concerns regarding quantification and statistics by referee #1).

I am therefore inviting you to revise the manuscript accordingly, and to resubmit it at your earliest convenience. Please also note the following points.

Please adjust the references to EMBO reports style. We can accommodate up to 8 main figures and 5 EV figures (see below). Please change your manuscript accordingly.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Important: All materials and methods should be included in the main manuscript file.

Regarding data quantification and statistics, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information must be provided in the figure legends.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

Please let us know whether you would like the review history and original referee comments from EMBO Journal to be included in the Peer Review Process File that we publish online with all accepted papers. For transfers between EMBO Press titles, which all share the Transparent Editorial Process, we would normally include the comments from the upstream submission, which could be valuable here for also showing your original/first submission date. On the other hand, given the very critical nature of the original referee comments, we would also understand if you opted to only include the review process history after transfer to EMBO reports in this case.

You are also able to opt completely out of this. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.


COMMENTS FROM EDITORIAL ADVISOR

The current manuscript by the Klevit laboratory and Riedl's Nature paper on the HOIP-E2 structure do overlap to a significant extent. However, I do believe that the Klevit paper adds important insight that would justify publication in EMBO Reports.

1. It demonstrates that an open E2~Ub conformation is used by multiple RBRs (in Lechtenberg et al., it was mostly inferred from sequence conservation, but only shown for HOIP).

2. It shows that the extended E2~Ub conformation exists in solution, and thus, is not stabilized by crystallography conditions.

3. It provides insight into why RBRs bind the E2~Ub in an open conformation (i.e. the U-box fusion experiment, an intriguing approach) - to prevent ubiquitylation reactions driven directly by the E2. In this regard, the authors should cite the first papers on LUBAC by Kazu Iwai's lab - they had always used E2-25K as an E2, but surprisingly saw formation of linear ubiquitin chains. I think this would be a nice example of why the intrinsic activity of the E2 would need to be suppressed.

I agree with reviewer 1 that the L104Q experiments, while showing that a closed conformation of the E2~Ub conjugate is not needed for RBR activity, does not explain why "HHARI RING1 does not enhance UbcH5~Ub reactivity towards free Lys". This needs to be reworded, even if the authors do not proceed to show why the closed conformation is required for aminolysis.

Thus, the findings of the Klevit paper will be interesting for many in the ubiquitin field, and they do

confirm and extend an important crystal structure. In my opinion, it is a manuscript that would be suitable for EMBO Reports.

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**1st Revision - authors' response**                                              20 May 2016

I am pleased to submit a revised manuscript for publication in <u>EMBO Reports</u> titled "Molecular Insights into RING-Between-RING Ub Transfer Mechanisms." We greatly appreciate your willingness to publish our work without it undergoing further review. We have revised the manuscript to respond to some remaining concerns and confusions highlighted in the last reviewers' comments from <u>EMBO Journal</u>. These are outlines in the "Responses to Reviewers" and attached to this letter.

We request that the review history and original referee comments from EMBO Journal be included in the Peer Review Process File.

We look forward to hearing from you concerning our contribution in the near future.

The following changes were made to revise our manuscript titled "Molecular Insights into RBR Ubiquitin Transfer Mechanisms"

1. Per suggestion from the advisor we incorporated and discussed findings by Kirisako *et al* (EMBO, 2006) that showed that the RBR E3 HOIP will overwrite intrinsic K48-linked chain building activity of E2-25K.
2. Previous work (Dou et al, 2012; Plechanovova et al, 2012; Pruneda et al, 2012) have shown that closed E2~Ub conformations are required for activation of the E2~Ub for aminolysis reactions. Although to date there is no thorough explanation for why closed E2~Ub states are required we believe that the lack of closed states (as presented here) is a reasonable explanation for the lack of activation of E2~Ub for aminolysis. It is correct that we do not explain why closed states are aminolysis reactive states and we changed the wording to reflect our findings.
3. Page 1, paragraph 2. We adjusted the wording here to reflect that RBR RING1 domains do not contain a critical conserved residue found in canonical RING domains. This observation provides one possible explanation for why RBR RING1 domains do not activate E2~Ub. Although it is true that Asp, Ser, and Thr residues could also form hydrogen bonds, fewer than half of the RBR RING1 domains contain a Asp/Ser/Thr at this position suggesting that the function of a hydrogen bonding residue is not conserved among RBR RING1s.
4. Page 18. As suggested by Referee 1, we changed the wording to reflect that the full linker between IBR and RING2 is contains a structured element called REP.
5. The figures were adjusted to the EMBO Reports format and edited based on comments from Referee 1. Additional adjustments were made (colors and fonts).
6. References were added and corrected were needed. Reference style was changed to EMBO Reports.

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**2nd Editorial Decision**                                                        24 May 2016

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

## EMBO PRESS

## YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ⬇

**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 consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

### 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 specific subsection in the methods section for statistics, reagents, animal models and human subjects.**

**In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).**

### B- Statistics and general methods

Please fill out these boxes ⬇ (Do not worry if you cannot see all your text once you press return)

| Question | Answer |
|---|---|
| 1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size? | NA |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used. | NA |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | NA |
| 3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe. | NA |
| For animal studies, include a statement about randomization even if no randomization was used. | NA |
| 4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe. | NA |
| 4.b. For animal studies, include a statement about blinding even if no blinding was done | NA |
| 5. For every figure, are statistical tests justified as appropriate? | NA |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. | NA |
| Is there an estimate of variation within each group of data? | NA |
| Is the variance similar between the groups that are being statistically compared? | NA |

### C- Reagents

| | |
|---|---|
| 6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right). | HA antibody: Life Tein, LT0422 (Fig5, EV5); GST antibody: Life Tein, LT0423 (Fig1,4,6); Flag antibody: Sigma Aldrich, F3165(Fig1; T7 antibody: EMD Millipore, 69522(Fig1) |
| 7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. | NA |

\* for all hyperlinks, please see the table at the top right of the document

## D- Animal Models

| | |
|---|---|
| 8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals. | NA |
| 9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments. | NA |
| 10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance. | NA |

## E- Human Subjects

| | |
|---|---|
| 11. Identify the committee(s) approving the study protocol. | NA |
| 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. | NA |
| 13. For publication of patient photos, include a statement confirming that consent to publish was obtained. | NA |
| 14. Report any restrictions on the availability (and/or on the use) of human data or samples. | NA |
| 15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable. | NA |
| 16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list. | NA |
| 17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines. | NA |

## F- Data Accessibility

| | |
|---|---|
| 18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>e. Proteomics and molecular interactions | NA |
| 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). | NA |
| 20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right). | NA |
| 21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section.<br><br>Examples:<br>**Primary Data**<br>Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462<br>**Referenced Data**<br>Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26<br>AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208 | NA |
| 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. | NA |

## 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 |