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The E3 ligase HOIP specifies linear ubiquitin chain assembly through its RING- IBR-RING domain and the unique LDD extension

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1st Editorial Decision 29 March 2012

Thank you again for submitting your manuscript on HOIP linear chain formation for our consideration. Three experts have now provided their comments, which you will find copied below. While all three referees agree on the interest and potential importance of your present analysis, they however raise a number of important issues that would require further work in order to substantiate the conclusions and insights from the study. These points and suggestions appear to be straightforward and well taken, and most of them are also raised in similar ways by more than one referee. In light of these evaluations, I would therefore like to invite you to prepare a revised manuscript that addresses both the textual/editorial/interpretational as well as the major experimental issues brought up by the reviewers. In particular, it will be important to strengthen the analysis of the 'LDD' domain and its role in chain formation as requested by all three reviewers, including determination of the non-covalent ubiquitin binding mode and attempts to trap a ubiquitin-HOIP thioester. Furthermore, the study would clearly be strengthened by further analysis of the proposed HOIP autoinhibition mechanism and the relevance of other LUBAC subunits/domains in HOIP regulation.

Should you be able to adequately address these main issues as well as the various other specific concerns clearly laid out by the reviewers, then we should be happy to consider a revised version further for publication. Please be reminded, however, that it is our policy to allow a single round of major revision only, and that it will therefore be important to diligently and comprehensively answer to all points at this stage in the process. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore

be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

Yours sincerely,

Editor
The EMBO Journal

Referee reports:

Referee #1 (Remarks to the Author):

Smit et al present a thorough and robust investigation into the mechanism of linear ubiquitin chain formation by the RBR ligases in LUBAC. The paper is clear, the experiments are high quality, and this report represents the first details of the mechanism of linear ubiquitin chain formation by the HOIP-HOIL-Sharpin Linear Ubiquitin Chain Assembly Complex.

The authors find that a truncation of HOIP is sufficient for linear ubiquitin chain assembly, suggesting the presence of an inhibitory module in the full-length protein.
They also find that a non-covalent interaction with ubiquitin is necessary for linear ubiquitin chain formation.
In addition, they find evidence for a thioester intermediate between HOIP and ubiquitin, and also evidence for an apparent E2-independent activity.
This is the first time these observations have been made for the LUBAC, and they provide important confirmation of observations made by several other groups for Parkin and HHARI, also RBR ligases.
They nicely show that the chain formation is likely to be in cis. These findings are new to the LUBAC field. These findings will be very valuable particularly to the LUBAC/linear ubiquitin/NFkB fields, with further interest to those researchers studying RBR ligases, and ubiquitination in general. As such, I fully support publication of a suitably revised manuscript in EMBO J.

However, I do have some reservations, mostly minor text changes, and two experimental concerns.

1. The title is a bit cryptic - could the authors define LDD in the title?

2. The authors mention Parkin, HHARI and RBR several times in the manuscript - these would be useful keywords to add for indexing.

3. The authors have overlooked previous studies in their introduction:

a. 3 papers defined Sharpin's involvement in LUBAC, not just Gerlach et al. This should also refer Ikeda et al and Tokunaga et al., both 2011.

b. Chaugule et al., 2011 reported a non-covalent interaction with ubiquitin being required for the

RBR ligase Parkin to build chains. This should be mentioned along with the reference to Wenzel et al describing a non-observed, but mechanistically inferred thioester intermediate. This non-covalent interaction with ubiquitin is a major part of the results and mechanism presented in Figure 7, and has been observed before in this family. Although this is noted in the discussion, the introduction in its current form suggests this is unknown.

c. The authors state that RING2 binds only one zinc (as observed in the structure of the C-terminal RING of HHARI in Capili et al., 2004). This should be referenced, but subsequent work described in Hristova et al., 2009, JBC, shows that the RING2 of another RBR ligase co-ordinates 2 zinc atoms. This should be mentioned.

d. Another of their findings is defining the minimal domain in HOIP for chain formation - the authors should cite in the introduction the known minimal requirements in other RBRs - IBR-R2 in Parkin, for example - Matsuda et al., 2006.

e. The sentence 'RING1 has a classical RING fold which is typically used for E2-E3 interactions' is not referenced - at least Zheng et al's seminal paper from the Pavletich group should be cited, and an updated citation for more general readers would also be useful.

4. I don't think it is as clear as suggested that RING E3 ligases have their chain type dictated by the E2. There simply aren't enough data on E2-E3 mechanisms (especially with substrates) to be dogmatic or didactic about it.

5. The final sentence of the introduction suggests this study provides 'novel' insight in(to?) the general mechanism for RBR mediated ubiquitin chain formation.....

While I understand the need to sell the results of one's endeavours, I think this is a bit disingenuous (and relates to points 3a-3e). As mentioned by the authors themselves at various points in the results section, E2-independent ubiquitination has been seen before by a member of the RBR family (Chew et al., PLoS ONE 2011), non-covalent interaction with Ub as a prerequisite for chains has been seen before in a member of this family (Chaugule et al., EMBOJ, 2011) and even the idea that a full length RBR is not active as a full-length protein (also Chaugule et al.). The formation of a low signal thioester intermediate has been seen before in an RBR family member (Wenzel et al, 2011), so from my reading of the manuscript, the true novelty is in the LINEAR ubiquitin chain formation, not the RBR family in general.

The end of the introduction should be revised to reflect the genuine novelty of the study.

6. On page 4, the authors suggest a potential auto-inhibition in full length HOIP. The authors could test this hypothesis by titrating full length HOIP into an assay containing RBR-LDD-HOIP, or R2-LDD-HOIP and observing whether the linear Ub chain formation is reduced. I understand that constructs missing the LDD are not well behaved enough to express, so that precludes the obvious experiment of adding HOIP without LDD to the R2-LDD alone.

7. On page 5 the authors state that the E2-independent activity of HOIP RBR-LDD was not affected by RING1 mutations (true for one double mutant but not the other) but then say this indicates a classical RING1 role. I got a bit confused here because in the UbcH5c blot, RING1 mutations do and do not affect chain formation, suggesting a key difference between UbcH5c and UbcH7. Can the authors address this, or clarify the apparent differences between the RING1 mutants and different E2s (lanes 2 and 3 in figure 2)?

8. I'm also a bit confused by the experiments in figure 4a. Wenzel et al., 2011 show that it is UbcH7 that defines the RBRs as RING-HECT hybrids, not the more promiscuous UbcH5c. In fact, UbcH5c is used as the E2 in almost all of the other experiments, but I would think that determining the formation of the thioester would require UbcH7, since Wenzel and Klevit suggest UbcH7 is an 'obligate' HECT-requiring E2. Surely it would make more sense to do those experiments with UbcH7?

9. Although it is acknowledged by the authors that the thioester is weak, these are the least convincing data in the paper. Can they trap the intermediate with a Cys-Ser mutant? I appreciate that

they state that all R2 cys mutants are defective in activity, but several of these mutant are double not single cysteine mutants, and Wenzel et al., identified potentially conserved cysteines within the R2. From the sequence alignment it looks as though it would be C885, a Cys to Ser mutation here may trap it. In supplementary fig 2, the authors indicate that they have made the Cys-Ser mutant but 'not determined' the formation of a thioester. This seems quite an omission. This would be a valuable addition to the manuscript as the current literature is restricted only to HHARI. Identifying the cysteine would provide a lot of support for the generic nature of the mechanism.

10. Page 8. The affinity for ubiquitin as determined by FP is low, even by ubiquitin biology standards. The authors also do not rule out that this binding is non-specific, only by showing a cysteine to alanine mutant of LDD binds less well. To do this, they should repeat the interaction experiment with NEDD8 and/or SUMO, or even the Ubl domain of HOIL-1L to determine whether the interaction with ubiquitin is specific to ubiquitin. Alternatively, they could try to identify the region of ubiquitin involved in the interaction (likely to be the I44 hydrophobic patch), and test a mutation. They must also control for the fluorophore as TAMRA is known to be quite hydrophobic.

11. I don't think the cellular data add anything to this manuscript, this is a good mechanistic in vitro investigation. It's hardly surprising to find that mutating the catalytic centre of something already shown to be required for NFkB activation in cells (Gerlach, Tokunaga, Ikeda etc) also impairs up the same process. If they are to be kept they should go in supplementary material. In any case, they should be referred to as cell culture experiments, not in vivo, this is transfection in HEK293 cells. In addition the delta UbA in figure 6 should be labelled as deltaUBA to be consistent with the nomenclature in the field and with supp fig 6.

12. There are a number of typos - toggling between American and English, and some instances of 'data was' which should be 'data were' (eg p.13). Each condition was tested in triplicate, or three times, but not 'in triplo'. Page 9. UbA should read UBA. Page 10. 'The need of a C-terminal' should read 'the need for a C-terminal...'

13. Could the authors change their figures to be consistent within each figure - I find the use of the wedge to show increasing time and increasing concentration in the same figure quite confusing. Maybe the time could be written, and the concentrations represented graphically. Or better still, all conditions written - the wedge suggests a smooth linear gradient, but for example in figure 1B the wedge denotes time points 0, 10, 20 and 40 minutes, which is not linear. And in 1C the wedge denotes concentrations of 0, 0.25, 0.5, 1, 2 and 4 micro-Molar, which is also not linear. Then 1F has a smaller wedge than other panels of the same figure, yet is denoting 15, 30, 60 and 120 minutes. The figures would be clearer with accurate details of the experimental conditions shown in the figures themselves.

Referee #2 (Remarks to the Author):

The manuscript by Smit et al addresses the mechanism of linear chain assembly by the HOIP, the active component of the LUBAC complex. The authors show that specificity for Met1-linked chains is encoded in the RBR module followed by a C-terminal uncharacterized ubiquitin binding domain. This is surprising since full-length HOIP in inactive, and requires HOIL-1L to activate it. The authors also show that it does not matter what E2 enzyme is used, in fact Ub dimer formation can be E2-independent in vitro. The C-terminal LDD is shown to be a Ub binding domain (but see below) and several Cys mutants are generated to propose a 2-step mechanism of chain assembly, as had been proposed recently for Parkin/HHARI (Wenzel et al, Nature 2011).
The manuscript is well written and the Figures are of very high quality. Many carefully analyzed biochemical aspects (E2 independence, discharge in cis, salt dependence, observation of 'charged' HOIP) will be interesting to people studying ubiquitin chain assembly. However, some parts of the manuscript should be improved before publication in EMBO J.

Main points:
1) The autoinhibition mechanism
Full-length HOIP is inactive while the RBR-LDD is fully active. HOIL1 is required to activate full-length HOIP. This biologically rather interesting finding is not characterized further, yet the authors should be able to do few straight-forward experiments to gain more biological insights for LUBAC.

Two questions arise:
a) HOIL-1 comprises a straight-forward domain structure comprising an NZF-Ubl module and an RBR domain. Which of these two modules is required? The NZF-UBL is supposed to bind HOIP, is this interaction sufficient to release autoinhibition? Or is it the RBR that competes with an intramolecular autoinhibition event in HOIP?

b) Does SHARPIN release the autoinhibition? Again, SHARPIN comprises just 2 halves, a N-terminal domain and a C-terminal NZF_UBL almost identical to HOIL. If SHARPIN was to activate HOIP, this would point to the common domain between HOIL and SHARPIN to be the activating principle, which would be an important insight.

2) The LDD
The identification of the LDD that may help position the acceptor Ub is the key finding of the paper, but its characterization is incomplete. The C-terminal region of HOIP is introduced early, and mutations are used throughout the manuscript from Fig. 2 onwards. It is claimed that the LDD orients the acceptor Ub and thus mediates linear chain formation. This has actually not been shown. What has been shown is that some residues in the LDD are important for chain formation and that an acceptor Ub may bind to LDD. Several issues arise.
a) The organization of the manuscript is not intuitive, and the reader wonders immediately what this ominous LDD is. The questions are: where is it (residues)? What does it look like? Is it conserved? The sequence alignment in Supp. Fig. 4 does not help much. It would make a lot of sense to include a sequence alignment to show the conservation, in the main Fig or as Supp Fig. 2, and introduce the C-terminal domain with a paragraph in the text early on.
b) Furthermore, the mutations in the LDD that disrupt HOIP activity are all Cys mutations, which is always dangerous in absence of validation of the protein fold. Why were these Cys chosen? There is no indication whether these are e.g. the most conserved residues. What does a threading analysis (PHYRE) show for this region?
c) A major concern is that the Cys mutations may affect folding / domain structure. The structural integrity of the constructs, in particular of the isolated LDD and LDD mutants (Fig. 1/5) should be shown, e.g. by 1D NMR experiments. The input for the isolated LDD and R2-LDD should be shown - is this protein as nice quality as the RBR-LDD (Supp Fig 2)?
d) PROSITE lists the C-terminal RING2 domain with different boundaries as compared to Supp Fig 4, extending to residue 930. The domain is called an IBR2 domain in PROSITE. Maybe the first Cys-rich part of the LDD (resi 895-930) is simply a structurally important part of RING2? This could explain why the RBR alone is insoluble? Can the annotated IBR2 fragment be expressed? What is the secondary structure prediction beyond residue 930? Is this region conserved, and required? It seems important to characterize this region further, and perform additional experiments where/how it binds to Ub, and clearly describe the rationale for the chosen domain boundaries, and the choice of mutations.
e) The data on Ub binding to the RBR-LDD C930A (binds) and LDD C930A (does not bind) is ambiguous. A Ub mutant (e.g. Ub I44A?) should be identified that disrupts binding to the wild-type LDD, to convincingly demonstrate importance of Ub-LDD interaction.
f) In an ideal world, the authors would map the LDD Ub binding site (e.g. by NMR) and identify non-Cys surface mutations that disrupt Ub binding. But this could be beyond the scope of this work.

3) Mechanism of chain formation
a) The separation of discharge and chain forming activity is nice. However, if the RING2 is there, and if the RING1 can discharge the Ub (Fig. 4C, bottom), why is the Ub not charging the RING2 (Fig. 4A)? Also this could be an effect of C916A and C930A in RING2 stability. The real issue is in Fig 4A which shows that all mutants, regardless whether they are in R1, R2 or LDD disallow Ub charging of the RBR - if the 2-step mechanism was correct, only R1 / IBR mutants should inhibit this, and other mutants should only inhibit charging if the actual Cys that is charged was mutated (or if the mutations disrupts the domain that is to be charged - see above). So far, the mutants in LDD suggest their importance in Ub charging of the RBR, not towards acceptor Ub binding.
b) There is also a faint activity of the LDD C930A mutant in fig. 2. If this activity could be enhanced by lower [NaCl] or longer incubation times it would be interesting to check if this mutant looses its specificity.

4) In vivo analysis
a) It would be nice to check if HOIP RBR-LDD only is constitutively active in NF B activation.

Minor comments
1) Abstract: "Growth factor induced NF-kB activation..." : not correct, since the LUBAC dependent processes are not growth factor induced (cytokine, DNA damage etc.).
2) p2: same problem, most NF-kB processes have now been shown to utilize LUBAC. Also cite Niu et al, EMBO J for DNA damage induced NF-kB. Several recent reviews by Iwai, Walczak and Dikic on this topic.
3) p3: Since the authors mention salt dependency, they should also comment on the pH in the main text.
4) p5: Is the E2 independent process still dependent on the formation of a thioester intermediate? Can it be blocked/quenched by DTT or bME?
5) The Fig legend of Fig S1C mentioned Ubch7 but this is not included. Fig S1C is very similar to Fig. 1D.
6) A reference for the design of the V701A mutation should be included.
7) The authors are encouraged to use gene nomenclature for E2 proteins (Ube2D, Ube2L3 etc) in an effort to simplify the literature (see Ye and Rape, Nat Rev Mol Cell Biol 2010).
8) All Figs : kDa labels (numbers) should be given for all blots
9) Fig. 4: Please define the asterisk
10) The discussion is good, however the UBD in Parkin is not supposed to be C-terminal to the RBR, but within the RBR (Chaugule 2011).

Referee #3 (Remarks to the Author):

This manuscript reports biochemical studies aimed at characterizing and defining the roles of domains within the RING-Between-RING (RBR) E3 ligase, HOIP. The data presented support two unexpected conclusions that provide new insights into the mechanism of linear poly-ubiquitin chain formation. First, HOIP's ligase activity is masked or inhibited in the full-length polypeptide. Removal of the entire N-terminal region up to the RBR domain or binding of the full-length protein by HOIL-1L unmasks the ligase activity. Surprisingly, the activity of the largely truncated HOIP construct retains its specificity for building linear poly-Ub chains, indicating that the determinant for this special activity lies somewhere within the RBR domain through the C-terminal. The second unexpected result is that a region C-terminal to the RBR domain is required for the linear chain building activity.

On the basis of the unmasking of activity by truncation or complex formation, the authors conclude that HOIP is auto-inhibited by its N-terminal region. While that is a formally logical conclusion, the available information do not distinguish whether the observed inhibition is functionally relevant or is an unintended consequence of expressing HOIP in the absence of its binding partners. The experimental observations could be due to the full-length protein being misfolded or ill-behaved, leading to its low activity. Expression of just the RBR-LDD domains could produce properly folded material. Likewise formation of the hetero-complex with HOIL-1L might do the same. Rigorous support for auto-inhibition would require adding the N-terminal region back in trans and observing inhibition. I'm not sure this is an important enough point to warrant doing the experiment, but the manuscript should more accurately present the possible explanations for the observation.

A large number of mutations were prepared and characterized for ligase activity, as presented in Figure 2. The results establish that ligase activity requires not only the canonical RBR, but also the LDD domain. There is little or no rationale provided in the text for the choice of residues that were mutated, although one familiar with the structures of RBRs can read the authors' minds. For the more general reader, the positions of (putative) Zn-liganding residues with the R1, IBR, and R2 domains should be identified, as mutation of these likely yield destabilized structure of the domain in which the mutated residue is found. In addition, the rationale behind how were other positions were chosen would clarify Figure 2. Similarly, the Cys residue in R2 that is predicted to serve as an active site, based on sequence alignments with HHARI and parkin, should be clearly identified. There is no explicit mention of this residue in the text, although the authors appear to have their eye on it, as they mutated it to both Ala and Ser. Presumably, the Cys885Ser mutant was created with the intention of observing formation of an oxy-ester derivative, but there is no mention of this in the text. Was this attempted? Finally, most or all of the mutations made in the LDD were to Cys residues. It would be helpful to have an MSA of the LDD domain presented as Supplemental

Information (Fig. S4B, perhaps) to ascertain how conserved these residues are.

Data presented in Figure 4 nicely delineate the steps required for transfer of Ub from the E2 active site to an acceptor Ub to form di-Ub. Experiments were performed with both UbcH5 and UbcH7 as E2. My recommendation here would be to switch which data are in the main text and which are in the Supplement. Because of the lack of intrinsic reactivity with lysine, the data with UbcH7 was cleaner and therefore more compelling.

A novel finding is the identification of the LDD as a domain required for chain building. The authors present data that show that the LDD binds Ub. They also present a somewhat confusing result showing that mutation of Cys930 in the LDD significantly decreases Ub binding in the context of LDD alone, but not in the context of the RBR+LDD. It is difficult to know what to make of this observation in the absence of any data that speaks to the stability or foldedness of the LDD. This observation, plus several others listed below, makes me wonder if it isn't more accurate to think of the LDD as an integral part of R2 or at least an extension of it, rather than an independent domain. Taking this proposal one step further, the authors' proposal that the LDD is responsible for the proper positioning of the acceptor Ub for linear chain formation would seem to imply that the LDD has a fixed orientation relative to the active site of R2 (in analogy to Ubc13/Mms2). Given the very short "linker" (if it is that) between what is currently thought of as the end of R2 and the beginning of LDD, it may be reasonable to consider the two "domains" potentially as one. This may explain why it is difficult to express the RBR without the LDD domain. Also, the "linker" is only nine residues long and its amino acid composition/sequence does not look all that linker-like, containing numerous aromatic and hydrophobic residues. In this respect, the R2 of parkin is likely to be extended to include additional residues (and even additional Zn-binding site(s)) relative to some other R2s. The bottom line is that there is not currently sufficient structural data to know with certainty what the boundaries of R2 domains are. Given the intriguing results in this paper, it would be prudent to leave the door open to the possibility of more variation in this domain than is currently defined.

In summary, this manuscript presents convincing data that significantly expand current knowledge of the linear-chain building E3, HOIP. The conclusions are for the most part well supported by the data and will be of interest to a wide readership. The authors should be encouraged to make changes to the manuscript to clarify and to enhance readability, providing clearer rationales for some of their experimental designs.

1st Revision - authors' response 20 June 2012

We thank the referees for their comments on the paper. Since several points were raised multiple times we first give an overview of the major points, before giving the point by point answers to the individual questions of the referees.

All three reviewers wanted us to improve the thioester formation between HOIP and ubiquitin. We have now optimized this assay by increasing the pH to pH 8.5 and lowering the NaCl concentration in the reactions. The optimized assays resulted in increased quantities of visible thioester. Further optimization was limited by the appearance of an auto-ubiquitinated species. Under the new reaction conditions the RING1 mutants and several LDD mutants form the ubiquitin~HOIP intermediate (Figure 4A, B, Supplementary figure S4A, B, C). The RING2 mutants are still completely impaired in ubiquitin~HOIP thioester formation in the new reaction conditions, confirming a central role for RING2 in ubiquitin chain formation.

Second, all reviewers wondered why full length HOIP is inhibited until it binds to HOIL-1L or Sharpin. Although this is not the focus of our manuscript, we performed some additional experiments to gain insight into this mechanism. Since the $HOIP^{RBR\text{-}LDD}$ is constitutively active, the N-terminal region of HOIP seems to have an inhibitory effect on the RBR-LDD in the full-length protein. We have tried to mimic the situation by adding either full length HOIP or different HOIP N-terminal constructs to the reaction with $HOIP^{RBR\text{-}LDD}$ and found that these could not inhibit the

activity (Supplementary figure S1H). Hence, the inhibition is more complicated than direct interaction and involves interactions and/or conformational changes that depend on the covalent linkage of the N-terminus to the RBR-LDD.

Finally, the referees asked if we could strengthen the LDD-ubiquitin interaction-data. Therefore, we performed more controls for the FP-assay with ubiquitin^{TAMRA}. First, we show that HOIP does not interact with free TAMRA-dye (Supplementary figure S5B), and the affinities for C-terminal and N-terminal labeled TAMRA-ubiquitin are comparable (Figure 5A, Supplementary figure S5A). These controls illustrate that the weak interaction between HOIP and the TAMRA-labeled ubiquitin is not an artifact of the TAMRA-dye.
Second, we validated the importance of the interaction between ubiquitin and HOIP in *in vitro* single cycle turnover assays by adding ^{biotin}ubiquitin as a competitor for acceptor ubiquitin in di-ubiquitin formation. The N-terminus of the ^{biotin}ubiquitin is blocked and can therefore not function as an acceptor for the ^{TAMRA}ubiquitin in the assays. The formation of di-ubiquitin, but not the formation of the HOIP~^{TAMRA}ubiquitin intermediate, was inhibited (Figure 5C and Supplementary figure S5C). These results confirm the importance of the interaction between the acceptor ubiquitin and HOIP, as was also shown by the inhibitory effect of the LDD on free ubiquitin chain formation (Figure 5B).
Third we tested if the ubiquitin hydrophobic patch is involved in acceptor ubiquitin binding to HOIP. The single and triple ubiquitin hydrophobic patch mutants could still receive ^{TAMRA}ubiquitin (Figure 5D, Supplementary figure S5D), indicating that the classical ubiquitin interaction site is not involved in linear ubiquitin chain formation.

In addition to these general points, we have addressed the various points raised by individual reviewers. Taken together, by addressing the referee comments the experimental data in the manuscript has been strengthened substantially.

Below we give point by point answers to the comments of the referees.

Referee #1 (Remarks to the Author):

1. The title is a bit cryptic - could the authors define LDD in the title?

Yes, we can see that this is the case. However, explicitly defining the LDD would require naming 'linear ubiquitin chains' twice within one title, which is unpleasant to read. Moreover, the maximum number of characters that are allowed in the title (100 characters, including spaces), virtually prevents this.

2. The authors mention Parkin, HHARI and RBR several times in the manuscript - these would be useful keywords to add for indexing.

This is a useful point. Since the number of keywords is limited we have replaced those keywords that already appeared in the abstract with Parkin and HHARI. (RBR also already appears in the abstract.)

3. The authors have overlooked previous studies in their introduction:
a. 3 papers defined Sharpin's involvement in LUBAC, not just Gerlach et al. This should also refer Ikeda et al and Tokunaga et al., both 2011.
b. Chaugule et al., 2011 reported a non-covalent interaction with ubiquitin being required for the RBR ligase Parkin to build chains. This should be mentioned along with the reference to Wenzel et al describing a non-observed, but mechanistically inferred thioester intermediate. This non-covalent interaction with ubiquitin is a major part of the results and mechanism presented in Figure 7, and has been observed before in this family. Although this is noted in the discussion, the introduction in its current form suggests this is unknown.
c. The authors state that RING2 binds only one zinc (as observed in the structure of the C-terminal RING of HHARI in Capili et al., 2004). This should be referenced, but subsequent work described in Hristova et al., 2009, JBC, shows that the RING2 of another RBR ligase co-ordinates 2 zinc atoms. This should be mentioned.
d. Another of their findings is defining the minimal domain in HOIP for chain formation - the authors should cite in the introduction the known minimal requirements in other RBRs - IBR-R2 in Parkin, for example - Matsuda et al., 2006.

e. The sentence 'RING1 has a classical RING fold which is typically used for E2-E3 interactions' is not referenced - at least Zheng et al's seminal paper from the Pavletich group should be cited, and an updated citation for more general readers would also be useful.

We thank the reviewer for the constructive remarks and we have made these adjustments.

4. I don't think it is as clear as suggested that RING E3 ligases have their chain type dictated by the E2. There simply aren't enough data on E2-E3 mechanisms (especially with substrates) to be dogmatic or didactic about it.

We agree with referee #1 that the mechanism by which E2-E3 combinations determine chain type specificity is not fully understood. Therefore, we have toned down the statement in the introduction, by describing that the E2s contribute (and not dictate) to the chain types that are formed in cooperation with RING E3 ligases. The examples that follow the statement make clear that there are different types of contributions between E2s and RING E3s to the chain type specificity.

5. The final sentence of the introduction suggests this study provides 'novel' insight in(to?) the general mechanism for RBR mediated ubiquitin chain formation.....

While I understand the need to sell the results of one's endeavours, I think this is a bit disingenuous (and relates to points 3a-3e). As mentioned by the authors themselves at various points in the results section, E2-independent ubiquitination has been seen before by a member of the RBR family (Chew et al., PLoS ONE 2011), non-covalent interaction with Ub as a prerequisite for chains has been seen before in a member of this family (Chaugule et al., EMBOJ, 2011) and even the idea that a full length RBR is not active as a full-length protein (also Chaugule et al.). The formation of a low signal thioester intermediate has been seen before in an RBR family member (Wenzel et al, 2011), so from my reading of the manuscript, the true novelty is in the LINEAR ubiquitin chain formation, not the RBR family in general.

The end of the introduction should be revised to reflect the genuine novelty of the study.

We thank the referee for pointing out that the end of the introduction could be improved. Indeed, the major novel insights are related to linear ubiquitin chain formation. Nevertheless, by combining the various assays within a single analysis we add to the strength of knowledge on the general RBR mechanism and extend it beyond HHARI and Parkin. We have changed the end of the introduction to reflect our findings more accurately.

6. On page 4, the authors suggest a potential auto-inhibition in full length HOIP. The authors could test this hypothesis by titrating full length HOIP into an assay containing RBR-LDD-HOIP, or R2-LDD-HOIP and observing whether the linear Ub chain formation is reduced. I understand that constructs missing the LDD are not well behaved enough to express, so that precludes the obvious experiment of adding HOIP without LDD to the R2-LDD alone.

We have performed the suggested experiment. The addition of full length HOIP into the chain formation reaction with $HOIP^{RBR\text{-}LDD}$ did not result in an inhibition of the chain formation (Supplementary figure S1H). We then purified two different constructs of the N-terminus (including the UBA domain) of HOIP and added a large excess of these constructs into the chain formation reaction with $HOIP^{RBR\text{-}LDD}$. Neither of these N-terminal HOIP constructs inhibited the chain formation by $HOIP^{RBR\text{-}LDD}$ (Supplementary figure S1H) (see general section above). Possibly, the covalent link between these domains and the RBR-LDD creates a high local concentration, or organizes a precise location and orientation of the N-terminus with respect to the RBR-LDD domain that is important for the auto-inhibition. Alternatively, the catalytic domain is kept in an inactive conformation in full length HOIP and undergoes an activating conformational change upon binding of HOIL-1L or Sharpin. We have added this discussion to the manuscript.

7. On page 5 the authors state that the E2-independent activity of HOIP RBR-LDD was not affected by RING1 mutations (true for one double mutant but not the other) but then say this indicates a classical RING1 role. I got a bit confused here because in the UbcH5c blot, RING1 mutations do and do not affect chain formation, suggesting a key difference between UbcH5c and UbcH7. Can

the authors address this, or clarify the apparent differences between the RING1 mutants and different E2s (lanes 2 and 3 in figure 2)?

The reviewer is correct, that the mutations at the site that coordinates the second zinc ion (C717,719A) cause a loss in activity with UbcH7 and not with UbcH5c. This indicates a difference in binding of UbcH5c and UbcH7 to the RING1 domain and consequently to the activity of this particular mutant, which we have now made more explicit in the text. Meanwhile, the mutations in the region of the first zinc ion (C699/K873A and V701A/I) cause a loss of activity with both UbcH5c and UbcH7, showing the general point that RING1 is essential in the E2-dependent activity of HOIP.

8. I'm also a bit confused by the experiments in figure 4a. Wenzel et al., 2011 show that it is UbcH7 that defines the RBRs as RING-HECT hybrids, not the more promiscuous UbcH5c. In fact, UbcH5c is used as the E2 in almost all of the other experiments, but I would think that determining the formation of the thioester would require UbcH7, since Wenzel and Klevit suggest UbcH7 is an 'obligate' HECT-requiring E2. Surely it would make more sense to do those experiments with UbcH7?

We agree with referee #1 that UbcH7 is specific towards cysteines, while UbcH5c is capable to release ubiquitin onto lysines and cysteines (Wenzel et al. 2011). However, in this case both release onto a cysteine, since reduction of the samples by βME removes the modification. This point is now made explicit in the text. We have added the UbcH7 experiment as Supplementary figure S4B. Since there is less background in the UbcH5c experiment we prefer to show the newly optimized Ubch5c experiment in the main text (Figure 4A).

9. Although it is acknowledged by the authors that the thioester is weak, these are the least convincing data in the paper. Can they trap the intermediate with a Cys-Ser mutant? I appreciate that they state that all R2 cys mutants are defective in activity, but several of these mutant are double not single cysteine mutants, and Wenzel et al., identified potentially conserved cysteines within the R2. From the sequence alignment it looks as though it would be C885, a Cys to Ser mutation here may trap it. In supplementary fig 2, the authors indicate that they have made the Cys-Ser mutant but 'not determined' the formation of a thioester. This seems quite an omission. This would be a valuable addition to the manuscript as the current literature is restricted only to HHARI. Identifying the cysteine would provide a lot of support for the generic nature of the mechanism.

As described in the general section, we have significantly improved the thioester formation experiments, confirming that RING1 and LDD mutants are still able to reach this state (Figure 4A, Supplementary figure S4A, B). The RING2 mutants that have been tested do still not form a HOIP~ubiquitin intermediate in the new conditions, supporting the proposed central role for RING2 in ubiquitin linkage formation. The C885S mutant was indeed tested in the thioester-formation assay, but did not form a HOIP~ubiquitin intermediate (Supplementary figure S4C). Consequently, we were able to strengthen the data for the central role of RING2 in the HOIP~ubiquitin intermediate formation, but the exact location of the intermediate on RING2 remains to be resolved.

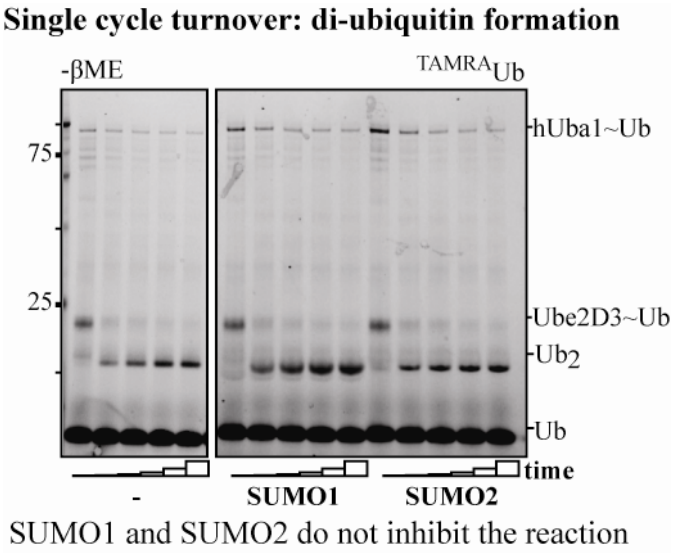
10. Page 8. The affinity for ubiquitin as determined by FP is low, even by ubiquitin biology standards. The authors also do not rule out that this binding is non-specific, only by showing a cysteine to alanine mutant of LDD binds less well. To do this, they should repeat the interaction experiment with NEDD8 and/or SUMO, or even the Ubl domain of HOIL-1L to determine whether the interaction with ubiquitin is specific to ubiquitin. Alternatively, they could try to identify the region of ubiquitin involved in the interaction (likely to be the I44 hydrophobic patch), and test a mutation. They must also control for the fluorophore as TAMRA is known to be quite hydrophobic.

This is an important point and we addressed it in a number of different ways:

- We added a number of controls to rule out non-specific binding of the hydrophobic TAMRA-dye to HOIP. First, we found that N-terminally and C-terminally TAMRA labeled ubiquitin binding with equal affinity, showing that the position of the TAMRA does not influence binding (Supplementary figure S5A).
- Second, free TAMRA-dye was tested in the FP-assay, revealing that the free TAMRA-dye does not interact with $HOIP^{RBR\text{-}LDD}$ or with $HOIP^{LDD}$ (Supplementary figure S5B), indicating that the interaction between HOIP and ^{TAMRA}Ubiquitin is not caused by a non-specific effect of the TAMRA-dye.

- To verify the functional importance of the binding, we added an experiment where we added 64µM N-terminally blocked ubiquitin (^{biotin}ubiquitin) into the single cycle turnover reaction. ^{Biotin}ubiquitin cannot serve as an acceptor, but it did inhibit the transfer of ^{TAMRA}ubiquitin onto wt-ubiquitin, showing that interaction between the acceptor ubiquitin and HOIP is important for function (Figure 5C).
- We also added 128µM SUMO1 and SUMO2 into the single cycle turnover assay (see below), but both proteins did not interfere with the di-ubiquitin formation, indicating that the HOIP-ubiquitin interaction is specific.
- Finally, we tested if the ubiquitin I44 hydrophobic patch is involved in the interaction between HOIP and the acceptor ubiquitin. Several different ubiquitin hydrophobic patch mutants (L8A, I44A, V70A, triple^{L8,I44,V70AAA}) were used as acceptor ubiquitin in discharge assays. All I44 hydrophobic patch mutants receive ^{TAMRA}ubiquitin, indicating that this site is not involved in the interaction with HOIP (Figure 5D, Supplementary figure S5D)

We agree with the reviewer that the observed affinity between HOIP^{LDD} and ubiquitin is low, which suggests that the LDD will provide additional functions beyond its role in docking ubiquitin, to the ubiquitin linkage formation. We have extended our discussion with this point in the manuscript.



SUMO1 and SUMO2 do not inhibit the reaction

11. I don't think the cellular data add anything to this manuscript, this is a good mechanistic in vitro investigation. It's hardly surprising to find that mutating the catalytic centre of something already shown to be required for NFkB activation in cells (Gerlach, Tokunaga, Ikeda etc) also impairs up the same process. If they are to be kept they should go in supplementary material. In any case, they should be referred to as cell culture experiments, not in vivo, this is transfection in HEK293 cells. In addition the delta UbA in figure 6 should be labeled as deltaUBA to be consistent with the nomenclature in the field and with supp fig 6.

We agree with referee #1 that the experiments in HEK293FT cells confirm previous published data on the catalytic role of the RBR domain. However, the importance of the LDD domain, which lies C-terminally of the RBR, has not been described before. The loss of NF-κB activation in the LDD mutant is a valuable addition to the paper to prove the significance of this domain as we characterized it *in vitro*. We have added pull-down data, showing that the mutants do interact with HOIL-1L in cells, to illustrate that the proteins are well folded and form the physiological complex (Supplementary figure S6A). For these reasons, we prefer to keep the panel in the main text. The experiments are now referred to as cell-based assays. In addition, we have made sure that the ΔUBA construct is referred to correctly throughout the manuscript.

12. There are a number of typos - toggling between American and English, and some instances of 'data was' which should be 'data were' (eg p.13). Each condition was tested in triplicate, or three

times, but not 'in triplo'. Page 9. UbA should read UBA. Page 10. 'The need of a C-terminal' should read 'the need for a C-terminal...'

We thank referee #1 for pointing out the linguistic mistakes. We have made these changes to improve the manuscript.

13. Could the authors change their figures to be consistent within each figure - I find the use of the wedge to show increasing time and increasing concentration in the same figure quite confusing. Maybe the time could be written, and the concentrations represented graphically. Or better still, all conditions written - the wedge suggests a smooth linear gradient, but for example in figure 1B the wedge denotes time points 0, 10, 20 and 40 minutes, which is not linear. And in 1C the wedge denotes concentrations of 0, 0.25, 0.5, 1, 2 and 4 micro-Molar, which is also not linear. Then 1F has a smaller wedge than other panels of the same figure, yet is denoting 15, 30, 60 and 120 minutes. The figures would be clearer with accurate details of the experimental conditions shown in the figures themselves.

We have attempted to make the figures more intuitive, following these suggestions. The wedges for the time-series have been changed into bars that represent the difference between the time-points more accurately and we have replaced the wedges for the concentration-series in the figures by the actual numbers.

Referee #2 (Remarks to the Author):

Main points:

1) The autoinhibition mechanism

Full-length HOIP is inactive while the RBR-LDD is fully active. HOIL1 is required to activate full-length HOIP. This biologically rather interesting finding is not characterized further, yet the authors should be able to do few straight-forward experiments to gain more biological insights for LUBAC. Two questions arise:

a) HOIL-1 comprises a straight-forward domain structure comprising an NZF-Ubl module and an RBR domain. Which of these two modules is required? The NZF-UBL is supposed to bind HOIP, is this interaction sufficient to release autoinhibition? Or is it the RBR that competes with an intramolecular autoinhibition event in HOIP?

b) Does SHARPIN release the autoinhibition? Again, SHARPIN comprises just 2 halves, a N-terminal domain and a C-terminal NZF_UBL almost identical to HOIL. If SHARPIN was to activate HOIP, this would point to the common domain between HOIL and SHARPIN to be the activating principle, which would be an important insight.

We agree with the referee that it is an important question to understand how HOIL-1L and Sharpin activate HOIP. Interestingly Yagi et al. (2012) recently characterized the functional binding between HOIP and HOIL-1L, showing that the interaction between the UBA domain of HOIP and the UBL domain of HOIL-1L is essential for NF-κB activation. It is unlikely that the RBR-domain of HOIL-1L is needed to activate HOIP, because Sharpin, which does not have a RBR domain, also functionally interacts via its UBL domain with HOIP (Sieber et al. 2012). Consequently, the interaction between the UBA domains of either HOIL-1L or Sharpin seem sufficient to activate HOIP. We have added the reference of Yagi et al. and Sieber et al. to make these points in the manuscript.

We have not studied the *in vitro* activating capacities of different constructs of HOIL-1L or Sharpin our selves, because we would like to keep the focus of the paper on HOIP. Instead, we choose to study role of the N-terminus of HOIP in keeping the catalytic domain in an inactive state in the absence of HOIL-1L or Sharpin, by adding different constructs of the HOIP N-terminus as described in the general section above.

2) The LDD

The identification of the LDD that may help position the acceptor Ub is the key finding of the paper, but its characterization is incomplete. The C-terminal region of HOIP is introduced early, and mutations are used throughout the manuscript from Fig. 2 onwards. It is claimed that the LDD orients the acceptor Ub and thus mediates linear chain formation. This has actually not been shown. What has been shown is that some residues in the LDD are important for chain formation and that an acceptor Ub may bind to LDD. Several issues arise.

a) The organization of the manuscript is not intuitive, and the reader wonders immediately what this ominous LDD is. The questions are: where is it (residues)? What does it look like? Is it conserved? The sequence alignment in Supp. Fig. 4 does not help much. It would make a lot of sense to include a sequence alignment to show the conservation, in the main Fig or as Supp Fig.
2, and introduce the C-terminal domain with a paragraph in the text early on.

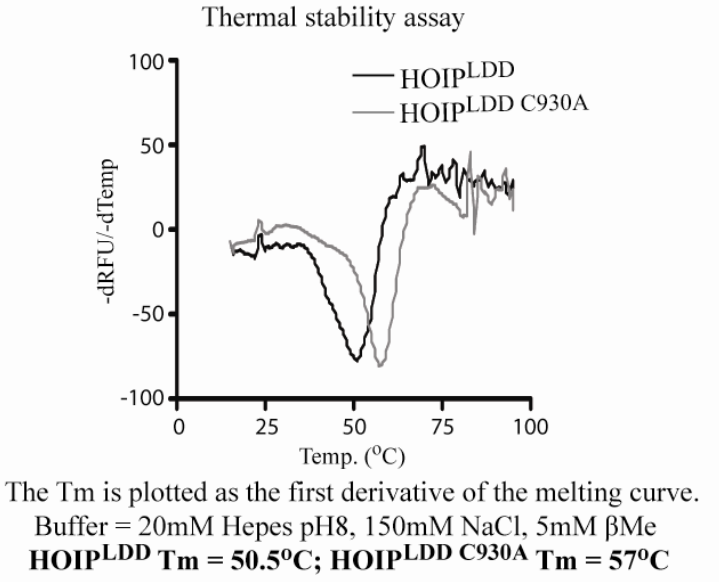
To help the reader we have given a more extended explanation when the LDD is first mentioned in the results and added a multi-sequence alignment as supplementary figure S1D. The LDD is not conserved between different RBR proteins; however between HOIP orthologs this domain is highly conserved. The residue numbers of the LDD are provided in figure 1 and de locations of the different cysteine mutants in the LDD are indicated in the multi-sequence alignment (S1D).

b) Furthermore, the mutations in the LDD that disrupt HOIP activity are all Cys mutations, which is always dangerous in absence of validation of the protein fold. Why were these Cys chosen? There is no indication whether these are e.g. the most conserved residues. What does a threading analysis (PHYRE) show for this region?

We have added a multi-sequence alignment (Supplementary figure S1D), that shows that the cysteines are well conserved in HOIP orthologs. Another reason to choose these residues was their importance in the adjacent RING domains To gain more insight in the possible function and secondary structure of the LDD, we have performed a threading analysis of this domain, as the referee suggested. However, there were no homologues of this domain picked up in neither a (PSI-)BLAST search, nor a threading analysis with PHYRE. We have made this explicit in the text.

c) A major concern is that the Cys mutations may affect folding / domain structure. The structural integrity of the constructs, in particular of the isolated LDD and LDD mutants (Fig. 1/5) should be shown, e.g. by 1D NMR experiments. The input for the isolated LDD and R2-LDD should be shown - is this protein as nice quality as the RBR-LDD (Supp Fig 2)?

The folding of mutated proteins is always a concern in *in vitro* experiments. As suggested by the referee, we have added a gel with the input samples and a gel filtration experiment for $HOIP^{LDD}$ and $HOIP^{LDD}$ C930A to illustrate the integrity of the different constructs (Supplementary figure S2C, D). In addition, a thermal stability assay confirmed that $HOIP^{LDD}$ is folded and that the C930A mutant does not destabilize the domain (see below).



The Tm is plotted as the first derivative of the melting curve.
Buffer = 20mM Hepes pH8, 150mM NaCl, 5mM βMe
$HOIP^{LDD}$ Tm = 50.5°C; $HOIP^{LDD\ C930A}$ Tm = 57°C

The RBR-LDD constructs are too large for 1D NMR. Therefore, we have addressed this point in a different way, by a pull-down experiment after expression in HEK293FT cells. The immunoprecipitation experiments show that HOIP RING2 and LDD mutants still interact with HOIL-1L (Supplementary figure S6A). Therefore, we conclude that the point mutations that we have introduced may cause local effects, but do not disrupt the complete folding of the proteins.

d) PROSITE lists the C-terminal RING2 domain with different boundaries as compared to Supp Fig 4, extending to residue 930. The domain is called an IBR2 domain in PROSITE. Maybe the first Cys-rich part of the LDD (resi 895-930) is simply a structurally important part of RING2? This could explain why the RBR alone is insoluble? Can the annotated IBR2 fragment be expressed? What is the secondary structure prediction beyond residue 930? Is this region conserved, and required? It seems important to characterize this region further, and perform additional experiments where/how it binds to Ub, and clearly describe the rationale for the chosen domain boundaries, and the choice of mutations.

The LDD is chosen as the region that is highly conserved between HOIP orthologs (supplemental figure S1D), but is not present in other RBR proteins (Supplemental figure S4D), which is in line with its unique role in promoting linear ubiquitin chains. Since the domain can fold by itself (Supplementary figures S2D and thermal stability experiments shown above for point 2C) and retains ubiquitin binding activity (Figure 5A, B), we do think it is functioning as a domain and is not part of RING2. We have chosen the domain borders based on the independent folding and on the sequence alignment.

Based on the alignment data of the different RBR proteins, the LDD sequence does not seem to be a general feature of the RING2 domains in other RBRs, which also suggests that it does not form one big domain with RING2. Nevertheless, the LDD sequence is never found outside the context of RING2 in HOIP and we were unable to create RING2 constructs that lack the LDD. Therefore it is likely to function in close connection to the RING2 domain. We make this suggestion in the manuscript.

We have not tried to express the specific IBR2 fragment; however we tested two fragments that are similar to IBRD2. The constructs did not express (699-923) or only yielded small quantities of instable protein (699-951), indicating that the IBR2 will most likely not result in the expression of soluble protein. In addition, the secondary structure prediction predicts a stretch of alpha-helixes between residues 993 and 1060, and also a RONN disorder prediction suggests that the region is structured, indicating that the full LDD is needed.

We were able to strengthen the data for the importance of the LDD for ubiquitin binding, as ^{biotin}ubiquitin, and not SUMO, competes with the acceptor ubiquitin for di-ubiquitin formation with ^{TAMRA}ubiquin (Figure 5C, discussed in the general section above, and see point 10 from referee 1). Since all LDD mutants are affected in ubiquitin linkage formation, we could not provide detailed insights in the exact binding modus of the ubiquitin to the LDD. The rational for all point mutants is now described throughout the paper and in supplementary figure S2A.

e) The data on Ub binding to the RBR-LDD C930A (binds) and LDD C930A (does not bind) is ambiguous. A Ub mutant (e.g. Ub I44A?) should be identified that disrupts binding to the wild-type LDD, to convincingly demonstrate importance of Ub-LDD interaction.

We have added several experiments and controls that strengthen the data for the importance of the interaction between HOIP and the acceptor ubiquitin (See general section above). The competition experiment between wt-ubiquitin and a N-terminally blocked ubiquitin (^{biotin}ubiquitin) showed that ^{biotin}ubiquitin can compete out the acceptor wild type ubiquitin in the reaction, despite the lack of its ubiquitin amino-terminus. Nevertheless, ^{biotin}ubiquitin did not inhibit HOIP~Ubiquitin thioester formation, nor did it interfere with E2~ubquitin discharge by HOIP$^{RBR-LDD}$ C930A, showing that ubiquitin interaction is essential during the LDD-mediated last step of ubiquitin transfer (figure 5C, Supplementary figure S5C) (see also point 10 of reviewer 1).

In order to gain further insights into the nature of the HOIP-ubiquitin interaction, we tested several ubiquitin Ile44 hydrophobic patch mutants as acceptor ubiquitin in single cylcle turnover assays. Surprisingly, we found that the ubiquitin hydrophobic patch is not relevant for this interaction (Figure 5D, Supplementary figure S5D)

f) In an ideal world, the authors would map the LDD Ub binding site (e.g. by NMR) and identify non-Cys surface mutations that disrupt Ub binding. But this could be beyond the scope of this work.

We agree with referee #2 that it would be very interesting to further characterize the ubiquitin-LDD binding by NMR, however we are not capable to address this point at the moment.

3) Mechanism of chain formation

a) The separation of discharge and chain forming activity is nice. However, if the RING2 is there, and if the RING1 can discharge the Ub (Fig. 4C, bottom), why is the Ub not charging the RING2 (Fig. 4A)? Also this could be an effect of C916A and C930A in RING2 stability. The real issue is in Fig 4A which shows that all mutants, regardless whether they are in R1, R2 or LDD disallow Ub charging of the RBR - if the 2-step mechanism was correct, only R1 / IBR mutants should inhibit this, and other mutants should only inhibit charging if the actual Cys that is charged was mutated (or if the mutations disrupts the domain that is to be charged - see above). So far, the mutants in LDD suggest their importance in Ub charging of the RBR, not towards acceptor Ub binding.

As discussed in the general section, we have optimized the thioester-formation and detected the HOIP~ubiquitin intermediate in RING1 and LDD mutants (Figure 4A, B, Supplementary figure S2A, S4A, B). We never detected the HOIP~ubiquitin intermediate on RING2 mutants, confirming its role as thioester acceptor.

The experimental evidence on the role of the LDD has been strengthened by the controls for the FP assay and the competition assay with ^{biotin}ubiquitin, as described in the general section above. The acceptor ubiquitin/ LDD interaction is essential for the last step of the ubiquitin linkage formation. However, since the observed affinity for ubiquitin is low, it seems likely that the LDD provides further contributions, either to the thioester charging on RING2 or by changing the HECT active site to provide the specificity to modify the ubiquitin amino-terminus. We have added the new evidence and discussion on the role of the LDD to our manuscript.

b) There is also a faint activity of the LDD C930A mutant in fig. 2. If this activity could be enhanced by lower [NaCl] or longer incubation times it would be interesting to check if this mutant looses its specificity.

It would be very interesting to identify a HOIP mutant that has lost the linear ubiquitin chain formation specificity, however the faint activity of the LDD C930A mutant was too low for the proposed specificity analysis.

4) In vivo analysis

a) It would be nice to check if HOIP RBR-LDD only is constitutively active in NF-kB activation.

We agree that it would be nice to test a constitutively active construct in cells. However, Tokunaga et al. (2009) showed that the deletion of the HOIP ZF-domain and the interference between HOIL-1L/HOIP interactions disrupts the recruitment of HOIP to NEMO and subsequently leads to a complete loss of NF-κB activation. Consequently, $HOIP^{RBR\text{-}LDD}$, which lacks the ZF and the UBA domain for HOIL-1L interaction, will not be recruited correctly to NEMO in cells, which would make the readout of the proposed experiment too ambiguous for interpretation. Therefore, we have not performed this experiment.

Minor comments

1) Abstract: "Growth factor induced NF-kB activation..." : not correct, since the LUBAC dependent processes are not growth factor induced (cytokine, DNA damage etc.).

The abstract has been changed to

2) p2: same problem, most NF-kB processes have now been shown to utilize LUBAC. Also cite Niu et al, EMBO J for DNA damage induced NF-kB. Several recent reviews by Iwai, Walczak and Dikic on this topic.

We thank referee #2 for pointing out the mistake in our manuscript. We have changed the description of NF-kB activation accordingly and the paper by Niu et al, EMBO J has been included in the references.

3) p3: Since the authors mention salt dependency, they should also comment on the pH in the main text.

The ubiquitin chain formation reactions take place at pH-values of pH7 or higher. We tested for the pH dependence and found that the reaction rates with the full length proteins are only mildly affected by increasing the pH-values up to pH9.5 (Supplementary figure S1C).

4) p5: Is the E2 independent process still dependent on the formation of a thioester intermediate? Can it be blocked/quenched by DTT or bME?

We could not visualize the thioester in an E2 independent manner, because this activity is too weak to be able to catch the very transient thioester intermediate. Consequently, we could not perform the proposed experiment.

5) The Fig legend of Fig S1C mentioned Ubch7 but this is not included. Fig S1C is very similar to Fig. 1D.

We thank the referee for pointing out the mistake in the figure legend. The original Figure 1D and Figure S1C showed the activity of HOIP, HOIL-1L and HOIP$^{RBR-LDD}$ in the presence of respectively UbcH7 and UbcH5c. The correct figure legends have been added to the figures.

6) A reference for the design of the V701A mutation should be included.

We have added a reference to Brzovic et al. 2003 for the design of this mutant.

7) The authors are encouraged to use gene nomenclature for E2 proteins (Ube2D, Ube2L3 etc) in an effort to simplify the literature (see Ye and Rape, Nat Rev Mol Cell Biol 2010).

We appreciate the issue on the nomenclature for E2 proteins and have changed all names into the gene nomenclature.

8) All Figs : kDa labels (numbers) should be given for all blots

The kDa labels have been added to each figure to improve the readability of the figures.

9) Fig. 4: Please define the asterisk

The asterisk indicated the faint HOIP~ubiquitin band. The figure has been updated with the optimized HOIP~ubiquitin thioester formation assays (Figure 4A, B, Supplementary figure S4A, B, C).

10) The discussion is good, however the UBD in Parkin is not supposed to be C-terminal to the RBR, but within the RBR (Chaugule 2011).

We thank referee #2 for pointing out this mistake and have corrected it in the text.

Referee #3 (Remarks to the Author):

On the basis of the unmasking of activity by truncation or complex formation, the authors conclude that HOIP is auto-inhibited by its N-terminal region. While that is a formally logical conclusion, the available information do not distinguish whether the observed inhibition is functionally relevant or is an unintended consequence of expressing HOIP in the absence of its binding partners. The experimental observations could be due to the full-length protein being misfolded or ill-behaved, leading to its low activity. Expression of just the RBR-LDD domains could produce properly folded material. Likewise formation of the hetero-complex with HOIL-1L might do the same. Rigorous support for auto-inhibition would require adding the N-terminal region back in trans and observing inhibition. I'm not sure this is an important enough point to warrant doing the experiment, but the manuscript should more accurately present the possible explanations for the observation.

We appreciate the fact that the difference in HOIL-1L independent activity of full length HOIP (not active) and HOIP$^{RBR-LDD}$ (active) can be explained in multiple ways. To gain a better understanding in the mechanism underlying this difference, we tested if full length HOIP and different constructs of the HOIP N-terminus could inhibit the chain formation reaction with HOIP$^{RBR-LDD}$ (Supplementary figure S1H), but saw no effect, as discussed in the general section above. We have made the point that alternative explanations are possible explicit in the text.

A large number of mutations were prepared and characterized for ligase activity, as presented in Figure 2. The results establish that ligase activity requires not only the canonical RBR, but also the LDD domain. There is little or no rationale provided in the text for the choice of residues that were mutated, although one familiar with the structures of RBRs can read the authors' minds. For the more general reader, the positions of (putative) Zn-liganding residues with the R1, IBR, and R2 domains should be identified, as mutation of these likely yield destabilized structure of the domain in which the mutated residue is found. In addition, the rationale behind how were other positions were

chosen would clarify Figure 2. Similarly, the Cys residue in R2 that is predicted to serve as an active site, based on sequence alignments with HHARI and parkin, should be clearly identified. There is no explicit mention of this residue in the text, although the authors appear to have their eye on it, as they mutated it to both Ala and Ser. Presumably, the Cys885Ser mutant was created with the intention of observing formation of an oxy-ester derivative, but there is no mention of this in the text. Was this attempted? Finally, most or all of the mutations made in the LDD were to Cys residues. It would be helpful to have an MSA of the LDD domain presented as Supplemental Information (Fig. S4B, perhaps) to ascertain how conserved these residues are.

The rationale for the design of the different HOIP mutations is now explained in the text, as well as in the figure legend of Supplementary figure S2A.

The cysteine (C885) that aligns with the thioester forming cysteine in HHARI was tested as a target for the thioester bond on HOIP (Supplementary figure S4C). Both C885A and C885S are defective in thioester formation. Possibly the assay is not sensitive enough to detect the less favorable oxyester-formation on the serine, or C885 might not be the site for thioester formation. Since all other RING2 mutants in HOIP are also defective in thioester formation, we have not been able to identify the exact site on the domain.

We now describe the LDD when first mentioned in the text and have added a multi-sequence alignment to illustrate the high conservation of the different cysteine residues (Supplementary figure S1D).

Data presented in Figure 4 nicely delineate the steps required for transfer of Ub from the E2 active site to an acceptor Ub to form di-Ub. Experiments were performed with both UbcH5 and UbcH7 as E2. My recommendation here would be to switch which data are in the main text and which are in the Supplement. Because of the lack of intrinsic reactivity with lysine, the data with UbcH7 was cleaner and therefore more compelling.

We understand the theoretical rationale for the recommendation to swap the two figures, but we feel that the UbcH5C figure illustrates the point more clearly, since the signals are stronger and thus it is easier to follow the thioester release and the formation of the di-ubiquitin. Since there is in practice no background lysine modification (as seen by the absence of di-ubiquitin formation without UbΔGly76) we prefer to keep the UbcH5C figure in the main text.

A novel finding is the identification of the LDD as a domain required for chain building. The authors present data that show that the LDD binds Ub. They also present a somewhat confusing result showing that mutation of Cys930 in the LDD significantly decreases Ub binding in the context of LDD alone, but not in the context of the RBR+LDD. It is difficult to know what to make of this observation in the absence of any data that speaks to the stability or foldedness of the LDD.

We agree with the referee that the quality of the proteins is essential for the interpretation of the experiments. Therefore, we illustrate the quality of $HOIP^{LDD}$ and $HOIP^{LDD}$ C930A in several ways. First, an analytical gel filtration shows that $HOIP^{LDD}$ and $HOIP^{LDD\ C930A}$ elute at the same size (Supplementary figure S2D), indicating that the point-mutation does not unfold the protein. Second, the foldedness of the $HOIP^{LDD}$ and $HOIP^{LDD\ C930A}$ was tested in a thermal stability experiment (see point 2c of referee 2), showing that the mutation even stabilizes the protein slightly, rather than destabilizing it. Finally, the effect of the mutation in the context of the full length protein was tested by co-immunoprecipitation after expression in human cells. HOIP C930A could still be pulled-down with HOIL-1L from cells, indicating that it is correctly located and folded (Supplementary figure S6A). Therefore, we conclude that the mutation does not cause major deficiencies in the folding of the protein.

Interestingly, the mutation did affect the interaction with ubiquitin and $HOIP^{LDD}$, but it did not disrupt the binding of ubiquitin to $HOIP^{RBR\text{-}LDD}$. Since the quality and foldedness of the proteins is good, we conclude that there might be a second interaction site for ubiquitin outside the LDD domain in $HOIP^{RBR\text{-}LDD}$. Nevertheless, the importance the interaction between the acceptor ubiquitin and the LDD is now supported by multiple experiments, as described in the general section above.

This observation, plus several others listed below, makes me wonder if it isn't more accurate to think of the LDD as an integral part of R2 or at least an extension of it, rather than an independent domain. Taking this proposal one step further, the authors' proposal that the LDD is responsible for the proper positioning of the acceptor Ub for linear chain formation would seem to imply that the

LDD has a fixed orientation relative to the active site of R2 (in analogy to Ubc13/Mms2). Given the very short "linker" (if it is that) between what is currently thought of as the end of R2 and the beginning of LDD, it may be reasonable to consider the two "domains" potentially as one. This may explain why it is difficult to express the RBR without the LDD domain. Also, the "linker" is only nine residues long and its amino acid composition/sequence does not look all that linker-like, containing numerous aromatic and hydrophobic residues. In this respect, the R2 of parkin is likely to be extended to include additional residues (and even additional Zn-binding site(s)) relative to some other R2s. The bottom line is that there is not currently sufficient structural data to know with certainty what the boundaries of R2 domains are. Given the intriguing results in this paper, it would be prudent to leave the door open to the possibility of more variation in this domain than is currently defined.

The RING2 is well conserved within the RBR family, whereas the C-terminal part that we labeled LDD can only be found in HOIP and not in other RBR proteins. Therefore, the LDD region is unique to HOIP, which is in line with the fact the HOIP is the only RBR protein that is known to mediate the formation of linear ubiquitin chains. The LDD can be purified separately, and maintains an ability to bind ubiquitin and interfere with the chain formation reaction, indicating that there are some independent functions. Nevertheless, it is likely to function in close cooperation with RING2, as we discuss in the manuscript. See also point 2d of reviewer 2.

Acceptance letter 11 July 2012

Thank you for submitting your revised manuscript on linear chain formation by HOIP's RBR-LDD for our consideration. After some delay we have now heard back from all three of the original referees, whose comments are copied below, and we shall in principle now be happy to accept the paper for publication in The EMBO Journal. As you will see, referees 2 and 3 are in essence satisfied with your revisions, while referee 1 retains a limited number of specific criticisms. Having further discussed these issues with one of the
other referees, I decided to no insist on further experimental efforts at this stage;
however I do feel that some additional, minor text revisions would be warranted, particularly regarding the naming and definition of the 'LDD', which all referees remained a bit hesitant about.

To address this, I would like to propose some minor edits to the abstract (see below), aimed at increasing the clarity and accessibility also in light of our broader readership. I would appreciate if you could consider them and let me know if you are happy for us to modify the abstract like this. I would further propose to alter the redundant phrases 'LDD domain' and 'LUBAC complex' throughout the manuscript from our side. Finally, in line with referee 1's continued reservations about the title and its accessibility, I would like to suggest a few alternative proposals below, and would appreciate your feedback on them. All of them are slightly longer than allowed by our guidelines but we would be able to make an exception here.

Once we will have sorted out these last modifications, we should then be able to swiftly proceed with formal acceptance and production of the study; therefore please get back to me at your earliest convenience.

Thank you.

With best regards
Editor

The EMBO Journal

Referee #1

(Remarks to the Author)

I still think the title is cryptic, and it still reads LDD Domain, which is linear chain determining domain DOMAIN. I think the authors should really be encouraged to find a more accessible title. They could substitute linear chain in the first instance with Met1-linked ubiquitin chains (which is a description more in keeping with the current nomenclature of K48-, K63-, K11- etc). Although the manuscript is improved with the revisions, I still think a general reader could be put off by the LDD in the title.

The authors have addressed most of my original points. However I have 2 remaining concerns. I really don't like papers being delayed and going through multiple rounds of revision, but I am concerned that the authors didn't do the controls I felt were important in my original review (point 10). My original comment states:

Page 8. The affinity for ubiquitin as determined by FP is low, even by ubiquitin biology standards. The authors also do not rule out that this binding is non-specific, only by showing a cysteine to alanine mutant of LDD binds less well. To do this, they should repeat the interaction experiment with NEDD8 and/or SUMO, or even the Ubl domain of HOIL-1L to determine whether the interaction with ubiquitin is specific to ubiquitin. Alternatively, they could try to identify the region of ubiquitin involved in the interaction (likely to be the I44 hydrophobic patch), and test a mutation. They must also control for the fluorophore as TAMRA is known to be quite hydrophobic.

In response the authors state that they have done the TAMRA control, which is great.

However, rather than measuring the interaction (or not) of NEDD8 and/or SUMO with HOIP-RBR-LDD (and HOIP-LDD), they add SUMO to their single turnover assay and conclude that a lack of interference with the formation of di-ubiquitin equals a lack of interaction of SUMO with HOIP. This is a reasonable interpretation, but does not really address the original concern which is whether or not the interaction with ubiquitin is specific to ubiquitin. The single turnover assay does not address this, and the fact that no ubiquitin mutant tested has any impact is also a concern (Reviewer 2 also makes the same point that 'a ubiquitin mutant should be identified that disrupts binding to the wild-type LDD, to convincingly demonstrate importance of the Ub-LDD interaction' and this has not been done in the revision).

I don't quite understand why the authors didn't do an FP interaction study of I44A (or whatever mutation)-ubiquitin and RBR-LDD (and LDD), and TAMRA-SUMO and RBR-LDD (and LDD)-the latter of which, if there was no detectable binding, would have controlled both for TAMRA and for the specificity in one experiment. This should be an afternoon's work, assuming they have the proteins.

Did the authors do these controls or not? I think as the manuscript stands, the authors still haven't ruled out the possibility that the interaction with ubiquitin is non-specific. This interaction is a major point of the paper and the inspiration for the mechanistic analysis.

Also, it is of some concern that the Cys930Ala mutation is significantly more thermostable than the wild-type fragment (data in response to reviewer 2, point 2c, 6.5 degree increase in stability). This could be interpreted as the wild-type fragment being less well-folded than the mutant, which may also give rise to the apparent, very low affinity interaction between ubiquitin and LDD (and loss of it in the more stable, potentially better-folded mutant). This makes it even more important to know whether or not the interaction between ubiquitin and LDD is specific.

Referee #2

(Remarks to the Author)
The authors have done an very good job in addressing most of my comments

adequately, and the manuscript is more accessible and easier to follow.
The manuscript has a large number of very exciting findings and technical advances. I am still uneasy about the global effects that the Cys mutations seem to have (and here, an increase in thermo-stability for a mutant does not alleviate my concerns, in fact I am more worried about what I am looking at when I see oligomer gel filtration profiles for RBR-LDD (Fig 3d)). However, I hope the authors will resolve this by doing what they do very well, ie get the structure of that thing. I am still concerned that a structure may show little boundary to justify the 'discovery of an LDD' as such, but time will tell whether this new domain annotation will be justified.
Overall, great work, I support publication in EMBO J.

Referee #3

(Remarks to the Author)
The authors have done a very careful and thoughtful revision of their manuscript that is responsive to the issues and questions raised in the first review. The paper now makes a strong and compelling case for their proposed model for linear chain formation by HOIP. The results and model will be of significant interest to readers and move the field ahead. I recommend the paper for publication in its current form.

