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Cryo-EM structure of the chain-elongating E3 ligase UBR5

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Review #1

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

This manuscript describes cryo EM structural analyses of human E3 ligase Ubr5 in dimeric and tetrameric states. Ubr5 belongs to structurally poorly characterized family of Hect E3 ligases and has important biological functions, e.g. in targeting transcription factors for proteasomal degradation. The manuscript therefore addresses an important subject of basic science and biomedical interest. Using in vitro ubiquitylation assays the authors show that purified Ubr5 forms ubiquitin chains on a substrate (akirin2), but also on a non-substrate (securin), provided the proteins are covalently fused to ubiquitin. The authors interpret this as a preference of Ubr5 for ubiquitin chain elongation over initiation. Consistently they show that Ubr5 forms free ubiquitin chains linked by Lys48 in vitro.
Whilst the structures of full-length Ubr5 are very interesting and important, this manuscript appears to be at a premature stage. The structural interpretation and models lack experimental validation and remain speculative. The presented activity assays are interesting but do not quite link up with the structural part which leaves the manuscript somewhat disconnected. In my view this manuscript requires considerably more work to correlate structure with function, as suggested below, but holds the potential of turning into a highly insightful story.

Conceptual comments:

1. Key observation is that a Ubr5 dimer assembles into higher-order oligomers. The authors speculate that this is functionally relevant, e.g. by the possibility of substrate ubiquitylation occurring within the central cavity of the ring shaped tetramer or ubiquitylation in cis and trans. However, neither significance of Ubr5 oligomerisation nor dynamics/determinants in solution is investigated.
- In line 100, the authors state that individual oligomeric species could not be separated but do not show data. Why can the species not be separated? Do they exchange? Can exchange be controlled by ionic strength/pH/temperature...?
The authors also suggest that the tetramer is transient (e.g. line 165). What is the evidence for this? Subunit exchange may be tested by mixing different species, e.g, containing labels/tags etc.
- The authors should design structure-based mutations, particularly within the small, tetrameric interface, and measure oligomerisation state of the mutants to correlate their cryo EM analyses with oligomeric states observed in solution.

- The authors should also subject individual oligomers (if required, by mutational stabilization of particular states) in activity assays to test their hypotheses.
2. Lines 160-163: "We performed extended 3D classification of the tetramer, which allowed us to confidently dock two models of UBR5 dimers. This revealed that the tetrameric assembly of UBR5 is formed by SBB2 domains of two opposite dimers (Figure 1I)." The idea that the SBB2 domains make up tetrameric interface should be experimentally validated.
3. Lines 311 onward: The authors speculate that oligomeric arrangements of Ubr5 allow for substrate modification in trans and cis, expanding the substrate repertoire. As part of results section, this should be experimentally addressed. Depending on the exchange behaviour of subunits within oligomers, it may be possible to use mixing experiments. Alternatively, they authors may consider comparing Ubr5 ubiquitylation efficiency towards substrates of different sizes, which may allow for better interpretation of the distance restraints they defined.
4. Figure 1J suggests that MLLE domain was modelled, yet the authors note in the text (line 190) that this domain is disordered.
5. The interpretation of the in vitro experiments comparing ubiquitylation of ubiquitin fused akirin2 (substrate) and ubiquitin fused securin (non-substrate) require a re-evaluation: The fact that even ubiquitin fused securin is efficiently ubiquitylated by Ubr5 in vitro shows that a fused ubiquitin molecule is sufficient to recruit a protein for modification by Ubr5 (likely via the UBA domain) in vitro. The specificity of Ubr5 for certain substrates in the cell must therefore follow different (unknown) mechanisms. It is possible that observed, additional affinity between Ubr5 and akirin2 (which is independent of ubiquitin) contributes to this. The available data, however, are insufficient to suggest a hierarchy of interactions (suggested in line 235-237). The interpretation should also be adapted in Figure 6 in which an order of binding events is postulated.
6. Fluorescently labelled deltaGG-ubiquitin is used to monitor free chain formation by Ubr5. Why is this setup used rather than a simple assay with full-length ubiquitin? The rationale/benefit should be clarified.
7. Conformation/functional significance of the plug loop should be validated by mutagenesis.
8. The mass spec data should be presented in a compact supplementary figure or table, in addition to comprehensive data table in Suppl. Table 2.
9. Statements without data backup should be phrased as hypotheses or experimentally validated, e.g.,
- line 27:"Using cryo-EM processing tools, we observe the dynamic nature of the domain movements of UBR5, which allows the catalytic HECT domain to reach engaged substrates."
- line 31:"This preference for ubiquitinated substrates permits UBR5 to function in several different signalling pathways and cancers".

- line 78: "This striking feature allows the positioning of substrate binding sites in close proximity to the catalytic HECT domain in cis or trans, expanding its substrate-recruiting capacities."

Methods section:

- The authors should provide information on how Ubr5 sequence was optimized (line 477).
- The authors should explain why different versions of Ubr5 were used for cryo-EM and activity assays.
- Given the importance of oligomerisation, the authors should explain which fraction of the gel filtration was used for activity assays. Depending on the reversibility of oligomerisation and if oligomerisation impacts activity, the authors should also specify what concentrations these fractions had and/or which concentration they were concentrated to.
- The authors should specify how and at which position cysteine was introduced for labelling of the deltaGG-ubiquitin version.
- The authors should specify which concentrations mass photometry measurements were performed at.
- A description of mass spectrometry measurements is missing.

Results/figures etc:

- Lines 88-91 require more precision: "...highly conserved" - compared to what? Some quantification would help here. "...the length is an average across all species". What is meant by "all species" (all species shown, all metazoans?)?
- Figure 1B shows appears to also show a monomer peak. The authors should label it and comment on it.
Can the authors comment on the right shoulder of tetramer peak which was not fitted?
- Figure Legends 1H, 1I, and 1J are missing
- In Figures 1G, 2A and 2B, colour labelling positive/negative is swapped.
- Line 215: "... have observed the formation of free chains". Here, a figure/figure reference is needed.
- Figure 5 C, D, Figure 6: The way models are drawn is very difficult to understand. Maybe the authors could find clearer way to illustrate their hypotheses?

Referees cross-commenting

Reviewer 2 noticed that the manuscript contains a sentence that, in paraphrased form, may have been adopted from a competing manuscript published on Biorxiv some days

earlier. According to the conventions of good scientific practice, the competing manuscript should be cited here.

2. Significance:

Significance (Required)

see above

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 3 and 6 months

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Review #2

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

This manuscript reported the CryoEM ring-like structure of the full-length human E3 assembly ligase UBR5, showing its assembly into a tetramer. The authors identified critical determinants for antiparallel homodimer and tetrameric assembly. They further described AKIRIN2 as UBR5 substrate and provided evidences of a preferential interaction and activity of UBR5 towards monoubiquitinated proteins. Based on these findings, they proposed UBR5 as chain-elongating E3 ligase.

CryoEM data are solid, and the model interpretation of the tetrameric structure provides a precise description of the domain composition of the protein that well fit with biochemical data. Additional experiments are suggested to corroborate few statements of the authors.
We believe they are realistic in terms of time and resource.

1. Authors should address the importance of tetramerization by mutating SBB2 at the tetramerization interface and comparing the mutant with wild type in mass photometry and ubiquitination assays. In silico analysis of the interaction interfaces (e.g by using PISA software) could be useful to select amino acids to be mutated. The authors suggested a role for oligomerization in catalysis and mutants are needed in order to define the real "functional unit" of the enzyme.
2. The authors used sucrose gradient sedimentation assay to prove UBR5 and substrate interaction (Fig. 3). Control experiment that showed UBR5 protein sedimentation in presence of GFP only is instead in Supplementary Fig. 3D. Unfortunately, in that panel the signal of UBR5 is not visible. Main figure should be revised showing proper controls of the experiment.
3. The authors need to better clarify the features of the AKIRIN-UBR5 interaction. According to the data, the enzyme is equally active on both AKIRIN-Ub and Securin-Ub, suggesting a Ub-specific engagement. What would be a correct explanation of these results? Is the UBA domain directly involved in this process? Testing the activity of a UBA-impaired mutant should help to solve this issue.
4. The authors identified a 25 aa sequence, called Plug loop, preceding the HECT domain. In the structure it is inserted between N and C-lobe subdomains of the HECT and appears to lock the enzyme in an open L-conformation. These structural findings are interesting, but no supported by experimental data. Which is the effect of the Plug loop deletion in a ubiquitination assay? Without further validation the last chapter of the results remains purely speculative and may better fit in the discussion.
5. The datasets are clearly affected by preferential orientation as showed by the angular distribution and 2D classes (reason why the authors correctly performed data collection with tilt). A comment on this is required in the experimental section. In addition, it is not clear whether the presented maps (Fig 1 and 2) derive from merging of the two datasets or only the model has been built using the two different datasets.
6. As a general comment, authors should enlarge panels in which structural details are

described, highlighting the side chain residues involved in binding interfaces. Fig. 5 and Fig. 6 are particularly small and incomplete. Most of the structural figures miss key labels needed for a proper understanding. E.g. among the others, numbering of the helix composing the armadillo domain.
- The overall organization of the figures is quite confusing. Pag. 7 Figure 2C should represent a "box stabilized by three zinc ions mediated by two histidine and seven cysteine residues" according to text citation, but none of these details is highlighted in the corresponding figure. The eye in Figure 1,2,4 does not mean much if a proper box is not linked to the actual site to be seen. In addition, arrows indicating the rotation axis is hard to interpret. Few panels miss the legend. Figure 1A and many other panels miss the reference in the text. More details below.

Additional points:

- Mass Photometry data need additional comments and labels. Please comment on the MP concentration used to analyze the samples. Being a dynamic system, you are probably seeing an equilibrium of species at 10 nM in MP. For better completeness of MP figures, labels that includes counts, % of species and sigma should be added to the nice representation of oligomers. Which condition/fraction represent the MP data showed in 1B?
- If Alphafold models are mentioned and used for model building, it would be nice to provide at least a pLDDTscore and ptm score. Since some details of the AF model are described in the text, an additional superposition of the AF model with the final model derived by EM would be useful to the community.
- A simple workflow describing the cryoEM data processing that includes how many particles have been used in each step is required, at least in the methods section. The authors need to show the cryoEM 2D classes of the dimer as well.
- Please add the domain boundaries in Figure 1A and highlight the domains on the alignment included in Supplemental Table 1.
- Pag. 8 please decide which abbreviation to use, either UBR or Ubr.
- Page 8, line 192. I found annoying to find the same sentence used by competitors who posted a bioRxiv paper 3 days before the one we are reviewing (doi.org/10.1101/2022.10.31.514604 page 4, line 135).
- In supp. 1C legend, "high concentration of NaCl" is a bit vague
- Complementary to Supp Fig 2A, a zoom in of the density map with traced model would be beneficial to show the actual map quality obtained.
- Pag. 6 lines 133-134, the helix residues involved in homodimerization are cited in the text, but not highlighted in the Figure 1.
- Figure 1 legend, panels H-I-J description are missing.

- Figure 3, panel B, meaning of the asterisk is not reported in the figure legend.
- Figure 4, 5 panels from A to E are cited in the text while figure reported only 4.

Referees cross-commenting

I think all the reviews are fairly consistent and agree with the comments raised by my colleagues with the one exception of Point 3 of Reviewer 1. The issue is certainly important yet the experiment suggested is not clear. I personally have troubles designing an informative experimental set-up.

2. Significance:

Significance (Required)

This paper presents the intriguing Cryo-EM structure of the full-length HECT E3 ligase UBR5.
As it stands, this work provides evidence of the existence of a tetrameric RING-like conformation that could represent the functional unit of the catalysis. Very little validation of the features identified in the Cryo-EM structure is given, thus the paper remains quite descriptive, but in any case interesting and informative for the ubiquitin field.

Considering that UBR5 is a quite competitive subject in these days (e.g. at least one additional Cryo-EM structure was posted in BioRxiv, doi.org/10.1101/2022.10.31.514604), I would positively consider this manuscript for publication if the authors reply in full to the issues raised.

My field of expertise: Ubiquitin regulation and interactions, biochemistry, biophysics and Cryo-EM.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

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Review #3

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

The manuscript presented the Cryo-EM structure of HECT E3 UBR5. Using Alphafold2 model of UBR5, the authors were able to dock and refine the structure model of full length UBR5. Interestingly, UBR5 exists as a homodimer and could potentially assemble into a larger oligomer based on SEC and Cryo-EM data. The antiparallel arrangement of the homodimer suggests that the C-terminal HECT domain could transfer ubiquitin in trans or in cis configuration. The tetrameric model reveals a ring-like structure with a large central cavity, presumably to accommodate large proteins/complexes. Using AKIRIN2 as a substrate, the authors demonstrated that UBR5 did not ubiquitinate AKIRIN2, but prefers ubiquitin modified AKIRIN2 as the substrate for ubiquitin chain elongation. Indeed, they observed that UBR5 preferentially ubiquitinates pre-ubiquitinated non-cognate substrate and free ubiquitin hinting that UBR5 is a chain elongating E3. Lastly they showed that UBR5 HECT contains a plug-loop that blocks C-lobe rotation and suggested that conformational change is necessary for ubiquitin transfer.

Comments:

1. Homodimerization and oligomerization are the novel aspect of this study but the manuscript lacks validation of the structure. The authors should provide

biochemical/mutagenesis analysis to support the dimerization interface observed in the structure. Also the model showed that SBB2 is involved in the tetramerization interface, could the authors verify this by designing a SBB2 deletion mutant?
2. Would be useful to show the docking of Alphafold2 model onto the Cryo-EM map prior to further model building and refinement in the supplementary data.
3. Please show the SDS-PAGE of purified UBR5 used for Cryo-EM study.
4. Figure referencing is not in order. For example Figure 1J was described before Figure 1I. Figure 2A,B mentioned after Figure 2C. Also some Figures are not properly referenced in the main text, e.g. p10 when describing ubiquitin chain formation of UbAKIRIN2 and UbSecurin. Please check throughout the manuscript.
5. Figure legends are missing for Figure 1H-1J
6. It was stated in p10 that there was no binding between UBR5 and UbSecurin in Figure S3C, but Figure S3C showed faint FAM-UbSecurin across the fractions. It would be useful to repeat this with FAM-UbSecurin alone to ensure the faint bands are background signal.
7. In p10, it was stated that UBR5 and UbAKIRIN2 interaction was enhanced in the presence of ubiquitin. How did the authors come to this observation? The sucrose gradients (Figure 3B) showed that UBR5 co-elutes with both AK2 and UbAK2. This reviewer is unclear whether the intensity of the bands can be used to evaluate the strength of the binding affinity. Was the experiment performed at the same protein component concentration/condition? The UBR5 appears to elute at different fractions from the two experiments.
8. The authors suggested that the UBA domain might bind ubiquitin and promote ubiquitination of UbAKIRIN2. It is noteworthy that prior studies on several HECT E3s showed that HECT domain alone can catalyze free ubiquitin chain assembly. Could it be possible that the HECT domain of UBR5 alone could catalyze the extension of UbAKIRIN2, UbSecurin or UbdeltaGG?
9. In Figures 3A and S3B, does the ubiquitin chain elongation occur only on the fused ubiquitin?
10. Figure 4E mentioned in the main text but is missing.
11. It is not clear from Figure 4 whether the plug-loop is blocking the rotation of C-lobe. The overlaid Figure 4 is quite busy, would be useful to show the UBR5 HECT domain alone with other HECT domain presented in the same orientation but in separate panels. With the plug-loop in the current configuration, does it block E2 binding and transthiolation reaction? Figure 5B seemingly suggested that plug-loop is blocking transthiolation but it is hard to visualize. The authors could enlarge Figure 5B and color the plug-loop differently.

2. Significance:

Significance (Required)

The manuscript provides the structural insight into the organization of UBR5. While the Cryo-EM data largely agrees with the Alphafold2 UBR5 model, this should not take away the significant effort in obtaining the large UBR5 protein structure. The structure reveals an unexpected homodimerization of UBR5 and in the assembly of larger oligomer. The biochemical analyses suggest that UBR5 is proficient in ubiquitin chain elongation. Overall the study provides a structural framework for understanding how UBR5 could function as an ubiquitin ligase. The findings will be of interest to scientist in the ubiquitin field and in understanding UBR5 biology.

Limitation: aside from the structure, the study lacks detailed mechanisms of how UBR5 catalyzes ubiquitin transfer. While few models for ubiquitin transfer were proposed, the study lacks suitable substrates to investigate the mechanism. The study showed that UBR5 can elongate ubiquitin chain, but it is not clear whether UBR5 could transfer ubiquitin to substrate.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Cannot tell / Not applicable

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No

Revision Plan

Manuscript number: RC-202201747
Corresponding author(s): Zuzana Hodakova, Nicholas G. Brown, David Haselbach

[The "revision plan" should delineate the revisions that authors intend to carry out in response to the points raised by the referees. It also provides the authors with the opportunity to explain their view of the paper and of the referee reports.

The document is important for the editors of affiliate journals when they make a first decision on the transferred manuscript. It will also be useful to readers of the reprint and help them to obtain a balanced view of the paper.

If you wish to submit a full revision, please use our "Full Revision" template. ***It is important to use the appropriate template to clearly inform the editors of your intentions.****]*

1. General Statements [optional]

This section is optional. Insert here any general statements you wish to make about the goal of the study or about the reviews.

Please see the attached cover letter.

2. Description of the planned revisions

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

This manuscript describes cryo EM structural analyses of human E3 ligase Ubr5 in dimeric and tetrameric states. Ubr5 belongs to structurally poorly characterized family of Hect E3 ligases and has important biological functions, e.g. in targeting transcription factors for proteasomal degradation. The manuscript therefore addresses an important subject of basic science and biomedical interest. Using in vitro ubiquitylation assays the authors show that purified Ubr5 forms ubiquitin chains on a substrate (akirin2), but also on a non-substrate (securin), provided the proteins are covalently fused to ubiquitin. The authors interpret this as a preference of Ubr5 for ubiquitin chain elongation over initiation. Consistently they show that Ubr5 forms free ubiquitin chains linked by Lys48 in vitro.
Whilst the structures of full-length Ubr5 are very interesting and important, this manuscript appears to be at a premature stage. The structural interpretation and models lack experimental validation and remain speculative. The presented activity assays are interesting but do not quite link up with the structural part which leaves the manuscript somewhat disconnected. In my view this manuscript requires considerably more work to correlate structure with function, as suggested below, but holds the potential of turning into a highly insightful story.

Revision Plan

We thank the reviewer for the very helpful comments and will implement the suggestions as outlined below.

Conceptual comments:
1) Key observation is that a Ubr5 dimer assembles into higher-order oligomers. The authors speculate that this is functionally relevant, e.g. by the possibility of substrate ubiquitylation occurring within the central cavity of the ring shaped tetramer or ubiquitylation in cis and trans. However, neither significance of Ubr5 oligomerisation nor dynamics/determinants in solution is investigated.
We intend to perform several experiments to assess the significance of UBR5 oligomerisation, as outlined below.

- In line 100, the authors state that individual oligomeric species could not be separated but do not show data. Why can the species not be separated? Do they exchange? Can exchange be controlled by ionic strength/pH/temperature...?
We performed several experiments to address this question, which can partially be found in the Supplemental material of the manuscript. We will reformulate these data more concisely in the revised manuscript. In summary:

Size exclusion chromatography (SEC) profiles show two peaks (Suppl. Fig.1A). The first peak is a mixture of oligomers (Suppl. Fig.1B), the second peak is mainly tetrameric (Fig 1B), as seen by mass photometry and EM. We do not obtain peaks for dimers or tetramers alone.

Regarding the stability of the oligomers in different buffer conditions, we measured the oligomerisation of the second SEC elution peak with a buffer containing 1M NaCl (Suppl. Fig1C) and observed similar oligomeric assemblies. At low pH (5.8), UBR5 was again present in both dimeric and tetrameric assemblies. We also measured a heated sample which promoted higher oligomerisation and resulted in a more heterogeneous sample. All mass photometry measurements are shown in the figure below, where i) represents the sample used for cryo-EM and ii) to iv) show different conditions (highlighted in bold). We will edit the colours and labels of this mock figure and implement it into Supplemental Figure1.

The authors also suggest that the tetramer is transient (e.g. line 165). What is the evidence for this? Subunit exchange may be tested by mixing different species, e.g, containing labels/tags etc.
- The authors should design structure-based mutations, particularly within the small, tetrameric interface, and measure oligomerisation state of the mutants to correlate their cryo EM analyses with oligomeric states observed in solution.

We have designed and are currently in the process of expressing mutants aimed at disrupting the tetramerisation interface (i.e., the SBB2 domain). We plan to measure the oligomeric state of this mutant using Mass photometry.

- The authors should also subject individual oligomers (if required, by mutational stabilization of particular states) in activity assays to test their hypotheses.

We will follow up our structural analysis described in the previous point with testing the ubiquitination activity of this mutant. Ultimately, coupling these mutants with ubiquitination assays will be used to assess the functional relevance of UBR5 oligomerization.

2) Lines 160-163: "We performed extended 3D classification of the tetramer, which allowed us to confidently dock two models of UBR5 dimers. This revealed that the tetrameric assembly of UBR5 is formed by SBB2 domains of two opposite dimers (Figure 1I)." The idea that the SBB2 domains make up tetrameric interface should be experimentally validated.
As mentioned above, we will purify the SBB2 deletion mutant and characterise it using Mass photometry and EM.

3) Lines 311 onward: The authors speculate that oligomeric arrangements of Ubr5 allow for substrate modification in trans and cis, expanding the substrate repertoire. As part of results section, this should be experimentally addressed. Depending on the exchange behaviour of subunits within oligomers, it may be possible to use mixing experiments. Alternatively, they authors may consider comparing Ubr5 ubiquitylation efficiency towards substrates of different sizes, which may allow for better interpretation of the distance restraints they defined.
We will try to test this hypothesis using full-length UBR5, SBB2 domain deletion mutant, and a dimerisation interface mutant in activity assays. It should be noted that reviewers 1 and 2 have differing opinions on ways to address this point, where reviewer 2 states an informative experimental set-up to answer this issue is difficult to design. Should our approach not be possible, we will discuss the oligomeric arrangement in the discussion section.

4) Figure 1J suggests that MLLE domain was modelled, yet the authors note in the text (line

190) that this domain is disordered.
The unresolved domains in Fig1J are described in the figure caption lines 409&410. We will revise the text to make the statements clearer.

5) The interpretation of the in vitro experiments comparing ubiquitylation of ubiquitin fused akirin2 (substrate) and ubiquitin fused securin (non-substrate) require a re-evaluation: The fact that even ubiquitin fused securin is efficiently ubiquitylated by Ubr5 in vitro shows that a fused ubiquitin molecule is sufficient to recruit a protein for modification by Ubr5 (likely via the UBA domain) in vitro. The specificity of Ubr5 for certain substrates in the cell must therefore follow different (unknown) mechanisms. It is possible that observed, additional affinity between Ubr5 and akirin2 (which is independent of ubiquitin) contributes to this. The available data, however, are insufficient to suggest a hierarchy of interactions (suggested in line 235-237). The interpretation should also be adapted in Figure 6 in which an order of binding events is postulated.
We will clarify the interpretation of our experiments. We are currently working on characterising the interaction between Akirin2 and UBR5 but do not yet have high-resolution data of the complex. This structure will be necessary to design mutants to adequately test how multivalency contributes to polyubiquitination. We will therefore describe the interaction of UBR5 and AKIRIN2 as a hypothesis it in the discussion section, as well as amend Figure 6.

6) Fluorescently labelled deltaGG-ubiquitin is used to monitor free chain formation by Ubr5. Why is this setup used rather than a simple assay with full-length ubiquitin? The rationale/benefit should be clarified.
We use fluorescently labelled ubiquitin delta GG for a cleaner read-out of our activity assays to characterise chain elongation. This ubiquitin with a C-terminal GG deletion can only serve as acceptor ubiquitin but cannot be attached to the growing chain. We use the Cy5 label to specifically detect only this mutant ubiquitin and observe further ubiquitin chain elongation specifically on this acceptor ubiquitin. We will expand our explanation of the use of this variant in line 244. We will also amend our Supplemental figure 3 to show a comparison of Ubiquitin delta GG and wild-type ubiquitin activity assays ,as shown below. (Ubi deltaGG assays in Figure3 are represented in the attached figure).

7) Conformation/functional significance of the plug loop should be validated by mutagenesis.
We have already designed and are currently in the process of expressing mutants of the plug loop. We aim to characterise these mutants using cryo-EM and activity assays.

8) The mass spec data should be presented in a compact supplementary figure or table, in addition to comprehensive data table in Suppl. Table 2.
We will represent the mass spec data in the form of a bar chart in Supplemental Figure 3.

7) Statements without data backup should be phrased as hypotheses or experimentally validated, e.g.,
- line 27:"Using cryo-EM processing tools, we observe the dynamic nature of the domain movements of UBR5, which allows the catalytic HECT domain to reach engaged substrates."
We rephrase to: Using several cryo-EM image processing tools, we observed multiple domains of UBR5 as highly flexible. We hypothesize that this dynamic nature allows the catalytic HECT domain to reach engaged substrates bound to different positions.

-line 31:"This preference for ubiquitinated substrates permits UBR5 to function in several different signalling pathways and cancers".
We rephrase to: This preference for ubiquitinated substrates may permit UBR5 to function in several different signalling pathways and cancers.

-line 78: "This striking feature allows the positioning of substrate binding sites in close proximity to the catalytic HECT domain in cis or trans, expanding its substrate-recruiting capacities."
We rephrase to: This striking feature allows the positioning of substrate binding sites in close proximity to the catalytic HECT domain in cis or trans, which may expand its substrate-recruiting capacities.

Methods section:
- The authors should provide information on how Ubr5 sequence was optimized (line 477).
The sequence was codon optimized to enable expression in mammalian cells to not include rare codons. This standard procedure was done by GENEart. We clarify this now in the text.

- The authors should explain why different versions of Ubr5 were used for cryo-EM and activity assays.
For activity assays, we use an N-terminally tagged UBR5 construct (termed “Strep-UBR5”), leaving the C terminus in its native state, which is essential for the activity of HECT ligases. The yields of this construct are low. Therefore, for higher yields (needed for cryo-EM), we use an N- and C- terminally tagged construct with His and Strep tags, respectively (termed “His-UBR5-Strep”). We will implement this comparison into the Results and Methods sections and clarify the use of the constructs in the figures and text.

Revision Plan

Both constructs mainly form a tetrameric assembly, shown in the mock figure below (left (i)-CryoEM sample, right (ii)-activity assay sample). We can implement the Strep-UBR5 mass photometry profile into Supplemental Figure 1.

- Given the importance of oligomerisation, the authors should explain which fraction of the gel filtration was used for activity assays. Depending on the reversibility of oligomerisation and if oligomerisation impacts activity, the authors should also specify what concentrations these fractions had and/or which concentration they were concentrated to.

For activity assays, we use a sample collected after Strep affinity chromatography which contains multiple oligomers. The samples are concentrated to either 2.5mg/ml or 1mg/ml. Final concentrations of UBR5 in activity assays were constant throughout experiments; 300nM. These points will be clarified in the Methods section of the revised manuscript.

As discussed previously in point 1, we have designed and are currently in the process of purifying mutations aimed at disrupting the tetramerisation interface (i.e., the SBB2 domain). After characterising this mutant with mass photometry, this mutant will be tested alongside full-length UBR5 to determine whether SBB2 domain deletion and, therefore, oligomerisation impacts activity.

- The authors should specify how and at which position cysteine was introduced for labelling of the deltaGG-ubiquitin version.

Cysteine was introduced directly in front of the ubiquitin delta GG sequence, placed after a TEV cleavage site. The full plasmid sequence is GST-TEV-"SGGS"linker-Cysteine-Ubiquitin_deltaGG, and this construct was expressed recombinantly in E.coli. The precise information on the design of this construct will be implemented in the Methods section of the revised manuscript.

- The authors should specify which concentrations mass photometry measurements were performed at.

Measurements were conducted using UBR5 samples at a final concentration of 100nM. We will specify this in the methods section.

- A description of mass spectrometry measurements is missing.

We will add the description of these measurements in the methods section.

Revision Plan

Results/figures etc:

- Lines 88-91 require more precision: "...highly conserved" - compared to what? Some quantification would help here. "...the length is an average across all species". What is meant by "all species" (all species shown, all metazoans?)?

We performed bioinformatic analyses to determine the conservation of UBR5 across all species with annotated proteome where it is found (only in metazoans). We selected 6 representative organisms and showed their full sequence alignments in Supplemental Table 1. We will clarify our analyses and observations in the text and make a clear link to the Supplemental Table 1, which shows, in part, the sequence alignments used for analysing the conservation of UBR5 across metazoans (with the human protein used in this study as a reference).

- Figure 1B shows appears to also show a monomer peak. The authors should label it and comment on it. This peak represents a species with low molecular weight (measured to be 161kDa, as seen on the labels above the peak in the figure below). Based on the purification gels, we believe these are additional contaminants found in the preps. Furthermore, their molecular weight does not match a monomeric protein (which would be 310kDa, and no such peak is observed). We will label the peak in our Supplemental figure 1 with the molecular weight clearly annotated and comment on it in our main text.

Can the authors comment on the right shoulder of tetramer peak which was not fitted?

The shoulder most likely represents dynamic species of UBR5 bound by co-purified contaminants. We only fitted the main peak found at this molecular weight, which represents the tetrameric assembly of UBR5. We can comment on the shoulder presence in the text of the results.

- Figure Legends 1H, 1I, and 1J are missing

We will add the respective figure legends in the revised manuscript.

- In Figures 1G, 2A and 2B, colour labelling positive/negative is swapped.

We will amend the colour bar to the correct labelling.

- Line 215: "... have observed the formation of free chains". Here, a figure/figure reference is needed.

We will add the Coomassie-stained gels of Figure 3A, and Supplemental Figures 3B,3D (same gels as shown in these figures), showing the depletion of ubiquitin as well as the formation of free chains. Additionally, this can also be seen in the mock figure attached to Point 6 above, which can be implemented into the manuscript.

- Figure 5 C, D, Figure 6: The way models are drawn is very difficult to understand. Maybe the authors could find clearer way to illustrate their hypotheses?

We will simplify the mentioned figures to illustrate our hypotheses in a clearer way.

Referees cross-commenting

Revision Plan

Reviewer 2 noticed that the manuscript contains a sentence that, in paraphrased form, may have been adopted from a competing manuscript published on Biorxiv some days earlier. According to the conventions of good scientific practice, the competing manuscript should be cited here.

We apologise that the reviewer perceived our phrasing on the positioning of the MLLE domain in the HECT N-lobe as a potential copy from a competing manuscript published in bioRxiv. This finding was unexpected mainly due to a disagreement with previous reports (such as the citation we provided). We, as well as our competitors, used phrasing commonly seen in literature to contradict these previous findings. Nevertheless, we rephrased the sentence to avoid any coincidental overlap to:
“An uncommon characteristic of the UBR5 HECT domain is the location of the MLLE motif in the N-lobe and not before the HECT domain, which disagrees with previous annotations.”

Reviewer #1 (Significance (Required)):

see above

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

This manuscript reported the CryoEM ring-like structure of the full-length human E3 assembly ligase UBR5, showing its assembly into a tetramer. The authors identified critical determinants for antiparallel homodimer and tetrameric assembly. They further described AKIRIN2 as UBR5 substrate and provided evidences of a preferential interaction and activity of UBR5 towards monoubiquitinated proteins. Based on these findings, they proposed UBR5 as chain-elongating E3 ligase.
CryoEM data are solid, and the model interpretation of the tetrameric structure provides a precise description of the domain composition of the protein that well fit with biochemical data. Additional experiments are suggested to corroborate few statements of the authors.
We believe they are realistic in terms of time and resource.
We thank the reviewer for the thoughtful and very constructive comments.

Revision Plan

1. Authors should address the importance of tetramerization by mutating SBB2 at the tetramerization interface and comparing the mutant with wild type in mass photometry and ubiquitination assays. In silico analysis of the interaction interfaces (e.g by using PISA software) could be useful to select amino acids to be mutated. The authors suggested a role for oligomerization in catalysis and mutants are needed in order to define the real "functional unit" of the enzyme.

We have designed variants aimed at disrupting the tetramerisation interface (i.e., the SBB2 domain) and are currently in the process of purifying them. We will characterise these mutants using mass photometry, cryo-EM, and in activity assays as suggested by the reviewer.

We tried to predict the tetramerisation interface using AlphaFold but did not achieve convincing results. From our own experience as well as the structural biology community, we know that predicting the interactions with AlphaFold often shows false negatives. Therefore, we cannot use such tools to design point mutations.

2. The authors used sucrose gradient sedimentation assay to prove UBR5 and substrate interaction (Fig. 3). Control experiment that showed UBR5 protein sedimentation in presence of GFP only is instead in Supplementary Fig. 3D. Unfortunately, in that panel the signal of UBR5 is not visible. Main figure should be revised showing proper controls of the experiment.

We apologise for the poor quality of the mentioned panel. We will revise the figure to contain all control gradients, which are always performed alongside the gradients represented in the figures, similar to the figure shown in Point 3.

3. The authors need to better clarify the features of the AKIRIN-UBR5 interaction. According to the data, the enzyme is equally active on both AKIRIN-Ub and Securin-Ub, suggesting a Ub-specific engagement. What would be a correct explanation of these results? Is the UBA domain directly involved in this process? Testing the activity of a UBA-impaired mutant should help to solve this issue.

The reviewer's interpretation agrees with our explanation of the results, which we will clarify in the text and test the UBA domain. In fact, we have purified Strep-UBR5 with the UBA domain deleted (UBR5delUBA) and tested its activity in ubiquitination assays (image below, left). We see that this mutant depletes ubiquitin at slower rates, and we are still investigating the precise mechanism.

We also tested the UBR5delUBA and UbAKIRIN2 interaction on sucrose gradients (right) and did not observe a disruption in binding, suggesting an additional ubiquitin binding site to the UBA domain (also under investigation).

4. The authors identified a 25 aa sequence, called Plug loop, preceding the HECT domain. In the structure it is inserted between N and C-lobe subdomains of the HECT and appears to lock the enzyme in an open L-conformation. These structural findings are interesting, but no supported by experimental data. Which is the effect of the Plug loop deletion in a ubiquitination assay? Without further validation the last chapter of the results remains purely speculative and may better fit in the discussion.
We have already designed and are currently in the process of expressing mutants of the plug loop. We aim to characterise these mutants using cryo-EM and activity assays.

5. The datasets are clearly affected by preferential orientation as showed by the angular distribution and 2D classes (reason why the authors correctly performed data collection with tilt). A comment on this is required in the experimental section. In addition, it is not clear whether the presented maps (Fig 1 and 2) derive from merging of the two datasets or only the model has been built using the two different datasets.
We will provide further clarification in the text as well as in an additional figure describing the processing pipeline and model building, which has also been suggested in a below comment.

6. As a general comment, authors should enlarge panels in which structural details are described, highlighting the side chain residues involved in binding interfaces. Fig. 5 and Fig. 6 are particularly small and incomplete. Most of the structural figures miss key labels needed for a proper understanding. E.g. among the others, numbering of the helix composing the armadillo domain.
We will make sure all panels are of the appropriate size, and all labels are added to make sure our figures are clear and readable. Additionally, we will number the helix composition of the armadillo domain.

• The overall organization of the figures is quite confusing. Pag. 7 Figure 2C should represent a "box stabilized by three zinc ions mediated by two histidine and seven cysteine residues" according to text citation, but none of these details is highlighted in the corresponding figure. The eye in Figure 1,2,4 does not mean much if a proper box is not linked to the actual site to be seen. In addition, arrows indicating the rotation axis is hard to interpret. Few panels miss the legend. Figure 1A and many other panels miss the reference in the text. More details below.
We will improve our labelling and figure organisation to make things clearer for the reader. We will revise the manuscript to ensure the proper flow of the text and the corresponding figures.

Additional points:

• Mass Photometry data need additional comments and labels. Please comment on the MP concentration used to analyze the samples. Being a dynamic system, you are probably seeing an equilibrium of species at 10 nM in MP. For better completeness of MP figures, labels that includes counts, % of species and sigma should be added to the nice representation of oligomers. Which condition/fraction represent the MP data showed in 1B?
We will clarify all details on OneMP measurements in the methods section. Further, we will provide raw images of measurements which include the required labels mentioned by the reviewer. We measure the samples at 100nM final concentration. Below is the image of the measurement shown in Fig1B with all labels. We will add labels to all our measurements as supplemental figure.

Revision Plan

• If Alphafold models are mentioned and used for model building, it would be nice to provide at least a pLDDTscore and ptm score. Since some details of the AF model are described in the text, an additional superposition of the AF model with the final model derived by EM would be useful to the community.
We will add the AlphaFold model of the full-length UBR5 fitted in our cryoEM map in Supplemental Figure 2, as shown below. We will further comment in the text that AlphaFold has never predicted UBR5 dimers or other oligomers.

• A simple workflow describing the cryoEM data processing that includes how many particles have been used in each step is required, at least in the methods section. The authors need to show the cryoEM 2D classes of the dimer as well.
We will prepare a figure showing the full processing pipeline, as mentioned in Point 5 and show 2D classes of the dimer in the revised manuscript.

• Please add the domain boundaries in Figure 1A and highlight the domains on the alignment included in Supplemental Table 1.
We will fill in this missing information.

• Pag. 8 please decide which abbreviation to use, either UBR or Ubr.
We used both as they represent either human (UBR) or yeast (Ubr) proteins. We will clarify this in the text, making sure abbreviations are consistent and clearly show which species we refer to.

• Page 8, line 192. I found annoying to find the same sentence used by competitors who posted a bioRxiv paper 3 days before the one we are reviewing (doi.org/10.1101/2022.10.31.514604 page 4, line 135).
We apologise that the reviewer perceived our phrasing on the positioning of the MLLE domain in the HECT N-lobe as a potential copy from a competing manuscript published in bioRxiv. This finding was unexpected mainly due to a disagreement with previous reports (such as the citation provided). We, as well as our competitors, used phrasing commonly seen in literature to contradict these previous findings. Nevertheless, we rephrased the sentence to avoid any coincidental overlap to:
“An uncommon characteristic of the UBR5 HECT domain is the location of the MLLE motif in the N-lobe and not before the HECT domain, which disagrees with previous annotations.”

Revision Plan

• In supp. 1C legend, "high concentration of NaCl" is a bit vague
We will clarify the use of “high salt” to be 1M NaCl.

• Complementary to Supp Fig 2A, a zoom in of the density map with traced model would be beneficial to show the actual map quality obtained.
We will introduce a density map with the traced model into Supplemental Fig2A.

• Pag. 6 lines 133-134, the helix residues involved in homodimerization are cited in the text, but not highlighted in the Figure 1.
We will highlight all residues mentioned in the text in the corresponding figure.

• Figure 1 legend, panels H-I-J description are missing.
We will add the respective figure legends in the revised manuscript.

• Figure 3, panel B, meaning of the asterisk is not reported in the figure legend.
We will add the meaning of the asterisk to the legend in the revised manuscript.

• Figure 4, 5 panels from A to E are cited in the text while figure reported only 4.
We will revise the manuscript to ensure the proper numbering of figure panels together with the text.

Referees cross-commenting

I think all the reviews are fairly consistent and agree with the comments raised by my colleagues with the one exception of Point 3 of Reviewer 1. The issue is certainly important yet the experiment suggested is not clear. I personally have troubles designing an informative experimental set-up.
We agree with reviewer 2 regarding Point 3 of reviewer 1. Currently, we lack an informative set-up to test such a strikingly complex concept and believe it is beyond the scope of the current manuscript.

Reviewer #2 (Significance (Required)):

This paper presents the intriguing Cryo-EM structure of the full-length HECT E3 ligase UBR5.
As it stands, this work provides evidence of the existence of a tetrameric RING-like conformation that could represent the functional unit of the catalysis. Very little validation of the features identified in the Cryo-EM structure is given, thus the paper remains quite descriptive, but in any case interesting and informative for the ubiquitin field.

Revision Plan

Considering that UBR5 is a quite competitive subject in these days (e.g. at least one additional Cryo-EM structure was posted in BioRxiv, doi.org/10.1101/2022.10.31.514604), I would positively consider this manuscript for publication if the authors reply in full to the issues raised.
My field of expertise: Ubiquitin regulation and interactions, biochemistry, biophysics and Cryo-EM.

We again thank the Reviewer for their insightful comments and suggestions!

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

The manuscript presented the Cryo-EM structure of HECT E3 UBR5. Using Alphafold2 model of UBR5, the authors were able to dock and refine the structure model of full length UBR5. Interestingly, UBR5 exists as a homodimer and could potentially assemble into a larger oligomer based on SEC and Cryo-EM data. The antiparallel arrangement of the homodimer suggests that the C-terminal HECT domain could transfer ubiquitin in trans or in cis configuration. The tetrameric model reveals a ring-like structure with a large central cavity, presumably to accommodate large proteins/complexes. Using AKIRIN2 as a substrate, the authors demonstrated that UBR5 did not ubiquitinate AKIRIN2, but prefers ubiquitin modified AKIRIN2 as the substrate for ubiquitin chain elongation. Indeed, they observed that UBR5 preferentially ubiquitinates pre-ubiquitinated non-cognate substrate and free ubiquitin hinting that UBR5 is a chain elongating E3. Lastly they showed that UBR5 HECT contains a plug-loop that blocks C-lobe rotation and suggested that conformational change is necessary for ubiquitin transfer.

Comments:
1. Homodimerization and oligomerization are the novel aspect of this study but the manuscript lacks validation of the structure. The authors should provide biochemical/mutagenesis analysis to support the dimerization interface observed in the structure. Also the model showed that SBB2 is involved in the tetramerization interface, could the authors verify this by designing a SBB2 deletion mutant?
We have already designed a variant aimed at disrupting the tetramerisation interface (i.e. the SBB2 domain) and are currently in the process of purifying them. We will characterise this mutant using MP and cryo-EM, as suggested by the reviewer.

2. Would be useful to show the docking of Alphafold2 model onto the Cryo-EM map prior to further model building and refinement in the supplementary data.
We will add the AlphaFold model of the full-length UBR5 fitted in our cryoEM map in Supplemental Figure 2, as shown below.

Revision Plan

3. Please show the SDS-PAGE of purified UBR5 used for Cryo-EM study.
We will introduce the following gel of purified UBR5 in Supplemental figure 1.

4. Figure referencing is not in order. For example Figure 1J was described before Figure 1I. Figure 2A,B mentioned after Figure 2C. Also some Figures are not properly referenced in the main text, e.g. p10 when describing ubiquitin chain formation of UbAKIRIN2 and UbSecurin. Please check throughout the manuscript. We will revise the manuscript to ensure the proper flow of the text and the corresponding figures. We will add/remove all references to figures which are incorrect.

5. Figure legends are missing for Figure 1H-1J
We will add the respective figure legends in the revised manuscript.

6. It was stated in p10 that there was no binding between UBR5 and UbSecurin in Figure S3C, but Figure S3C showed faint FAM-UbSecurin across the fractions. It would be useful to repeat this with FAM-UbSecurin alone to ensure the faint bands are background signal.
As requested, we will repeat and implement the gradients and respective controls in figures to ensure an accurate representation of the binding. We will also quantify the banding patterns to determine the level of background binding compared to control gradients.

7. In p10, it was stated that UBR5 and UbAKIRIN2 interaction was enhanced in the presence of ubiquitin. How did the authors come to this observation? The sucrose gradients (Figure 3B) showed that UBR5 co-elutes with both AK2 and UbAK2. This reviewer is unclear whether the intensity of the bands can be used to evaluate the strength of the binding affinity. Was the experiment performed at the same protein component concentration/condition? The UBR5 appears to elute at different fractions from the two experiments.

We will clarify in the text and methods that experiments were performed with the same protein concentrations and same gradient set-up and were performed side-by-side using the same centrifugation settings. We can improve the figure by quantifying the shift of AKIRIN2's position in the gradient.

8. The authors suggested that the UBA domain might bind ubiquitin and promote ubiquitination of UbAKIRIN2. It is noteworthy that prior studies on several HECT E3s showed that HECT domain alone can catalyze free ubiquitin chain assembly. Could it be possible that the HECT domain of UBR5 alone could catalyze the extension of UbAKIRIN2, UbSecurin or UbdeltaGG?
We will compare the activity of full-length UBR5 and an isolated UBR5 HECT domain. Based on the preliminary UBA domain deletion mutant, we expect a decreased rate of ubiquitination.

9. In Figures 3A and S3B, does the ubiquitin chain elongation occur only on the fused ubiquitin?
The majority of ubiquitination occurs on the fused ubiquitin. We will attach the full mass spec data and represent the mass spec data in the form of a simple bar chart in Supplemental Figure 3, together with the data requested in Point8 by reviewer 1.

10. Figure 4E mentioned in the main text but is missing.
We will amend all figures and their respective references in text which are incorrect.

11. It is not clear from Figure 4 whether the plug-loop is blocking the rotation of C-lobe. The overlaid Figure 4 is quite busy, would be useful to show the UBR5 HECT domain alone with other HECT domain presented in the same orientation but in separate panels. With the plug-loop in the current configuration, does it block E2 binding and transthiolation reaction? Figure 5B seemingly suggested that plug-loop is blocking transthiolation but it is hard to visualize. The authors could enlarge Figure 5B and color the plug-loop differently.
We will amend figures 4 and 5B to clarify our hypothesis and make sure all panels are of the appropriate size and colour to make the figures clear and readable.

Reviewer #3 (Significance (Required)):

The manuscript provides the structural insight into the organization of UBR5. While the Cryo-EM data largely agrees with the Alphafold2 UBR5 model, this should not take away the significant effort in obtaining the large UBR5 protein structure. The structure reveals an unexpected homodimerization of UBR5 and in the assembly of larger oligomer. The biochemical analyses suggest that UBR5 is proficient in ubiquitin chain elongation. Overall the study provides a structural framework for understanding how UBR5 could function as an ubiquitin ligase. The findings will be of interest to scientist in the ubiquitin field and in understanding UBR5 biology.

Limitation: aside from the structure, the study lacks detailed mechanisms of how UBR5 catalyzes ubiquitin transfer. While few models for ubiquitin transfer were proposed, the study lacks suitable substrates to investigate the mechanism. The study showed that UBR5 can elongate ubiquitin chain, but it is not clear whether UBR5 could transfer ubiquitin to substrate.
We propose that UBR5 is an efficient chain elongator. Aside from the AKIRIN2 binding and ubiquitination experiments, we performed IP-MS experiments to identify substrates for chain initiation and found several interactors, such as the DSIF complex. However, the DSIF complex was not ubiquitinated by UBR5 (see below). While we cannot exclude the possibility that our recombinant systems lack certain factors or post-translational modifications necessary for efficient ubiquitination of substrates *in vivo*, which we will be mentioned as a limitation of our study in the text, UBR5 acts as a potent chain elongator.

Revision Plan

3. Description of the revisions that have already been incorporated in the transferred manuscript

Please insert a point-by-point reply describing the revisions that were already carried out and included in the transferred manuscript. If no revisions have been carried out yet, please leave this section empty.

4. Description of analyses that authors prefer not to carry out

Please include a point-by-point response explaining why some of the requested data or additional analyses might not be necessary or cannot be provided within the scope of a revision. This can be due to time or resource limitations or in case of disagreement about the necessity of such additional data given the scope of the study. Please leave empty if not applicable.

We notice that reviewer 1 and 2 have differing opinions on ways to address Point 3 where reviewer 2 states an informative experimental set-up to answer the issue (cis/trans mechanisms of ubiquitination) is difficult to design. The experiments required to answer this point in full might fall beyond the scope of the current study.

We would therefore propose to move the text and figure related to Point 3 of reviewer 1 (discussing “oligomeric arrangements of Ubr5 and substrate modification in trans and cis”) to our Discussion section.

Dr. David Haselbach
IMP - Research Institute of Molecular Pathology
Campus-Vienna-Biocenter 1
Vienna 1030
Austria

21st Dec 2022

Re: EMBOJ-2022-113348
Cryo-EM structure of the chain-elongating E3 ligase UBR5

Dear David and Nick,

Thank you for transferring your manuscript from Review Commons to The EMBO Journal. I have now had the chance to read your study, as well as to go through the referee reports and your responses to them. Given the interest of the subject and the overall encouraging referee feedback, we would be happy to consider an adequately revised version further for EMBO Journal publication. In this respect, it appears that the main concern echoed by all three reviewers was clearly that the structural interpretation and models still lack sufficient experimental validation and remain to some extent speculative. This would therefore be the key issue to address during revision, and I realize that your revision plan does appear promising for eventually satisfying the reviewers. I would therefore invite you to revise the manuscript along the lines suggested in your response letter.

When preparing a revised manuscript, please try to adhere to the guidelines listed below and in our Guide to Authors as closely as possible, as this should greatly facilitate our assessment at the time of resubmission - in particular regarding the completion of our author checklist, the inclusion of editable text files and individual figures, the formatting references and the conversion of "supplemental" material into Expanded View and/or Appendix content. Please also note that it is our policy to allow only a single round of (major) revision, making it important to comprehensively answer all criticisms at this point - if this should require more time than our standard three-months deadline, I would be happy to discuss an extension of the revision time, during which our 'scooping protection' (meaning that competing work appearing elsewhere in the meantime will not affect our considerations of your study) would of course remain valid. Please do not hesitate to contact me should you have any further questions at this stage.

Thank you again for the opportunity to consider this study for The EMBO Journal. I look forward to receiving your revision.

With kind regards,

Hartmut

Hartmut Vodermaier, PhD
Senior Editor, The EMBO Journal
h.vodermaier@embojournal.org

*** PLEASE NOTE: All revised manuscript are subject to initial checks for completeness and adherence to our formatting guidelines. Revisions may be returned to the authors and delayed in their editorial re-evaluation if they fail to comply to the following requirements (see also our Guide to Authors for further information):

1) Every manuscript requires a Data Availability section (even if only stating that no deposited datasets are included). Primary datasets or computer code produced in the current study have to be deposited in appropriate public repositories prior to resubmission, and reviewer access details provided in case that public access is not yet allowed. Further information: embopress.org/page/journal/14602075/authorguide#dataavailability

2) Each figure legend must specify
- size of the scale bars that are mandatory for all micrograph panels
- the statistical test used to generate error bars and P-values
- the type error bars (e.g., S.E.M., S.D.)
- the number (n) and nature (biological or technical replicate) of independent experiments underlying each data point
- Figures may not include error bars for experiments with n<3; scatter plots showing individual data points should be used instead.

3) Revised manuscript text (including main tables, and figure legends for main and EV figures) has to be submitted as editable

text file (e.g., .docx format). We encourage highlighting of changes (e.g., via text color) for the referees' reference.

4) Each main and each Expanded View (EV) figure should be uploaded as individual production-quality files (preferably in .eps, .tif, .jpg formats). For suggestions on figure preparation/layout, please refer to our Figure Preparation Guidelines: http://bit.ly/EMBOPressFigurePreparationGuideline

5) Point-by-point response letters should include the original referee comments in full together with your detailed responses to them (and to specific editor requests if applicable), and also be uploaded as editable (e.g., .docx) text files.

6) Please complete our Author Checklist, and make sure that information entered into the checklist is also reflected in the manuscript; the checklist will be available to readers as part of the Review Process File. A download link is found at the top of our Guide to Authors: embopress.org/page/journal/14602075/authorguide

7) All authors listed as (co-)corresponding need to deposit, in their respective author profiles in our submission system, a unique ORCiD identifier linked to their name. Please see our Guide to Authors for detailed instructions.

8) Please note that supplementary information at EMBO Press has been superseded by the 'Expanded View' for inclusion of additional figures, tables, movies or datasets; with up to five EV Figures being typeset and directly accessible in the HTML version of the article. For details and guidance, please refer to:
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In the interest of ensuring the conceptual advance provided by the work, we recommend submitting a revision within 3 months (21st Mar 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

Link Not Available

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Rev_Com_number: RC-2022-01747
New_manu_number: EMBOJ-2022-113348
Corr_author: Haselbach
Title: Cryo-EM structure of the chain-elongating E3 ligase UBR5

Revision Plan

Review COMMONS

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Manuscript number: RC-202201747
Corresponding author(s): Zuzana Hodakova, Nicholas G. Brown, David Haselbach

[The "revision plan" should delineate the revisions that authors intend to carry out in response to the points raised by the referees. It also provides the authors with the opportunity to explain their view of the paper and of the referee reports.

The document is important for the editors of affiliate journals when they make a first decision on the transferred manuscript. It will also be useful to readers of the reprint and help them to obtain a balanced view of the paper.

If you wish to submit a full revision, please use our "Full Revision" template. ***It is important to use the appropriate template to clearly inform the editors of your intentions.****]*

1. General Statements [optional]

This section is optional. Insert here any general statements you wish to make about the goal of the study or about the reviews.

Please see the attached cover letter.

We use colour-coding throughout this Revision plan document. We colour-code our response to the reviewers' comments in orange, and represent copy-pasted sections of the manuscript using double quotation marks and text in blue.

2. Description of the planned revisions

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

This manuscript describes cryo EM structural analyses of human E3 ligase Ubr5 in dimeric and tetrameric states. Ubr5 belongs to structurally poorly characterized family of Hect E3 ligases and has important biological functions, e.g. in targeting transcription factors for proteasomal degradation. The manuscript therefore addresses an important subject of basic science and biomedical interest. Using in vitro ubiquitylation assays the authors show that purified Ubr5 forms ubiquitin chains on a substrate (akirin2), but also on a non-substrate (securin), provided the proteins are covalently fused to ubiquitin. The authors interpret this as a preference of Ubr5 for ubiquitin chain elongation over initiation. Consistently they show that Ubr5 forms free ubiquitin chains linked by Lys48 in vitro.
Whilst the structures of full-length Ubr5 are very interesting and important, this manuscript appears to be at a premature stage. The structural interpretation and models lack experimental

validation and remain speculative. The presented activity assays are interesting but do not quite link up with the structural part which leaves the manuscript somewhat disconnected. In my view this manuscript requires considerably more work to correlate structure with function, as suggested below, but holds the potential of turning into a highly insightful story.

We thank the Reviewer for their very helpful comments. We have implemented the suggestions and edits in the revised manuscript, as outlined below:

Conceptual comments:

1) Key observation is that a Ubr5 dimer assembles into higher-order oligomers. The authors speculate that this is functionally relevant, e.g. by the possibility of substrate ubiquitylation occurring within the central cavity of the ring shaped tetramer or ubiquitylation in cis and trans. However, neither significance of Ubr5 oligomerisation nor dynamics/determinants in solution is investigated.

We have expressed UBR5 mutants to disrupt both dimerisation and tetramerisation/higher oligomerisation. In brief, we confirm the role of the dimerisation helix with two mutants of this interface, using mass photometry, in lines 156-166:

"To confirm the role of the helix in UBR5 dimerisation, we expressed mutants aimed at disrupting this interface using insect cell expression system (represented as (i) with each construct). We first characterised and compared full-length proteins ($UBR5^{wt}$ and $UBR5^{wt(i)}$) using SEC, ThermoFluor and MP to confirm their oligomerisation properties and stability (Appendix Fig S1B,C, Fig EV1D). We subsequently purified and characterised two mutants; a deletion of the entire dimerisation helix (residues 1912-1931), $UBR5^{\Delta DH(i)}$ and a UBR5 mutant with three charge-reversing point mutations of the key residues of this interface, $UBR5^{DHmutant(i)}$;1914 (R>D),1926 (R>D) and 1931 (E>R). Both mutants displayed a change in their oligomeric properties, with monomers becoming the predominant species (Fig 2C)."

-We also show that the SBB2 domains of UBR5 mediate contacts between two dimers in a tetrameric assembly by generating an SBB2-deletion mutant, and characterise it using mass photometry and cryo-EM, in lines 183-197:

"SBB domains were often found to facilitate oligomerisation via β strand hydrogen bonding (Youkharibache et al., 2019). To identify if these motifs form the tetramerisation interface of UBR5, we performed extended 3D classification of the tetramer to obtain a more rigid density. We could not assign the atomic coordinates of these domains due to low resolution, but we confidently docked in two UBR5 dimers. This revealed that this assembly is indeed facilitated by SBB2 domains of two opposite dimers (Fig 2D). We confirmed the role of SBB2 domains in UBR5 tetramerisation by expressing a UBR5 mutant with an SBB2 domain deletion ($UBR5^{\Delta SBB2}$) and analysing its oligomeric state using MP. This revealed an almost exclusively dimeric assembly of UBR5 (Fig 2E). We observed a small peak with a molecular weight of a tetramer. This peak is of considerably lower abundance than the dimeric peak, but it cannot be excluded that UBR5 dimers display weak binding properties to each other even in the absence of SBB domains. We further subjected $UBR5^{\Delta SBB2}$ to cryo-EM analysis. 2D classification of this mutant revealed an exclusive dimeric assembly, further confirming the role of SBB2 domains in contacts between two dimers (Fig 2F)."

- In line 100, the authors state that individual oligomeric species could not be separated but do not show data. Why can the species not be separated? Do they exchange? Can exchange be controlled by ionic strength/pH/temperature...?

We expanded our characterisation of UBR5 purification and mass photometry measurements. We further clarified measurements previously poorly explained in Supplemental material of the manuscript (MP measurements of UBR5 sample at 1M salt) and expanded our characterisation of UBR5 oligomerisation at different conditions, in lines 118-123:

Revision Plan

Review COMMONS

"We found a range of oligomers was formed in a repeating order of two. Monomeric UBR5 was not detected (Fig 1A,B, Fig EV1B). The oligomers remained stable across a range of buffer conditions and possibly adopt higher oligomers with increased temperature (Fig EV1C). These results suggested that UBR5 forms stable dimers as the building block and functional unit of all oligomers."

The authors also suggest that the tetramer is transient (e.g. line 165). What is the evidence for this? Subunit exchange may be tested by mixing different species, e.g, containing labels/tags etc.
We tried to address the transiency of the oligomers. We re-applied our UBR5 samples of peak1 of size exclusion chromatography columns but did not observe a change in oligomeric states. We observe changes in oligomers upon heating the sample (Figure EV1), suggesting there might be subunit exchange, but we currently lack an experimental set up to fully test this concept.

- The authors should design structure-based mutations, particularly within the small, tetrameric interface, and measure oligomerisation state of the mutants to correlate their cryo EM analyses with oligomeric states observed in solution.
We have expressed an UBR5-delSBB2 mutant and characterised its altered oligomeric state using mass photometry. We further visualised the now dimeric assembly using cryo-EM 2D. These results are now implemented in Figure2 and lines lines 183-197, as mentioned in point1.

- The authors should also subject individual oligomers (if required, by mutational stabilization of particular states) in activity assays to test their hypotheses.
We performed activity assays with mutants of the dimerisation and tetramerisation interfaces. Activity assays are now implemented in Figure 3 and the respective Extended view figure, Figure EV3.

2) Lines 160-163: "We performed extended 3D classification of the tetramer, which allowed us to confidently dock two models of UBR5 dimers. This revealed that the tetrameric assembly of UBR5 is formed by SBB2 domains of two opposite dimers (Figure 1I)." The idea that the SBB2 domains make up tetrameric interface should be experimentally validated.
We have expressed the UBR5-delSBB2 mutant and characterised its altered oligomeric state using mass photometry. We further visualised the now dimeric assembly using cryo-EM 2D. These results are now implemented in Figure2 and lines 183-197, as mentioned in point1.

3) Lines 311 onward: The authors speculate that oligomeric arrangements of Ubr5 allow for substrate modification in trans and cis, expanding the substrate repertoire. As part of results section, this should be experimentally addressed. Depending on the exchange behaviour of subunits within oligomers, it may be possible to use mixing experiments. Alternatively, they authors may consider comparing Ubr5 ubiquitylation efficiency towards substrates of different sizes, which may allow for better interpretation of the distance restraints they defined.
We have addressed our hypothesis using three substrates described in this study. Additionally, we have tested full-length and oligomerisation-impaired mutants in activity assays but have not observed a decrease in activity. We therefore cannot precisely determine the function of oligomerisation.

It should be noted that reviewers 1 and 2 have differing opinions on ways to address this point, where reviewer 2 states an informative experimental set-up to answer this issue is difficult to design. As we have not observed significant impact of mutations to the oligomerisation interface, we discussed the oligomeric arrangement in the discussion section in a more hypothetical manner.

4) Figure 1J suggests that MLLE domain was modelled, yet the authors note in the text (line 190) that this domain is disordered.

We describe the MLLE domain with greater care in lines 245-249, explaining which regions are resolved and which remain unresolved.

"AlphaFold2 predicted UBR5 structure suggested this domain is an insertion in the HECT domain, separated with 40-amino acid long unstructured linkers on each side. Whilst we cannot resolve this domain due to its flexible nature, we could partially build the linkers which connect MLLE to the HECT domain, showing they are an extension of the N-lobe of the HECT domain (Fig EV2A-iv)."

Additionally, we highlight which domains are unresolved using gray boxes in the sequence schematic in Figure1H, and represent the hypothetical positions of unresolved domains, such as MLLE, in EV2 instead of a main Figure.

5) The interpretation of the in vitro experiments comparing ubiquitylation of ubiquitin fused akirin2 (substrate) and ubiquitin fused securin (non-substrate) require a re-evaluation: The fact that even ubiquitin fused securin is efficiently ubiquitylated by Ubr5 in vitro shows that a fused ubiquitin molecule is sufficient to recruit a protein for modification by Ubr5 (likely via the UBA domain) in vitro. The specificity of Ubr5 for certain substrates in the cell must therefore follow different (unknown) mechanisms. It is possible that observed, additional affinity between Ubr5 and akirin2 (which is independent of ubiquitin) contributes to this. The available data, however, are insufficient to suggest a hierarchy of interactions (suggested in line 235-237). The interpretation should also be adapted in Figure 6 in which an order of binding events is postulated.

We clarified the interpretation of our experiments both in the results and discussion sections. Overall, we distinguish between binding and chain-elongating functions of UBR5 better. We clarify that any primed substrate serves as a good substrate for chain elongation, and that monoubiquitination may not be enough for strong binding, but may require multivalent binding.

Furthermore, we aimed to disrupt the multivalent interactions of UbAKIRIN2 to UBR5 to gain a deeper understanding of the interaction. We unexpectedly observed that UBR5 potentially possesses another ubiquitin binding site other than the UBA domain, as UBA-deletion mutant was still able to bind and ubiquitinate UbAKIRIN2. We have not yet identified a binding site of AKIRIN2 to UBR5.

Taken together, we expand our discussion on the role of these interactions between UBR5 and AKIRIN2, and UBR5 and ubiquitinated substrates in discussion lines 458 – 460:

"Whilst the interaction of UBR5 with AKIRIN2 may also serve a more specialised function, an avidity binding mechanism could be a means of selection preference of certain substrates"

6) Fluorescently labelled deltaGG-ubiquitin is used to monitor free chain formation by Ubr5. Why is this setup used rather than a simple assay with full-length ubiquitin? The rationale/benefit should be clarified.

Revision Plan

We moved this experiment to Extended View figure as it represented the same finding as our UbAKIRIN2 fusion-containing activity assay in Figure 3. We explained the use of Ubiquitin deltaGG

"To further confirm the chain-elongating properties of UBR5 in the absence of substrate, we tested a Cy5-labelled ubiquitin with a deletion of the two C-terminal glycine residues (^{Cy5}UbΔGG). We use this mutant ubiquitin to obtain clear assay read-outs, as it can only serve as a ubiquitin acceptor." Lines 282-286

and additionally introduced a more informative bottom panel to our activity assays, which monitors ubiquitin pool depletion, with the aim to monitor UBR5 activity even if it remains inactive on a substrate.

7) Conformation/functional significance of the plug loop should be validated by mutagenesis.
We purified UBR5 with a deletion of the plug loop and assessed its activity in ubiquitination assays. We observe a similar rate of chain elongation, and using ThermoFluor we observe that this feature stabilises the protein and thus may aid in protein folding. Results of our experiments are implemented in Figure 4, ThermoFluor measurements in Figure EV1.
We additionally tried to characterise this mutant using cryo-EM, but could not reach consensus 2D classes, possibly due to destabilisation of the protein.

8) The mass spec data should be presented in a compact supplementary figure or table, in addition to comprehensive data table in Suppl. Table 2.
We introduced a compact representation of chain type formation in Figure EV3H.

7) Statements without data backup should be phrased as hypotheses or experimentally validated, e.g.,
- line 27:"Using cryo-EM processing tools, we observe the dynamic nature of the domain movements of UBR5, which allows the catalytic HECT domain to reach engaged substrates."
We rephrased to : "Using cryo-EM processing tools, we observe the dynamic nature of the UBR5 catalytic domain, which we postulate is important for its enzymatic activity" Lines 27-29.

-line 31:"This preference for ubiquitinated substrates permits UBR5 to function in several different signalling pathways and cancers".
We rephrased to: "This preference for ubiquitinated substrates and several distinct domains for protein-protein interactions may explain how UBR5 is linked to several different signalling pathways and cancers" Lines 30-33.

-line 78: "This striking feature allows the positioning of substrate binding sites in close proximity to the catalytic HECT domain in cis or trans, expanding its substrate-recruiting capacities."
We removed this sentence from the text and instead address the *cis* and *trans* mechanisms as a hypothesis in the discussion section.

Methods section:
- The authors should provide information on how Ubr5 sequence was optimized (line 477).

Revision Plan

We clarify this in line 484-486: "The codon-optimised sequence of full-length human UBR5 was purchased from Twist Bioscience as DNA fragments and cloned into pMEPI plasmid"

- The authors should explain why different versions of Ubr5 were used for cryo-EM and activity assays.

We clarified the use of two constructs in the results sections, (lines 112-115)"

"We expressed two full-length UBR5 constructs in HEK293T cells; an N- and C-terminally tagged construct, hereafter referred to as UBR5, and UBR5 with a native C terminus, hereafter referred to as UBR5^{wt}, to avoid potential changes to UBR5's activity by modifying its C terminus (Kim & Huibregtse, 2009).

We describe their alike behaviour on SEC and mass photometry in Fig EV1.

We further explained the construct sequence in the respective Methods section.

- Given the importance of oligomerisation, the authors should explain which fraction of the gel filtration was used for activity assays. Depending on the reversibility of oligomerisation and if oligomerisation impacts activity, the authors should also specify what concentrations these fractions had and/or which concentration they were concentrated to.

We clarify the use of UBR5 sample in the Methods section, lines 624-625:

"HisUBA1 at 0.3 µM, UBCH5b at 2.5 µM, UBR5^{wt} at 0.2µM or 0.3 µM (as indicated in figure captions) , wtUbiquitin at 20 µM, the substrate at 2 µM. " and all relevant captions. Additionally, we characterised the impact of olimerisation-defective mutants on ubiquitination activity, as shown in Figure 3, which show that the effect is minimal.

- The authors should specify how and at which position cysteine was introduced for labelling of the deltaGG-ubiquitin version.

We expanded the description of UbdeltaGG construct in our methods section: "Ubiquitin with a deletion of the two C-terminal residues, GG (Ub$^{\Delta GG}$), was cloned into a pGEX-TEV vector, preceded by a single cysteine for labelling, a TEV cleavage site and a GST tag (GST-TEV-Cys-Ub$^{\Delta GG}$)", Lines 515-517

- The authors should specify which concentrations mass photometry measurements were performed at.

We now specify in the respective methods section: "Each measurement was performed at a final protein concentration of 100nM." line 693-694.

- A description of mass spectrometry measurements is missing.

We added the respective methods section, lines 703-715

Results/figures etc:

- Lines 88-91 require more precision: "...highly conserved" - compared to what? Some quantification would help here. "...the length is an average across all species". What is meant by "all species" (all species shown, all metazoans?)?

We made text edits with respect to the conservation of UBR5. We now refer to these alignments throughout the manuscript where relevant, for example Lines 153-155, describing the conservation of residues forming the dimerisation interface.

"Residues forming this interaction are highly conserved across multiple species, ranging from C.*elegans* to humans species (Appendix Fig S3-Dark green stars)."

Revision Plan

We additionally annotated UBR5 domain boundaries in the sequence alignment with respect to the mentions in the text.

We show sequence alignments of 6 representative species in Appendix Figure 3 and explain in the figure title “representative subset of species”. We further explain the generation of these alignments in the respective methods section, lines 648-654

- Figure 1B shows appears to also show a monomer peak. The authors should label it and comment on it.

We introduced a table in Figure1, Figure 1A, which lists the molecular weights expected for each oligomeric state for a better understanding of the measured species by the readers. We clarify the observed oligomers in lines 118-120:

“We found a range of oligomers was formed in a repeating order of two. Monomeric UBR5 was not detected (Fig 1A,B, Fig EV1B).”

and annotate the low molecular weight peaks in respective figure captions, such as in Figure 1:

“Contaminant peaks and shoulders are annotated with “C”. Full mass measurements are listed in Appendix Table S1”

Additionally, we list measured molecular weights of all peaks from all mass photometry measurements in this manuscript in Appendix Table3.

Can the authors comment on the right shoulder of tetramer peak which was not fitted?

Please see above

- Figure Legends 1H, 1I, and 1J are missing

We added all missing figure legends to the revised manuscript.

- In Figures 1G, 2A and 2B, colour labelling positive/negative is swapped.

We amended the colour bar to the correct labelling.

- Line 215: "... have observed the formation of free chains". Here, a figure/figure reference is needed.

To depict that the enzyme is active but does not ubiquitinate the added substrate, we introduced panels in all our activity assay figures, which show free ubiquitin pool depletion. We also compare activity assays with and without an E3 present in Figure EV3G. We rephrased the text accordingly “substrates were not ubiquitinated by UBR5, despite pool of free ubiquitin being depleted “ Line 272-273

- Figure 5 C, D, Figure 6: The way models are drawn is very difficult to understand. Maybe the authors could find clearer way to illustrate their hypotheses?

We simplified the figures and introduced additional supporting elements to illustrate our hypotheses in a clearer way.

Referees cross-commenting

Revision Plan

Reviewer 2 noticed that the manuscript contains a sentence that, in paraphrased form, may have been adopted from a competing manuscript published on Biorxiv some days earlier. According to the conventions of good scientific practice, the competing manuscript should be cited here.

We apologise that the reviewer perceived our phrasing on the positioning of the MLLE domain in the HECT N-lobe as a potential copy from a competing manuscript published in bioRxiv. This finding was unexpected mainly due to a disagreement with previous reports (such as the citation we provided). We, as well as our competitors, used phrasing commonly seen in literature to contradict these previous findings. Nevertheless, we rephrased the sentence to avoid any coincidental overlap, and extended our MLLE domain annotations respectively:
"Contrary to initial annotations (Deo et al., 2001), AlphaFold2 predicted UBR5 structure suggested this domain is an insertion in the HECT domain, separated with 40-amino acid long flexible linkers on each side. Whilst we cannot resolve this domain due to its flexible nature, we could partially build the linkers which connect MLLE to the HECT domain, showing they are an extension of the N-lobe of the HECT domain" Lines 244-249

Reviewer #1 (Significance (Required)):

see above

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

This manuscript reported the CryoEM ring-like structure of the full-length human E3 assembly ligase UBR5, showing its assembly into a tetramer. The authors identified critical determinants for antiparallel homodimer and tetrameric assembly. They further described AKIRIN2 as UBR5 substrate and provided evidences of a preferential interaction and activity of UBR5 towards monoubiquitinated proteins. Based on these findings, they proposed UBR5 as chain-elongating E3 ligase.
CryoEM data are solid, and the model interpretation of the tetrameric structure provides a precise description of the domain composition of the protein that well fit with biochemical data. Additional experiments are suggested to corroborate few statements of the authors.
We believe they are realistic in terms of time and resource.
We thank the reviewer for the thoughtful and very constructive comments.

1. Authors should address the importance of tetramerization by mutating SBB2 at the tetramerization interface and comparing the mutant with wild type in mass photometry and ubiquitination assays. In silico analysis of the interaction interfaces (e.g by using PISA software) could be useful to select amino acids to be mutated. The authors suggested a role for oligomerization in catalysis and mutants are needed in order to define the real "functional unit" of the enzyme.
We have expressed UBR5 mutants to disrupt both dimerisation and tetramerisation/higher oligomerisation. In brief, we confirm the role of the dimerisation helix with two mutants of this interface, using mass photometry, in lines 156-166:

"To confirm the role of the helix in UBR5 dimerisation, we expressed mutants aimed at disrupting this interface using insect cell expression system (represented as (i) with each construct). We first characterised and compared full-length proteins ($UBR5^{wt}$ and $UBR5^{wt(i)}$) using SEC, ThermoFluor and MP to confirm their oligomerisation properties and stability (Appendix Fig S1B,C, Fig EV1D). We subsequently purified and characterised two mutants; a deletion of the entire dimerisation helix (residues 1912-1931), $UBR5^{\Delta DH(i)}$ and a UBR5 mutant with three charge-reversing point mutations of the key residues of this interface, $UBR5^{DHmutant(i)}$;1914 (R>D),1926 (R>D) and 1931 (E>R). Both mutants displayed a change in their oligomeric properties, with monomers becoming the predominant species (Fig 2C)."

-We show that SBB2 domains mediate contacts between two dimers in a tetrameric assembly by generating an SBB2-deletion mutant and characterise it using mass photometry and cryo-EM, lines 183-197:

"SBB domains were often found to facilitate oligomerisation via β strand hydrogen bonding (Youkharibache et al., 2019). To identify if these motifs form the tetramerisation interface of UBR5, we performed extended 3D classification of the tetramer to obtain a more rigid density. We could not assign the atomic coordinates of these domains due to low resolution, but we confidently docked in two UBR5 dimers. This revealed that this assembly is indeed facilitated by SBB2 domains of two opposite dimers (Fig 2D). We confirmed the role of SBB2 domains in UBR5 tetramerisation by expressing a UBR5 mutant with an SBB2 domain deletion ($UBR5^{\Delta SBB2}$) and analysing its oligomeric state using MP. This revealed an almost exclusively dimeric assembly of UBR5 (Fig 2E). We observed a small peak with a molecular weight of a tetramer. This peak is of considerably lower abundance than the dimeric peak, but it cannot be excluded that UBR5 dimers display weak binding properties to each other even in the absence of SBB domains. We further subjected $UBR5^{\Delta SBB2}$ to cryo-EM analysis. 2D classification of this mutant revealed an exclusive dimeric assembly, further confirming the role of SBB2 domains in contacts between two dimers (Fig 2F)."

We characterised the activity of all oligomerisation-defective mutants, which are now implemented in Figure 3 and Figure EV3 and respective text, lines 329-337

"The striking revelation that UBR5 assembles into dimers and tetramers prompted the question if these features regulate ubiquitination activity of the enzyme. We therefore subjected all our oligomerisation mutants ($UBR5^{\Delta SBB}$, $UBR5^{\Delta DH}$, $UBR5^{HelixMutant}$) to ubiquitination assays of ^{GFP}UbAKIRIN2 (Fig 3G). We observed a similar rate of ubiquitin depletion and substrate ubiquitination of wild-type and dimerisation-impaired UBR5 mutants. Interestingly, disrupting the tetramerisation interface seemed to subtly enhance the activity of UBR5. Oligomerisation of UBR5 thus does not appear to be a crucial factor for its intrinsic activity of ubiquitin chain elongation."

2. The authors used sucrose gradient sedimentation assay to prove UBR5 and substrate interaction (Fig. 3). Control experiment that showed UBR5 protein sedimentation in presence of GFP only is instead in Supplementary Fig. 3D. Unfortunately, in that panel the signal of UBR5 is not visible. Main figure should be revised showing proper controls of the experiment.

We apologise for the poor quality of the mentioned panel. We have introduced control gradients alongside our gradients showing substrate interactions and made sure all panels have visible signal. These gradients are implemented in Figure 3 and Figure EV3.

3. The authors need to better clarify the features of the AKIRIN-UBR5 interaction. According to the data, the enzyme is equally active on both AKIRIN-Ub and Securin-Ub, suggesting a Ub-specific engagement. What would be a correct explanation of these results? Is the UBA domain directly involved in this process? Testing the activity of a UBA-impaired mutant should help to solve this issue.

We expressed two mutants with a defective UBA domain – a UBA-deletion mutant, UBR5delUBA, and two point mutations in the UBA domain to prevent ubiquitin engagement based on previous studies (Kozlov et al 2007). We show the following:

"sucrose gradient experiments of full-length and $UBR5^{\Delta UBA}$ revealed that the interaction of ^{GFP}UbAKIRIN2 was unaffected (Fig 3F). "Line 309-311

And characterised a defect in processivity of the two mutants in lines 315-325:

"In the early ubiquitination time points, $UBR5^{\Delta UBA}$ displayed a defect in the added ubiquitin chain length (Fig 3E). With longer time points, $UBR5^{\Delta UBA}$ built polyubiquitin chains and depleted the free ubiquitin pool but at a significantly slower rate than $UBR5^{wt}$. To

assess if the defect arises from lower local ubiquitin concentrations, as a result of decreased E2~Ub recruitment, we gradually increased the E2 concentration in our ubiquitination assays to try overcome this defect. However, this increase only partially restored the ubiquitination capabilities of $UBR5^{\Delta UBA}$ compared to wild type (Fig EV3I). We further confirmed the defect stems from its inability to engage with ubiquitin by mutating two key residues of the UBA domain which, based on a previous study, bind ubiquitin grooves ($UBR5^{V196K,L224K}$) (EV2A-i)"

4. The authors identified a 25 aa sequence, called Plug loop, preceding the HECT domain. In the structure it is inserted between N and C-lobe subdomains of the HECT and appears to lock the enzyme in an open L-conformation. These structural findings are interesting, but no supported by experimental data. Which is the effect of the Plug loop deletion in a ubiquitination assay? Without further validation the last chapter of the results remains purely speculative and may better fit in the discussion.

We purified UBR5 with a deletion of the plug loop and assessed its activity in ubiquitination assays. In brief, this mutant retains activity but appears less stable, described in lines 375-383:

To test whether this loop plays a crucial role in UBR5 activity, we generated a deletion mutant of the plug loop ($UBR5^{\Delta PL}$) and subjected it to activity assays. We observed rapid ubiquitin chain formation, showing it is not essential for UBR5 intrinsic activity (Fig 4F). Instead, we noticed $UBR5^{\Delta PL}$ is unstable over time. We analysed the mutant using ThermoFluor, which showed two melting temperatures; an unfolding behaviour suggestive of loss of protein folding co-operativity (Chari et al., 2015) (Fig EV1C). This feature could therefore serve during protein assembly as a folding aid to the HECT domain, with future structural experiments necessary to elucidate its precise role.

5. The datasets are clearly affected by preferential orientation as showed by the angular distribution and 2D classes (reason why the authors correctly performed data collection with tilt). A comment on this is required in the experimental section. In addition, it is not clear whether the presented maps (Fig 1 and 2) derive from merging of the two datasets or only the model has been built using the two different datasets.

We expanded our description of the cryo-EM processing pipeline by introducing an Appendix Figure 2 containing all steps undertaken to generate the maps. We further explain the use of the maps in the respective methods sections, Lines 687-688

6. As a general comment, authors should enlarge panels in which structural details are described, highlighting the side chain residues involved in binding interfaces. Fig. 5 and Fig. 6 are particularly small and incomplete. Most of the structural figures miss key labels needed for a proper understanding. E.g. among the others, numbering of the helix composing the armadillo domain.

We have enlarged and properly labelled all figures, and highlight important structural details with greater care (for example, numbering of the armadillo repeats, now implemented in Figure EV2)

• The overall organization of the figures is quite confusing. Pag. 7 Figure 2C should represent a "box stabilized by three zinc ions mediated by two histidine and seven Ateine residues" according to text citation, but none of these details is highlighted in the corresponding figure. The eye in Figure 1,2,4 does not mean much if a proper box is not linked to the actual site to be seen. In addition, arrows indicating the rotation axis is hard to interpret. Few panels miss the legend. Figure 1A and many other panels miss the reference in the text. More details below.

We corrected our labelling and figure organisation to make things clearer for the reader. We revised our manuscript to ensure the proper flow of the text , figures and their respective citations.

Revision Plan

Additional points:

• Mass Photometry data need additional comments and labels. Please comment on the MP concentration used to analyze the samples. Being a dynamic system, you are probably seeing an equilibrium of species at 10 nM in MP. For better completeness of MP figures, labels that includes counts, % of species and sigma should be added to the nice representation of oligomers. Which condition/fraction represent the MP data showed in 1B?

We have provided the reader with all raw counts, sigma values and molecular weights measured for all mass photometry measurements in the Appendix Table 3. We list the values recorded as a table for clarity reasons – placing all labels into the main figures made the figures hard to read and full.

To simplify the figures for the readers, we introduced a table in Figure1, Figure 1A, which lists the molecular weights expected for each oligomeric state for a better understanding of the measured species shown in our manuscript.

• If Alphafold models are mentioned and used for model building, it would be nice to provide at least a pLDDTscore and ptm score. Since some details of the AF model are described in the text, an additional superposition of the AF model with the final model derived by EM would be useful to the community.

We introduced the AF model of full-length UBR5 fitted in our cryo-EM map, and an overlay of the AlphaFold2-predicted model overlayed with the dimeric UBR5 model obtained in our study. Both are implemented into Appendix Figure S1 and respective text, in lines 12-131:

"Docking in four copies of monomeric UBR5 predicted structure from AlphaFold2 into our cryo-EM map fully occupied the density (Appendix Fig S1A), consistent with our MP measurements. Notably, our structure predictions of UBR5 dimers or tetramers using AlphaFold2 did not predict sensible assemblies (Appendix Figure S1B)."

The pLDDTscore is depicted below (B-factor colouring of the AlphaFol2-predicted model of UBR5) + the same model colour coded from N to C terminus (Blue to red)

Revision Plan

• A simple workflow describing the cryoEM data processing that includes how many particles have been used in each step is required, at least in the methods section. The authors need to show the cryoEM 2D classes of the dimer as well.
We expanded our description of the cryo-EM processing pipeline by introducing an Appendix Figure 2 containing all steps undertaken to generate our final reconstructions, including micrograph and particle counts. We observe dimeric UBR5 in our mass photometry measurements but not in our 2D classes. The dimeric species could be classified together with the tetrameric particles. Furthermore, we would not expect 2D classes based on the relative abundance of the dimer compared to the tetramer.

• Please add the domain boundaries in Figure 1A and highlight the domains on the alignment included in Supplemental Table 1.
We added domain boundaries to the mentioned figure (now Appendix Figure S3). All domains have the identical colour-coding throughout the manuscript.

• Pag. 8 please decide which abbreviation to use, either UBR or Ubr.
We clarify in text we align yeast UBR2 to UBR5, and annotated all UBR proteins and UBR boxes with capital letter abbreviations.

• Page 8, line 192. I found annoying to find the same sentence used by competitors who posted a bioRxiv paper 3 days before the one we are reviewing (doi.org/10.1101/2022.10.31.514604 page 4, line 135).
We apologise that the reviewer perceived our phrasing on the positioning of the MLLE domain in the HECT N-lobe as a potential copy from a competing manuscript published in bioRxiv. This finding was unexpected mainly due to a disagreement with previous reports (such as the citation we provided). We, as well as our competitors, used phrasing commonly seen in literature to contradict these previous findings. Nevertheless, we rephrased the sentence to avoid any coincidental overlap, and extended our MLLE domain annotations respectively:
"Contrary to initial annotations (Deo et al., 2001), AlphaFold2 predicted UBR5 structure suggested this domain is an insertion in the HECT domain, separated with 40-amino acid long flexible linkers on each side. Whilst we cannot resolve this domain due to its flexible nature, we could partially build the linkers which connect MLLE to the HECT domain, showing they are an extension of the N-lobe of the HECT domain" Lines 244-249

• In supp. 1C legend, "high concentration of NaCl" is a bit vague
We clarified the use of "high salt" to be 1M NaCl.

• Complementary to Supp Fig 2A, a zoom in of the density map with traced model would be beneficial to show the actual map quality obtained.
We introduced an enlarged view of the full local resolution estimations of both maps used for model building (Appendix Figure S2).

• Pag. 6 lines 133-134, the helix residues involved in homodimerization are cited in the text, but not highlighted in the Figure 1.

We highlighted all residues and motifs mentioned in the text in the respective figures and figure captions.

• Figure 1 legend, panels H-I-J description are missing.
We revised the manuscript to ensure all figures have their respective legends.

• Figure 3, panel B, meaning of the asterisk is not reported in the figure legend.
We have revised Figure 3 and the elements in the figure. The asterisk represented a contaminant, most likely a degradation product or free GFP from the purification of the GFP-fused recombinant substrates. GFP does not bind UBR5 (shown in EV3) and is not important for this assay. We therefore show only the migration of full-length AKIRIN2. The full gels are submitted together with the manuscript.

• Figure 4, 5 panels from A to E are cited in the text while figure reported only 4.
We revised the manuscript to ensure all figures have their respective legends.

Referees cross-commenting

I think all the reviews are fairly consistent and agree with the comments raised by my colleagues with the one exception of Point 3 of Reviewer 1. The issue is certainly important yet the experiment suggested is not clear. I personally have troubles designing an informative experimental set-up.
We have tested full-length and oligomerisation-impaired mutants in activity assays to gain insights into Point3 of Reviewer 1. As we have not observed a significant change to the enzyme activity, we cannot precisely determine the function of oligomerisation.
We agree with reviewer 2 regarding Point 3 of reviewer 1. Currently, we lack an informative set-up to test such a strikingly complex concept and believe it is beyond the scope of the current manuscript.

Reviewer #2 (Significance (Required)):

This paper presents the intriguing Cryo-EM structure of the full-length HECT E3 ligase UBR5.
As it stands, this work provides evidence of the existence of a tetrameric RING-like conformation that could represent the functional unit of the catalysis. Very little validation of the features identified in the Cryo-EM structure is given, thus the paper remains quite descriptive, but in any case interesting and informative for the ubiquitin field.
Considering that UBR5 is a quite competitive subject in these days (e.g. at least one additional Cryo-EM structure was posted in BioRxiv, doi.org/10.1101/2022.10.31.514604), I would positively consider this manuscript for publication if the authors reply in full to the issues raised.
My field of expertise: Ubiquitin regulation and interactions, biochemistry, biophysics and Cryo-EM.

We again thank the Reviewer for their insightful comments and suggestions! We followed the

issues raised and hope we provide sufficient validation of our UBR5 cryo-EM structure in our revised manuscript.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

The manuscript presented the Cryo-EM structure of HECT E3 UBR5. Using Alphafold2 model of UBR5, the authors were able to dock and refine the structure model of full length UBR5. Interestingly, UBR5 exists as a homodimer and could potentially assemble into a larger oligomer based on SEC and Cryo-EM data. The antiparallel arrangement of the homodimer suggests that the C-terminal HECT domain could transfer ubiquitin in trans or in cis configuration. The tetrameric model reveals a ring-like structure with a large central cavity, presumably to accommodate large proteins/complexes. Using AKIRIN2 as a substrate, the authors demonstrated that UBR5 did not ubiquitinate AKIRIN2, but prefers ubiquitin modified AKIRIN2 as the substrate for ubiquitin chain elongation. Indeed, they observed that UBR5 preferentially ubiquitinates pre-ubiquitinated non-cognate substrate and free ubiquitin hinting that UBR5 is a chain elongating E3. Lastly they showed that UBR5 HECT contains a plug-loop that blocks C-lobe rotation and suggested that conformational change is necessary for ubiquitin transfer.

Comments:

1. Homodimerization and oligomerization are the novel aspect of this study but the manuscript lacks validation of the structure. The authors should provide biochemical/mutagenesis analysis to support the dimerization interface observed in the structure. Also the model showed that SBB2 is involved in the tetramerization interface, could the authors verify this by designing a SBB2 deletion mutant?

We have expressed UBR5 mutants to disrupt both dimerisation and tetramerisation/higher oligomerisation. In brief, we confirm the role of the dimerisation helix with two mutants of this interface, using mass photometry, in lines 156-166:

"To confirm the role of the helix in UBR5 dimerisation, we expressed mutants aimed at disrupting this interface using insect cell expression system (represented as (i) with each construct). We first characterised and compared full-length proteins (UBR5^{wt} and UBR5$^{wt(i)}$) using SEC, ThermoFluor and MP to confirm their oligomerisation properties and stability (Appendix Fig S1B,C, Fig EV1D). We subsequently purified and characterised two mutants; a deletion of the entire dimerisation helix (residues 1912-1931), UBR5$^{\Delta DH(i)}$ and a UBR5 mutant with three charge-reversing point mutations of the key residues of this interface, UBR5$^{DHmutant(i)}$;1914 (R>D),1926 (R>D) and 1931 (E>R). Both mutants displayed a change in their oligomeric properties, with monomers becoming the predominant species (Fig 2C)."

-We show that SBB2 domains mediate contacts between two dimers in a tetrameric assembly by generating an SBB2-deletion mutant and characterise it using mass photometry and cryo-EM, in lines 183-197:

"SBB domains were often found to facilitate oligomerisation via β strand hydrogen bonding (Youkharibache et al., 2019). To identify if these motifs form the tetramerisation interface of UBR5, we performed extended 3D classification of the tetramer to obtain a more rigid density. We could not assign the atomic coordinates of these domains due to low resolution, but we confidently docked in two UBR5 dimers. This revealed that this assembly is indeed facilitated by SBB2 domains of two opposite dimers (Fig 2D). We confirmed the role of SBB2 domains in UBR5 tetramerisation by expressing a UBR5 mutant with an SBB2 domain deletion (UBR5$^{\Delta SBB2}$) and analysing its oligomeric state using MP. This revealed an almost exclusively dimeric assembly of UBR5 (Fig 2E). We observed a small peak with a molecular weight of a tetramer. This peak is of considerably lower abundance than the dimeric peak, but it cannot be excluded that UBR5 dimers display weak binding properties to each other even in the absence of SBB domains. We further subjected

UBR5$^{\Delta SBB2}$ to cryo-EM analysis. 2D classification of this mutant revealed an exclusive dimeric assembly, further confirming the role of SBB2 domains in contacts between two dimers (Fig 2F)."

2. Would be useful to show the docking of Alphafold2 model onto the Cryo-EM map prior to further model building and refinement in the supplementary data.
We introduced the AF model of full-length UBR5 fitted in our cryo-EM map into Appendix Figure S1.

3. Please show the SDS-PAGE of purified UBR5 used for Cryo-EM study.
We introduced a panel showing purified UBR5 samples as panels under activity assays to represent levels of the enzyme. These panels contain full-length and mutant UBR5 proteins.

4. Figure referencing is not in order. For example Figure 1J was described before Figure 1I. Figure 2A,B mentioned after Figure 2C. Also some Figures are not properly referenced in the main text, e.g. p10 when describing ubiquitin chain formation of UbAKIRIN2 and UbSecurin. Please check throughout the manuscript. We revised the manuscript to ensure the proper flow of figures, their respective citations in text, and their legends.

5. Figure legends are missing for Figure 1H-1J
We revised the manuscript to ensure the proper flow of figures, their respective citations in text, and their legends.

6. It was stated in p10 that there was no binding between UBR5 and UbSecurin in Figure S3C, but Figure S3C showed faint FAM-UbSecurin across the fractions. It would be useful to repeat this with FAM-UbSecurin alone to ensure the faint bands are background signal.
We revised the experiment mentioned by the reviewer. We see weak binding of UbSecurin. We implement this finding in the text Lines 294-295
"We observed a strong shift in the gradient when AKIRIN2 carried a ubiquitin modification (Fig 3F). In contrast, very little UbSECURIN^{FAM} formed a complex with UBR5 (Fig EV 3F)."
We have additionally introduced control gradients alongside our gradients showing substrate interactions and made sure all panels have visible signal. These gradients are implemented in Figure 3 and Figure EV3.

7. In p10, it was stated that UBR5 and UbAKIRIN2 interaction was enhanced in the presence of ubiquitin. How did the authors come to this observation? The sucrose gradients (Figure 3B) showed that UBR5 co-elutes with both AK2 and UbAK2. This reviewer is unclear whether the intensity of the bands can be used to evaluate the strength of the binding affinity. Was the experiment performed at the same protein component concentration/condition? The UBR5 appears to elute at different fractions from the two experiments.
We clarify the set-up of this experiment in the respective methods section, Line 641-642
"All compared gradients were performed in parallel using the same input protein concentrations (40pmol for each input protein)."
Using these gradients, we assess relative binding strength of individual constructs, but we cannot obtain affinity measurements.

8. The authors suggested that the UBA domain might bind ubiquitin and promote ubiquitination of UbAKIRIN2. It is noteworthy that prior studies on several HECT E3s showed that HECT domain alone can catalyze free ubiquitin chain assembly. Could it be possible that the HECT domain of UBR5 alone could catalyze the extension of UbAKIRIN2, UbSecurin or UbdeltaGG?

We have expressed the isolated HECT domain, and observed significantly reduced activity, which is in line with previous findings comparing HECT domain of HUWE1 with its full-length counterpart (Hunkeler et al 2021). These findings are now implemented in Figure 4 and lines 383-387

We additionally expressed and tested the activity of the isolated UBR5 HECT domain. We observed ubiquitination of UbAKIRIN2 only after increasing the reaction temperature to 37°C (Fig 4G). The activity of the isolated HECT domain was significantly lower than for full-length UBR5 and all mutants.

We have also expressed UBR5 with a UBA deletion, and UBR5 with two point mutations that impair ubiquitin binding, and show that these mutants have a decreased activity. These findings are now implemented in Figure 3 and Figure EV3.

9. In Figures 3A and S3B, does the ubiquitin chain elongation occur only on the fused ubiquitin?

The majority of ubiquitination occurs on the fused ubiquitin. We clarify this now in text, lines 287-289 and the respective figure:

"Further mass spectrometry analysis of ubiquitinated ^{GFP}UbAKIRIN2 and ^{Cy5}UbΔGG demonstrated UBR5 is predominantly specific for K48-linked chains. Only a minor proportion of AKIRIN2 was ubiquitinated (Figure EV3H)"

10. Figure 4E mentioned in the main text but is missing.

We revised the manuscript to ensure figures have their respective citations in text.

11. It is not clear from Figure 4 whether the plug-loop is blocking the rotation of C-lobe. The overlaid Figure 4 is quite busy, would be useful to show the UBR5 HECT domain alone with other HECT domain presented in the same orientation but in separate panels. With the plug-loop in the current configuration, does it block E2 binding and transthiolation reaction? Figure 5B seemingly suggested that plug-loop is blocking transthiolation but it is hard to visualize. The authors could enlarge Figure 5B and color the plug-loop differently.

We have revised the figure containing the plug loop (Figure 4), showing only UBR5 with the position of the plug loop between the N and C lobes, which we also labelled. We revised our schematics figure (now Figure 6), enlarged the panels and colour-coded regions in a more clear way.

This enzyme shows unstable behaviour after purification, which we characterised using ThermoFluor. Whilst we can confirm it remains active, enzymatic assays with this protein remain tricky ; the enzyme degrades over time or upon freezing aliquots. We therefore do not have a clear answer on whether it blocks E2 binding or transthiolation.

We have tried to characterise the conformation of the HECT domain of this but we could not obtain sensible 2D classes of the mutant (as shown below), most likely due to its instability.

Revision Plan

Reviewer #3 (Significance (Required)):

The manuscript provides the structural insight into the organization of UBR5. While the Cryo-EM data largely agrees with the Alphafold2 UBR5 model, this should not take away the significant effort in obtaining the large UBR5 protein structure. The structure reveals an unexpected homodimerization of UBR5 and in the assembly of larger oligomer. The biochemical analyses suggest that UBR5 is proficient in ubiquitin chain elongation. Overall the study provides a structural framework for understanding how UBR5 could function as an ubiquitin ligase. The findings will be of interest to scientist in the ubiquitin field and in understanding UBR5 biology.

Limitation: aside from the structure, the study lacks detailed mechanisms of how UBR5 catalyzes ubiquitin transfer. While few models for ubiquitin transfer were proposed, the study lacks suitable substrates to investigate the mechanism. The study showed that UBR5 can elongate ubiquitin chain, but it is not clear whether UBR5 could transfer ubiquitin to substrate.
Ubiquitin transfer by a full-length HECT E3 ligases is one of the most important and long-standing open questions of the field for over a decade. Whilst we are highly interested in answering this question, we believe this remains to be beyond the scope of this study. Nevertheless, we believe that our manuscript brings value to understanding the details of this mechanism. We report the first full-length UBR5 structure and describe many of its previously uncharacterised domains. We further show biochemical data to disrupt many of its interfaces which can be beneficial in future studies. We discuss our findings and implications to substrate selection and respective UBR5 activity in our discussion section, Line429-435, 445-449. We propose UBR5 is efficient at elongating ubiquitin chains, but also discuss this function in relation to its substrate recruitment.

Additionally, to the data presented in the manuscript, we have performed further experiments to characterise the interactome of UBR5. We observed multiple substrates many of which are in good agreement with the recently submitted preprint by Mark et al. We have tested one of our top hits in an activity assay with recombinant proteins and did not observe substrate ubiquitination.
We exclude this data from our manuscript as we think it does not bring additional value to the study presented.

Revision Plan

3. Description of the revisions that have already been incorporated in the transferred manuscript

We have carried out all revisions asked by the reviewers, aside from Point3 of reviewer 1 (see below).
All revisions are replied to in full, as listed in the sections above. Where changes were implemented, we refer to the respective figure or text lines.

4. Description of analyses that authors prefer not to carry out

Please include a point-by-point response explaining why some of the requested data or additional analyses might not be necessary or cannot be provided within the scope of a revision. This can be due to time or resource limitations or in case of disagreement about the necessity of such additional data given the scope of the study. Please leave empty if not applicable.

We notice that reviewer 1 and 2 have differing opinions on ways to address Point 3 where reviewer 2 states an informative experimental set-up to answer the issue (cis/trans mechanisms of ubiquitination) is difficult to design. The experiments required to answer this point in full might fall beyond the scope of the current study.

Whilst we have attempted to answer this hypothesis (using oligomerisation impaired mutants), we have not observed a significant decrease in activity and therefore cannot precisely determine the function of oligomerisation. We therefore discuss these arrangements in the discussion section and the respective figure, Figure 5, as a hypothesis.

Dr. David Haselbach
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Vienna 1030
Austria

25th May 2023

Re: EMBOJ-2022-113348R
Cryo-EM structure of the chain-elongating E3 ligase UBR5

Dear David,

Thank you for submitting your revised manuscript to The EMBO Journal. It has now been re-reviewed by all three original Review Commons referees (see comments below), who -I am happy to say- were fully satisfied with the revisions. In this light, we shall be happy to accept the manuscript for publication, as soon as a few remaining editorial points listed below have been addressed:

- Please make sure to reference and discuss the related recent work by Wang et al (Structure 2023), which had already been available as preprint for some time.

- In the Data Availability section, please include direct hyperlinks to the deposited datasets at the respective repositories; and make sure that all deposited datasets will become publicly accessible now as quickly as possible.

- Please upload each EV Movie as one separate ZIP archive file, which should contain the respective movie file (in a universally accessible format) as well as a text file with the according label (Movie EV1/2/3...) and the corresponding legend for the respective movie.

I am therefore returning the manuscript to you for a final round of minor revision, to allow you to make these adjustments and upload all modified files. Once we will have received them, we should be ready to swiftly proceed with formal acceptance and production of the manuscript.

Yours sincerely,

Hartmut Vodermaier

Hartmut Vodermaier, PhD
Senior Editor, The EMBO Journal
h.vodermaier@embojournal.org

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- Figures may not include error bars for experiments with n<3; scatter plots showing individual data points should be used instead.

3) Revised manuscript text (including main tables, and figure legends for main and EV figures) has to be submitted as editable text file (e.g., .docx format). We encourage highlighting of changes (e.g., via text color) for the referees' reference.

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5) Point-by-point response letters should include the original referee comments in full together with your detailed responses to them (and to specific editor requests if applicable), and also be uploaded as editable (e.g., .docx) text files.

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In the interest of ensuring the conceptual advance provided by the work, we recommend submitting a revision within 3 months (23rd Aug 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

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Referee #1:

The authors have provided additional experiments to address my concerns. I support the publication of the manuscript.

Referee #2:

My points have been sufficiently addressed.

Referee #3:

I found the authors' revisions to be satisfactory in addressing my concerns, and I appreciate the improvements made in the new version of the manuscript

All editorial and formatting issues were resolved by the authors.

Dr. David Haselbach
IMP - Research Institute of Molecular Pathology
Campus-Vienna-Biocenter 1
Vienna 1030
Austria

14th Jun 2023

Re: EMBOJ-2022-113348R1
Cryo-EM structure of the chain-elongating E3 ligase UBR5

Dear David,

Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

Your article will be processed for publication in The EMBO Journal by EMBO Press and Wiley, who will contact you with further information regarding production/publication procedures and license requirements. You will also be provided with page proofs after copy-editing and typesetting of main manuscript and expanded view figure files.

Should you be planning a Press Release on your article, please get in contact with embojournal@wiley.com as early as possible, in order to coordinate publication and release dates.

Thank you again for this contribution to The EMBO Journal and congratulations on a successful publication! Please consider us again in the future for your most exciting work.

With kind regards,

Hartmut

Hartmut Vodermaier, PhD
Senior Editor, The EMBO Journal
h.vodermaier@embojournal.org

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Corresponding Author Name: Zuzana Hodakova, Nicholas G. Brown, David Haselbach
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Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
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