nature portfolio

Peer Review File

K48-ubiquitin-dependent proteases cut-up post-ER proteins

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript submitted by Minard and co-workers focuses on post-endoplasmic reticulum proteins and how K48-linked polyubiquitination and K63-linked polyubiquitination affect lysosomal degradation (in the case of K63-linkage) as well as shearing from the membrane (in the case of K48-linkage). Two ubiquitin-activated proteases have been studied in detail: Ddi1 and Rbd2. The data of the authors support the established fact that the site used for polyubiquitination (K48 and K63 as used here) encode the fate for proteins affected. The authors have made use of a repertoire of different experimental methodologies belonging to molecular biology, biochemistry, and biophysics to address the scientific objective of activating proteases by the ubiquitin machinery.

I will focus in my review on the results obtained by high-resolution NMR spectroscopy in the following as it represents my expertise regarding the experimental repertoire used by the authors.

The NMR data obtained in this study are presented in Figure 3D, E in the main part of the manuscript as well as in Supplemental Figure 4.

Overall, I have to say that I am surprised about the superficiality of the presentation of the NMR data, especially when submitting the work to such a respective Journal like Nature Communications.

Specifically:

The data presented in Figure 3D, E (left) aim to report on the interaction between 15N isotopically enriched ubiquitin and HDD-RVB domains from Sc (D) and human (E).

- The manuscript does not comprise the correct information about the analytical function the authors have used to fit (continuous line in the plots) the experimental data. The statement “… fitted to a standard quadratic equation …” is not only superficial but also wrong.

- A reliable analysis of an NMR spectroscopic titration experiment relies on accurate referencing of the dimensions used for data acquisition. Which efforts have the authors made in this respect?

- Which cross peaks arising in 2D 1H-15N HSQC spectra have been used for the two individual plots shown in panel D and E? What is the rationale for the specific color coding used here (note: color coding used in Supplemental Figure 4 cannot be identified; see also below)?

- Labeling of y-axes misses completely, labeling of x-axes is incorrect. In fact, to quantitatively analyze an NMR titration experiment, the concentration of the ligand (here: HDD-RVB domains) has to be presented, the concentration of the target protein (here: ubiquitin) is contained in the fitting function. Additionally, neither the parameter nor the unit is presented on the x-axes. Which information is additionally encoded in using circles as triangles in panel D, E (no information found in the legend accompanying this Figure)?

- The legend of Figure 3 states “residues of Ub that underwent the largest chemical shift perturbation are mapped in red onto the structure of Ub”. Which specific cut-off value of the perturbation of the chemical shift has been used? Additionally, a separate plot presenting residue-specific perturbations of chemical shifts is highly recommended.

- Data presented in Figure 3 report on an NMR titration experiment. The authors have solely analyzed changes in chemical shifts upon adding of an HDD-RVB domain to ubiquitin. Unfortunately, line width and signal heights of resonance signals arising in 2D 1H-15N HSQC have not been analyzed by the authors.

- The authors have not mentioned the binding model (e.g. one-to-one binding, …) they have used for interpreting the NMR spectroscopic data. The stoichiometry represents an important parameter when performing an interaction study.

- The title of Supplemental Figure reads as “HSQC analysis of 15N-Ub in the presence of the HDD-RVP domains from ScDdi1 and human DDI2” even panel A presents a SDS-PAGE.

- Panel B of Supplemental Figure 4 presents rather small sections of the entire spectral ranges the 2D 1H-15N HSQC NMR spectra comprise. It is highly recommended to show the entirely acquired data set this is, if required, followed by smaller sections. Additionally, cross peaks originating in 2D NMR correlation spectra are shown as contour lines. Unfortunately, the authors present border strips only.

- General: I am not convinced that the authors have not used D2O in their NMR experimental work.

Reviewer #2

(Remarks to the Author)
In this paper, authors report that K63-linked, but not K48-linked, polyubiquitination induces MVB sorting and lysosomal degradation of membrane proteins in S. cerevisiae. They also report that K48-polyUb substrates are degraded after leaving the ER via a pathway, termed CUT-UP (Cleavage of Ubiquitinated Targets by Ubiquitin-activated Proteases), which induces shearing of membrane proteins. They also report that K48-polyUb substrates are processed by the proteasome and by the two Ub-activated proteases Ddi1, a conserved cytosolic ubiquilin that generates cytosolic fragments and Rbd2, a rhomboid protease of Golgi/endosomes but also acting on the vacuole, which produces lumenal fragments. Finally, they report that the catalytic core of Ddi1, consisting of the HDD-RVP domain, is sufficient for Ub-dependent proteolysis, and that its activity is enhanced by auxiliary ubiquitin binding domains (atypical UBL domain and a UBA domain). They conclude that polyUb chains linked by different residues lead to different fates for post-ER proteins, and that there are two proteases that target ubiquitinated integral membrane proteins.

The topic is interesting, and the paper provides novel insights into our understanding of the mechanisms responsible for the degradation of membrane proteins. The paper combines elegant strategies with solid biochemical and morphological data. In general, I find the data convincing, with some relatively minor reservations below.

My main comment – or concern – is that it would be appropriate and helpful to illustrate conditions where native candidate proteins follow these Ub-dependent (Rbd2- or Ddi1-dependent) CUT-UP, protein shearing degradation pathways (see model in Fig 7).

Other comments

1) Nowhere in the text (Methods, etc.) is it stated that anti-GFP E3 ligases are HA-tagged, as seen on the blots Fig 1E. It should be mentioned at least in the Fig legend.

2) It is stated that strains carrying K6R, K11R, K27R, K29R K33R, or K63R as their only form of Ub, all degraded Vps10-NG-AID and formed Ddi1-dependent cytosolic fragments (Fig 3B), but that Vps10-NG-AID degradation and formation of Ddi1-dependent fragments were slightly impaired in the K63R mutant strain. However, the data are not clear-cut and K63R does not appear to be that different from other strains.

3) The authors mention that the loss of both Rbd2 and Ddi1 together with MG132 treatment prevent the formation of truncated fragments, and that a HMW product then forms that remains in the SDS-PAGE stacking gel. The HMW product, which I assume ran at the position of the top arrowhead in Fig 5A, is barely visible, and the total amount of this high MW product does not seem to be roughly equivalent to the starting amount of full-length protein. Where is the boundary between stacking and separating gels?

Reviewer #3

(Remarks to the Author)
This study establishes a role for the intramembrane protease Rbd2 and the ubiquilin Ddi1 in processing ubiquitinated transmembrane proteins at post-ER compartments (Golgi, endosomes, vacuole/lysosomes). The authors further demonstrate that K48-linked chains are the preferred signal for engaging these Ub-dependent proteases, while K63-linked chains funnel the same transmembrane protein substrate into ESCRT-dependent MVB formation and consequently lysosomal/vacuolar degradation. The direct comparison of the signal on the same substrate type is key to compare the effect of the Ub chain types.
These data have important implications for the proteostasis and ubiquitin field.

Major points

Please check for correct terminology i.e. when to use the term Ub-activated protease. To my understanding ubiquitination facilitates substrate targeting for Rbd2, however does not activate the protease activity per se. Similarly, the term 'amplified' in the abstract, when referring to the effect of the UBA and UBL domains on the activity of Ddi1 should be reconsidered.

Line 256 – in this study K63 and K48 chains were compared. Selectivity of the ESCRT machinery for K63 cannot be concluded from these data alone. Other chain types may be processed/engaged by the ESCRT machinery. Please clarify.

Line 269 – which data show effects on cell growth? Is cell growth affected under the applied conditions and if so, how was this taken into consideration when interpreting degradation data?
Line 269 – the degradation of the construct is not blocked but rather strongly impaired. Please rephrase.

Line 381/Figure 3C – Based on supplemental figure 3, the expression level of the Rad23-DDI1 chimera is not at all comparable to wt. Based on the presented data, quantification of the clip under these conditions should be omitted. The rescue with pTEF-WT shows high variability. Could the authors please comment on as to why that could be the case?

Do the authors expect all constructs tested in Figure 3C to have K48 chain type specificity?

Figure 4B – what does the fragment, which appears between 50-70 kDa in the absence of RBD2 activity correspond to? Is this fragment also seen on the gels shown in panel A? The fragment also appears in Figure 5.

In Figure 4C – which difference was quantified here? How do negative values arise in the quantification? Could this be related to the Western blot signal being out of the linear range?

Line 485 – Did the authors attempt to quantify the levels of the model substrates by other methods than Western blot? Imaging- or flow cytometry-based quantification of the fluorescence signal of the model substrate or a deubiquitination prior to SDS-PAGE may help to clarify the question whether the degradation of the model substrate completely blocked.

Lines 498-504 – please rephrase this paragraph for clarity. In what way was the Rbd2-lumenal product variable? How do you interpret this effect?

Figure 6 – why does the Figure legend refer specifically to endocytic substrates? The pathway could equally target secretory substrates.

Could the authors comment on whether a single K48 modified substrate protein is processed by both proteases DDI1 and RBD2? Do they expect the different proteases/degradation pathways to act in parallel on a single substrate protein?

Is p97 required for the proteasome to engage the model substrate or does the proteasome act directly on the membrane?

Minor points

For clarity please refer to indole-3-acetic acid by IAA OR Aux in Figures and text.

Lines 242-252 duplicate the previous paragraph.

Line 289 – was mCherry or RFP inserted? Please clarify.

Line 348 – which additional mechanisms could underlie the degradation of the construct? Please elaborate.

Line 358 – should be Nrf1.

Figure 3B – please refer to the strain that was used to generate data in panel B in the figure legend.

Line 379 – should be HA-tagged DDI1 variants.

Line 444 – Cdc48/p97 is not a Ub-adaptor protein. The AAA unfoldase cannot bind Ub on its own but requires adaptor proteins (UFD1/NPL4) to engage with ubiquitinated substrates. Cdc48/p97 works with a host of adaptors, some of which are the SHP box adaptors. Please clarify in the text.

Please highlight the vacuole/delimiting membrane, ER, and other cellular organelles where appropriate in fluorescence microscopy images for people not familiar with imaging studies in yeast.

The comprehensive literature review in the Supplement is a great resource. Possibly consider presenting in Table format in the main text.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Minard and co-workers submitted a revised version of their manuscript entitled “K48-ubiquitin-dependent proteases cut-up post-ER proteins”.

Generally, they have addressed the points raised in my previous review. However, I recommend to fix the following items before publication (numbering used here refers to the numbering used in the rebuttal letter):

2. The data presented in Figure 3D, E (left) aim to report on the interaction between 15N isotopically enriched ubiquitin and HDD-RVB domains from Sc (D) and human (E). The manuscript does not comprise the correct information about the analytical function the authors have used to fit (continuous line in the plots) the experimental data. The statement “... fitted to a standard quadratic equation ...” is not only superficial but also wrong.
Response: In the methods section, we now fully describe how the data in Figure 3D, E were fitted to solve for the dissociation constant of the interaction of 15N-Ub and the HDD-RVP domain of yeast Ddi1 and human DDI2. The change in peak intensity of Ub L71 was assumed to reflect the fraction of ubiquitin bound to Ddi1 (Fielding, 2007). The change in peak intensity (ΔI) was calculated as the difference in peak intensity in the absence (Ifree) and presence (Ibound) of Ddi1, then divided by the peak intensity in the absence of Ddi1 (see equation (1)).
(eq 1)
The changes in peak intensity were fitted to the single-site binding model described in equation (2), using a least-squares fit calculated with GraphPad Prism to find the dissociation constant Kd and maximal concentration of bound protein [PLmax].
(eq 2)
…

In Figure 3D, E [PL]/[Pt] is plotted on the Y axis (Bound Fraction) and [L] is on the X axis (µM).
---> The phrase “… changes in peak intensity were fitted to the single-site binding model” shall not be used. You always fit the model to your data, never the other way around.
- 4. Which cross peaks arising in 2D 1H-15N HSQC spectra have been used for the two individual plots shown in panel D and E? What is the rationale for the specific color coding used here (note: color coding used in Supplemental Figure 4 cannot be identified; see also below)?
Response: We used Ub L71 peak intensity data for the two individual plots. We chose L71 because it is located in the binding interface and exhibits relatively large peak intensity changes. Originally, the color coding corresponded to the concentration of the HDD-RVP domain and matched the contours of the HSQC 15N-Ub spectra shown in the Supplemental Figure 4. We have now changed the figures in response. Full contour plots of 15N-Ub HSQC (-/+) HDD-RVP are now shown in Supplemental Figure 5. The titration curves are now simplified without color in Figure 3D,E
--> I recommend weakening the statement “L71 … exhibits relatively large peak intensity changes”. As seen in Supplemental Figure 5C, D, weighted CSP values of about 0.03 ppm cannot generally be classified as “large” changes.

Overall, I congratulate the authors to a nice piece of work.

Reviewer #2

(Remarks to the Author)
In this revised version of their article, the authors have adequately addressed most of my concerns. The article has been significantly improved, and I have no further questions or comments.

Reviewer #3

(Remarks to the Author)
All questions raised in my previous review were thoroughly addressed and I was excited to see the new additional data. Thank you for pointing me to the publication I missed, describing the direct interaction of VCP with ubiquitin.
While reading the revised version I noticed one typo in the description of the catalytic dead Rbd2 in the text.

I congratulate the authors to this very exciting and excellent manuscript!

Response to Reviewer's suggestions and comments

Reviewer 1

The manuscript submitted by Minard and co-workers focuses on post-endoplasmic reticulum proteins and how K48-linked polyubiquitination and K63-linked polyubiquitination affect lysosomal degradation (in the case of K63-linkage) as well as shearing from the membrane (in the case of K48-linkage). Two ubiquitin-activated proteases have been studied in detail: Ddi1 and Rbd2. The data of the authors support the established fact that the site used for polyubiquitination (K48 and K63 as used here) encode the fate for proteins affected. The authors have made use of a repertoire of different experimental methodologies belonging to molecular biology, biochemistry, and biophysics to address the scientific objective of activating proteases by the ubiquitin machinery.

I will focus in my review on the results obtained by high-resolution NMR spectroscopy in the following as it represents my expertise regarding the experimental repertoire used by the authors.

1. The NMR data obtained in this study are presented in Figure 3D, E in the main part of the manuscript as well as in Supplemental Figure 4. Overall, I have to say that I am surprised about the superficiality of the presentation of the NMR data, especially when submitting the work to such a respective Journal like Nature Communications.

Response: We thank the reviewer for their critical feedback. In response, we have expanded the presentation of the NMR data, which demonstrate that the central part of yeast and human Ddi1 (HDD-RVP domains) binds mono-ubiquitin in HSQC experiments with ^{15}N-Ub. These findings provide useful mechanistic insight into Ddi1's-ubiquitin-dependent activity. At the same time, we would like to emphasize that the NMR analysis constitutes a secondary focus of the manuscript, whereas the central message is to demonstrate the existence of an unexpected Ub-linkage-specific mechanism for degrading post-ER membrane proteins by two underappreciated enzymes.

2. The data presented in Figure 3D, E (left) aim to report on the interaction between 15N isotopically enriched ubiquitin and HDD-RVB domains from Sc (D) and human (E). The manuscript does not comprise the correct information about the analytical function the authors have used to fit (continuous line in the plots) the experimental data. The statement "… fitted to a standard quadratic equation …" is not only superficial but also wrong.

Response: In the methods section, we now fully describe how the data in Figure 3D, E were fitted to solve for the dissociation constant of the interaction of ^{15}N-Ub and the HDD-RVP domain of yeast Ddi1 and human DDI2. The change in peak intensity of Ub L71 was assumed to reflect the fraction of ubiquitin bound to Ddi1 (Fielding, 2007). The change in peak intensity (ΔI) was calculated as the difference in peak intensity in the absence (I_{free}) and presence (I_{bound}) of Ddi1, then divided by the peak intensity in the absence of Ddi1 (see equation (1)).

$$\Delta I = \left(\frac{I_{free} - I_{bound}}{I_{free}}\right) \equiv [\mathrm{PL}]/[P_t] \qquad (1)$$

The changes in peak intensity were fitted to the single-site binding model described in equation (2), using a least-squares fit calculated with GraphPad Prism to find the dissociation constant K_d and maximal concentration of bound protein [PL_{max}].

$$[\mathrm{PL}]/[P_t] = [\mathrm{PL}_{\max}] \frac{(\mathrm{K_d}+[L]+[P_t]) - (\sqrt{(\mathrm{K_d}+[L]+[P_t])^2 - (4[L][P_t])}}{2[P_t]} \quad (2)$$

Where [P_t], [L], and [PL] refer to:

- the total molar protein concentration of the protein ^{15}N-Ub, which had a fixed concentration during titration.
- the titrating ligand Ddi1 concentration.
- the bound ubiquitin-Ddi complex.

In Figure 3D, E [PL]/[P_t] is plotted on the Y axis (Bound Fraction) and [L] is on the X axis (μM).

3. A reliable analysis of an NMR spectroscopic titration experiment relies on accurate referencing of the dimensions used for data acquisition. Which efforts have the authors made in this respect?

Response: This is now stated in the NMR methods section. The ^{1}H chemical shifts are referenced to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). The ^{15}N chemical shifts were indirectly referenced to DSS using the Bruker software protocol.

4. Which cross peaks arising in 2D 1H-15N HSQC spectra have been used for the two individual plots shown in panel D and E? What is the rationale for the specific color coding used here (note: color coding used in Supplemental Figure 4 cannot be identified; see also below)?

Response: We used Ub L71 peak intensity data for the two individual plots. We chose L71 because it is located in the binding interface and exhibits relatively large peak intensity changes. Originally, the color coding corresponded to the concentration of the HDD-RVP domain and matched the contours of the HSQC ^{15}N-Ub spectra shown in the Supplemental Figure 4. We have now changed the figures in response. Full contour plots of ^{15}N-Ub HSQC (-/+) HDD-RVP are now shown in Supplemental Figure 5. The titration curves are now simplified without color in Figure 3D,E

5. Labeling of y-axes misses completely, labeling of x-axes is incorrect. In fact, to quantitatively analyze an NMR titration experiment, the concentration of the ligand (here: HDD-RVB domains) has to be presented, the concentration of the target protein (here: ubiquitin) is contained in the fitting function. Additionally, neither the parameter nor the unit is presented on the x-axes. Which information is additionally encoded in using circles as triangles in panel D, E (no information found in the legend accompanying this Figure)?

Response: The new figures (3D and 3E) are labelled as: Y axis is 'bound fraction'; X-axis is concentration of the HDD-RVP domain in μM.

6. The legend of Figure 3 states "residues of Ub that underwent the largest chemical shift perturbation are mapped in red onto the structure of Ub". Which specific cut-off value of the perturbation of the chemical shift has been used? Additionally, a separate plot presenting residue-specific perturbations of chemical shifts is highly recommended.

Response: The cut-off values of chemical shift perturbations quantified by $((0.2\Delta N^2 + \Delta H^2)^{1/2})$ that were used to map the largest CSPs onto the Ub structure are now designated in Supplemental Figure 5 and explained in the Methods section that specified we used a 0.5 SD above the mean CSP.

7. Data presented in Figure 3 report on an NMR titration experiment. The authors have solely analyzed changes in chemical shifts upon adding of an HDD-RVP domain to ubiquitin. Unfortunately, line width and signal heights of resonance signals arising in 2D 1H-15N HSQC have not been analyzed by the authors.
Response: We now clarify this in the NMR methods section. We have analyzed the data using both chemical shift perturbation and peak intensities (heights), knowing peak height is inversely proportional to the linewidth.

8. The authors have not mentioned the binding model (e.g. one-to-one binding, ...) they have used for interpreting the NMR spectroscopic data. The stoichiometry represents an important parameter when performing an interaction study.
Response: As mentioned in response to point 2, we now clarify that the binding model used was a stoichiometry of 1:1 in the NMR methods section

9.
• The title of Supplemental Figure reads as "HSQC analysis of ^{15}N-Ub in the presence of the HDD-RVP domains from ScDdi1 and human DDI2" even panel A presents a SDS-PAGE.
• Panel B of Supplemental Figure 4 presents rather small sections of the entire spectral ranges the 2D 1H-15N HSQC NMR spectra comprise. It is highly recommended to show the entirely acquired data set this is, if required, followed by smaller sections. Additionally, cross peaks originating in 2D NMR correlation spectra are shown as contour lines. Unfortunately, the authors present border strips only.
Response: Thank you for the suggestion. These data are now presented in Supplemental Figure 5 that shows the full spectra, a zoomed in section where more conventional contour plats are presented, and the magnitude of chemical shift perturbations for residues of ^{15}N-Ub when bound to HDD-RVP.

10. General: I am not convinced that the authors have not used D2O in their NMR experimental work.
Response: In the NMR methods section, we now clearly state that all NMR samples contained 10% D2O.

Reviewer 2
In this paper, authors report that K63-linked, but not K48-linked, polyubiquitination induces MVB sorting and lysosomal degradation of membrane proteins in S. cerevisiae. They also report that K48-polyUb substrates are degraded after leaving the ER via a pathway, termed CUT-UP (Cleavage of Ubiquitinated Targets by Ubiquitin-activated Proteases), which induces shearing of membrane proteins. They also report that K48-

polyUb substrates are processed by the proteasome and by the two Ub-activated proteases Ddi1, a conserved cytosolic ubiquilin that generates cytosolic fragments and Rbd2, a rhomboid protease of Golgi/endosomes but also acting on the vacuole, which produces lumenal fragments. Finally, they report that the catalytic core of Ddi1, consisting of the HDD-RVP domain, is sufficient for Ub-dependent proteolysis, and that its activity is enhanced by auxiliary ubiquitin binding domains (atypical UBL domain and a UBA domain). They conclude that polyUb chains linked by different residues lead to different fates for post-ER proteins, and that there are two proteases that target ubiquitinated integral membrane proteins.

The topic is interesting, and the paper provides novel insights into our understanding of the mechanisms responsible for the degradation of membrane proteins. The paper combines elegant strategies with solid biochemical and morphological data. In general, I find the data convincing, with some relatively minor reservations below.

1. My main comment – or concern – is that it would be appropriate and helpful to illustrate conditions where native candidate proteins follow these Ub-dependent (Rbd2- or Ddi1-dependent) CUT-UP, protein shearing degradation pathways (see model in Fig 7).
Response: We thank the reviewer for their interest in our work. We agree that an important next step is to identify the native substrates of this pathway, the proteotoxic conditions under which they engaged, and the biological consequence of their loss. We highlight this point in the Discussion 'Further studies are needed to identify the natural substrates of CUT-UP pathway and physiological conditions which activate it.' However, pursuing this direction with the same rigor as the present work would require substantial groundwork and, in effect, constitute a new and distinct chapter of investigation. Notably the targeted discovery of protease substrates is a technically challenging undertaking, as reflected by the limited number of known substrates for Ddi1 and Rbd2 and more generally for rhomboid proteases. Technical advances such as using substrate-traps (PMID: 35173328) may accelerate progress, but these techniques would still need to be adapted and validated in our hands before we could apply them to the CUT-UP pathway.

In the current study, our aim is to provide a clear example of the "ubiquitin code" in action and to identify two underappreciated enzymes with newly defined roles in protein degradation. We provide the first characterization of Ddi1 and Rbd2 enzymatic activity in yeast, with evidence supporting a general role for these enzymes in processing K48-linked ubiquitinated proteins. Our newly included data further demonstrate that Ddi1 can process cytosolic proteins and that, in the absence of Ddi1 and Rbd2, the cytosolic domain of our reporter is processed by the proteasome in a manner independent of Cdc48. We also addressed reviewer 3's question regarding the additional Ddi1-dependent product that appears when Rbd2 is absent. Finally, using CUT-UP pathway mutants, we extended our findings to show that the ubiquitin code is strict for MVB sorting, since it is not engaged by K48-ubiquitinated Vps10, even when the alternative CUT-UP pathway is disrupted.

2. Nowhere in the text (Methods, etc.) is it stated that anti-GFP E3 ligases are HA-tagged, as seen on the blots Fig 1E. It should be mentioned at least in the Fig legend.

Response: This omission has been corrected. The methods are revised to state "The E3 ligases were fused C-terminally to four tandem **HA** epitopes and the VHH-domain cABGFP4 nanobody (Saerens et al., 2005)."; and the Results are revised to state "The Ub-ligases (Figure 1A-B) included HECT- and RING- catalytic domains fused to an **HA**-tagged α-GFP nanobody …" . The legend for Fig. 1 now reads: "A) K63-Ub-ligases were engineered by fusing an **HA**-tagged GFP nanobody to the RING domain of Pib1, two tandem RING domains fo RNF4, the HECT domain of Rsp5, and a chimera of the Rsp5 and E6AP HECT. … Immunoblots (α-GFP, α-**HA**) of Vps10-GFP and the indicated **HA**-tagged GFP nanobody-Ub ligases after 1h of doxycycline treatment in PEP4 and pep4Δ cells."

3. It is stated that strains carrying K6R, K11R, K27R, K29R K33R, or K63R as their only form of Ub, all degraded Vps10-NG-AID and formed Ddi1-dependent cytosolic fragments (Fig 3B), but that Vps10-NG-AID degradation and formation of Ddi1-dependent fragments were slightly impaired in the K63R mutant strain. However, the data are not clear-cut and K63R does not appear to be that different from other strains.
Response: Thank you for pointing this out as an issue to call out to the reader. The yeast strains carrying single Ub K>R mutants were obtained from David Toczyski's lab, who originally published them in eLife (doi.org/10.7554/eLife.42955). The K63R strain, while not described in detail in that work, came to us with the caveat that it may have growth defects and is unusually canavanine-sensitive even when *CAN1* is deleted. Upon reviewing the data we found that this strain consistently expressed lower levels of the Vps10-NG-AID (Supplemental Figure 3B), and for this dataset overall (across all samples for all strains) a low level of Vps10-NG-AID in any K>R mutant was associated with less degradation (Supplemental Figure 3C). Given the strain's phenotypes and low expression, we think the degradation defect is most likely an indirect effect, and have therefore presented these observations without overstating their importance. The main question we wished to address was whether any non-K48 linkage was required for degradation, and none were.

4. The authors mention that the loss of both Rbd2 and Ddi1 together with MG132 treatment prevent the formation of truncated fragments, and that a HMW product then forms that remains in the SDS-PAGE stacking gel. The HMW product, which I assume ran at the position of the top arrowhead in Fig 5A, is barely visible, and the total amount of this high MW product does not seem to be roughly equivalent to the starting amount of full-length protein. Where is the boundary between stacking and separating gels?
Response: We used SurePAGE commercial gradient gels for these studies, which have a short "stacker" portion whose position we did not record. In earlier work, we routinely trimmed the top of the gel just below the wells; however, we found that omitting this trimming allowed detection of the HMW species. Accordingly, we can only indicate the position of the top MW marker rather than a stacking/separating boundary. We have revised the Methods text to clarify: "To detect the high molecular weight forms of Vps10 (Figure 6-8) the entire gel including the loading lanes was transferred."
To make the high-MW species more visible, we reconfigured Figure 5, 8 and Supplemental Figure 6 to include two exposures: a lighter one showing overall degradation and a darker one showing the high-MW species.

We also now demonstrate that a cytosolic NG-AID fusion protein is cleaved not only by the proteasome but also by Ddi1, and that combined inhibition of Ddi1 and the proteasome results in a high-MW smear with laddering consistent with poly-Ub chain attachment (Figure 3F).

To better determine whether the starting protein is fully recovered when Ddi1, Rbd2, and the proteasome are absent/inhibited, we performed a deubiquitination assay to collapse the high-MW band. We reasoned that the high-MW forms diluted the signal by spreading across a broad size range, which could have made quantification and transfer from the gel unreliable. To address this, we deubiquitinated Vps10 to collapse the high MW products and improve detection. To deubiquitinate Vps10, we used an ATP-depletion protocol (2-deoxyglucose, fluoride, and azide) to halt ATP-dependent processes such as ubiquitination while allowing deubiquitination by endogenous ubiquitin-peptidases to proceed. This collapsed the high-MW smear for both cytosolic NG-AID and RFP-Vps10-NG-AID (Figure 3F and 6A). Quantification of western blots of RFP-Vps10-NG-AID revealed that NG signal was fully recovered, and RFP signal was fully recovered after ATP depletion (Figure 7A).

We further assessed the extent of recovery of RFP-VPS10-NG-AID using flow cytometry. This revealed that Neon Green fluorescence was largely recovered in the *ddi1Δ rbd2Δ* mutant upon proteasome inhibition (Supplemental Figure 6B), however, mCherry expression was too low to be detected by this method. Together, these approaches support the conclusion that loss of Ddi1 and Rbd2, combined with proteasome inhibition, leads to robust stabilization of the parent protein.

In addition to the above findings, the ATP depletion assay also revealed that Ddi1 generated multiple cleavage products. Ddi1 generated multiple clipped products and since their sizes were unaffected by ATP depletion, the sizes were unlikely due to differences in polyubiquitin chain length, but instead from cleavage at multiple sites.

Reviewer 3

1. Please check for correct terminology i.e. when to use the term Ub-activated protease. To my understanding ubiquitination facilitates substrate targeting for Rbd2, however does not activate the protease activity per se. Similarly, the term ‘amplified’ in the abstract, when referring to the effect of the UBA and UBL domains on the activity of Ddi1 should be reconsidered.

Response: We agree with the reviewer that this is a point of biochemical precision, with “ubiquitin-activated protease” indicating that the catalytic domain of a protease is activated by ubiquitin binding, whereas “ubiquitin-dependent” could encompass the former model as well as a model where ubiquitin-binding targets the substrate to an enzyme with an already active catalytic domain. In the Discussion we consider both mechanisms. In the case of Ddi1, *in vitro* studies show that the recombinant catalytic domain can only act on ubiquitinated substrates. Therefore, some mechanism must exist to activate its proteolytic RVP domain, which we hypothesize involves direct ubiquitin binding given that the HDD-RVP domain binds ubiquitin. In the case of Rbd2, the lack of *in vitro* data and the unknown mechanism of its interaction with ubiquitin make it unclear whether ubiquitin facilitates substrate binding, activation of Rbd2, or both.

We also appreciate the reviewer's point about the term "amplify" and now use "enhance" to describe the effect of the UBA and UBL domains on Ddi1 activity.

2. Line 256 – in this study K63 and K48 chains were compared. Selectivity of the ESCRT machinery for K63 cannot be concluded from these data alone. Other chain types may be processed/engaged by the ESCRT machinery. Please clarify.
Response: We agree this is the wrong conclusion. The text has been changed to " These data indicate that K48- and K63-Ub ligases engaged distinct degradative pathways, demonstrating a functional Ub code for post-ER proteins and that the ESCRT/MVB pathway selectively excludes K48-ubiquitinated cargo."

We also have additional data demonstrating this point in the revised manuscript. Since it was possible that rather than the ESCRT/MVB pathway actively rejecting K48-ubiquitinated cargo, the cargo was simply more efficiently engaged by the CUT-UP pathway, we tested whether disrupting the CUT-UP pathway caused K48-Ub cargo to undergo MVB sorting. As shown in Figure 6B, even when Ddi1 and Rbd2 are absent and proteasome activity is inhibited, RFP-Vps10-NG-AID does not undergo MVB sorting. We demonstrated this by activating lumenal vacuolar proteases, which generate a processed form of NeonGreen (Vac-NG) from NG-tagged MVB substrates. K48-ubiquitinated RFP-Vps10-NG-AID did not produce a vacuolar-processed form of NeonGreen and instead accumulated in high-molecular-weight forms.

3. Line 269 – which data show effects on cell growth? Is cell growth affected under the applied conditions and if so, how was this taken into consideration when interpreting degradation data? Line 269 – the degradation of the construct is not blocked but rather strongly impaired. Please rephrase.
Response: We have clarified these points in the revised manuscript.
• For conditions using MG132 inhibitor, we now use the term "inhibited" rather than "blocked."
• We added Supplementary Figure 1B, which shows that overnight growth is strongly inhibited by MG132 only in the *pup1T30A pre3T20A pdr5Δ* strain, but not in the *PUP1 PRE3 pdr5Δ* strain.
• This figure also shows that in the short term (2 h), the $pup1^{T30A}$ $pre3^{T20A}$ *pdr5Δ* strain increased its OD_{600} to the same extent whether MG132 is present or not. This 2 h window encompasses all experiments reported here. Since no short-term growth defect was observed from MG132 treatment, there was no need to adjust degradation measurements for potential reduced cell numbers. These data demonstrate that inhibiting all three catalytic sites was necessary to achieve meaningful inhibition of proteasome activity. By inhibiting the proteasome thoroughly, we were able to reveal other proteases in CUT-UP pathway.

We also investigated whether degradation of a cytosolic substrate occurred independently of the proteasome via Ddi1. Fig 3F demonstrates that Ddi1 deletion prevented clipping of a cytosolic NeonGreen-AID and led to the accumulation of high molecular weight species of

NeonGreen-AID. These data reveal that Ddi1 cooperates with the proteasome to clip cytosolic substrates as well as membrane substrates.

4. Line 381/Figure 3C – Based on supplemental figure 3, the expression level of the Rad23-DDI1 chimera is not at all comparable to wt. Based on the presented data, quantification of the clip under these conditions should be omitted. The rescue with pTEF-WT shows high variability. Could the authors please comment on as to why that could be the case?
Response: We now note in the text that the expression level of the Ubl_{Rad23}–Ddi1 chimera is lower than that of wildtype Ddi1. We have retained the quantitation in Figure 3, as the construct was still expressed and its activity was measured with multiple independent experiments. By explicitly stating the difference in expression, we allow readers to interpret the data with this caveat in mind.

Regarding the variability in the *pTEF-WT* rescue, we agree with the reviewer that it is notable. This construct overexpresses Ddi1, and was made in case orthologous Ddi1 proteins expressed poorly in yeast. *pTEF-WT* overexpressed Ddi1-HA in all five replicates; in two replicates it exhibited no change in Ddi1 activity and in three replicates it exhibited increased activity. However, our assay was not optimized to detect increases in Ddi1 activity since we assessed activity at a single time point (60 min after SCF^{TIR1} induction) when a large proportion of Vps10 is degraded (Figure 1F). It's unclear why in some replicates more Ddi1 fragments were recovered, there might be unanticipated secondary proteostasis adaptions in response to Ddi1 overexpression, or this could just reflect technical and biological variability. A more detailed study would be required to assess dose dependence of Ddi1 on its biological activity. Importantly, with the exception of the Ubl_{Rad23}–Ddi1 chimera, the variants expressed to similar levels as their wildtype control.

5. Do the authors expect all constructs tested in Figure 3C to have K48 chain type specificity?
Response: This is an interesting question that we think relates two other questions, to what extent is Ddi1 (or Rbd2) specifically activated by Ub linkages other than K48, and what domains confer K48-Ub specificity. We think these questions are best addressed in future studies focused on the biochemical mechanisms of Ddi1 and Rbd2.

While our current data provide strong evidence that K48 linked ubiquitination does not support MVB sorting, even in the absence of the CUT UP pathway, the reciprocal question, whether other ubiquitin chain types might serve as signals for Ddi1 or Rbd2 dependent cleavage, remains unresolved. Our preliminary experiments however, indicate that ubiquitinated Mup1 GFP in an ESCRT mutants is highly stabilized, and does not divert into Ddi1 or Rbd2 dependent degradation.

It is also unclear how K48 ubiquitinated substrates are recognized by Ddi1, given that the ubiquitin binding elements it employs, including the HDD RVP core domain characterized here, bind mono ubiquitin in vitro. As noted by the reviewer, a key mechanistic question is whether this apparent chain type preference is conferred by the HDD RVP domain, or other Ub binding modules. Addressing this will require improved assays sensitive enough to detect the low level of activity associated with the HDD RVP core and with alternative polyUb chains. This assay should perhaps use a cytosolic

substrate, that is not subject to MVB, ERAD, or EGAD pathways. Although these mechanistic questions are beyond the scope of the current study, they represent an important direction for future work to define how Ddi1 achieves its ubiquitin dependence and any specificity toward ubiquitin chain topology.

6. Figure 4B – what does the fragment, which appears between 50-70 kDa in the absence of RBD2 activity correspond to? Is this fragment also seen on the gels shown in panel A? The fragment also appears in Figure 5.

Response: We thank the reviewer for this astute observation. Additional experiments, now included in Figure 7, focus on this extra band, termed RFPp55, which is derived from the lumenal domain of the RFP-Vps10-NG-AID reporter protein. As shown in Figure 7A, it accounts for only ~1% of the total parental protein after ubiquitination and proteasome inhibition are triggered, but this proportion increases when Rbd2 is deleted. This suggests the existence of a backup pathway that can be engaged when Rbd2 is absent.

RFPp55 is not observed in the absence of Ddi1 (either in *ddi1Δ* alone or in the *rbd2Δ ddi1Δ* double mutant treated with MG132). This indicates that the lumenal domain either gains access to the cytosol to become a Ddi1 substrate, or that Ddi1 acts indirectly on the machinery processing RFPp55. We considered the Tul1/Dsc complex as a candidate for cytosol export activity, given recent studies suggesting it can act as a translocation channel for some post-ER ubiquitinated proteins. However, RFPp55 was still produced in a *dsc2Δ* mutant. Likewise, we replaced the wild-type *CDC48* allele in our reporter strain with either *cdc48-2* or *cdc48-3*, simultaneously inactivating the endogenous *CDC48*, to assess any dependence on Cdc48; at the restrictive temperature (37 °C), RFPp55 production persisted in *cdc48-ts* mutants. This makes it unlikely that the fragment is generated via ER retrotranslocation through known Cdc48-dependent machinery. Finally, we used a tetracycline-sensitive riboswitch to shut off translation of newly synthesized reporter for 1 h before inducing ubiquitination to ensure RFPp55 was generated from a post-ER population rather than a newly synthesized one in the ER. Overall, these findings suggest an interesting backup or parallel processing pathway to Rbd2, which becomes more apparent in its absence.

7. In Figure 4C – which difference was quantified here? How do negative values arise in the quantification? Could this be related to the Western blot signal being out of the linear range?

Response: Negative values arose because the data are expressed as the difference in soluble RFP between the “copper and auxin treated” and “untreated” conditions, with the “untreated” value subtracted from the “treated” value. In some *rbd2*Δ samples, slightly lower levels of soluble RFP were detected in the treated condition compared to the untreated condition, resulting in a negative value. Overall, in *rbd2*Δ samples no increase in soluble fragments were observed, and thus the difference between “untreated” and “treated” condition was zero with minor biological and technical variation between replicates.

8. Line 485 – Did the authors attempt to quantify the levels of the model substrates by other methods than Western blot? Imaging- or flow cytometry-based quantification of the fluorescence signal of the model substrate or a deubiquitination prior to SDS-PAGE may help to clarify the question whether the degradation of the model substrate completely blocked.

Response: Thank you for this suggestion. Reviewer 2's fourth question also asked whether RFP-Vps10-NG-AID degradation was completely blocked by inhibiting the proteasome in cells lacking Rbd2 and Ddi1. We were unable to successfully isolate high–molecular weight forms of RFP–Vps10–NG–AID under native lysis conditions in order to add recombinant deubiquitinating enzymes. Instead, we rapidly depleted ATP using sodium azide (electron transport inhibitor), sodium fluoride (enolase inhibitor), and 30 mM 2-deoxyglucose (glycolysis inhibitor that consumes ATP) to exhaust ATP stores. This approach was intended to prevent new ATP-dependent ubiquitination and degradation while allowing endogenous deubiquitinating enzymes to remove ubiquitin chains. Under these conditions, the high–molecular weight smear collapsed, and quantification of these blots showed near-complete preservation of RFP–Vps10–NG–AID immunoreactivity following SCF^{TIR1}-mediated ubiquitination when MG132 was present in *rbd2Δ ddi1Δ* mutants (Figure 6A).

We also performed flow cytometry to complement these observations (Supplementary Figure 6B). These data showed degradation of NG fluorescence was strongly inhibited in MG132-*treated rbd2Δ ddi1Δ* mutants comparable to that observed in immunoblots of ATP-depleted cells. Unfortunately, the RFP signal was too low to measure by flow cytometry.

9. Lines 498-504 – please rephrase this paragraph for clarity. In what way was the Rbd2-lumenal product variable? How do you interpret this effect?

Response: We now clarify that the production of the Rbd2-dependent lumenal fragment is reduced when Ddi1 is lacking and cells are treated with MG132. Although this may be interpreted as indicating that Rbd2 processing occurs after the cytosolic domain is processed, we call attention to the observation that in fact full-length Vps10 is still degraded in an Rbd2-dependent manner, indicating that Rbd2 activity does not rely on proteasomal or Ddi1 activity. A potential explanation for this trend is that perhaps loss of Ddi1 increases the ability of Rbd2 to process RFP–Vps10–NG–AID in the TGN, which could lead to more secretion of the lumenal domain and a decrease in what is recovered in immunoblots. However, given that the effect is small, we do not think it warrants detailed speculation or additional experimentation.

10. Figure 6 – why does the Figure legend refer specifically to endocytic substrates? The pathway could equally target secretory substrates.

Response: We agree. The text in the results and the figure legends (previously those for 6 and 7) reference the broader secretory/endocytic endomembrane system.

11. Could the authors comment on whether a single K48 modified substrate protein is processed by both proteases DDI1 and RBD2? Do they expect the different proteases/degradation pathways to act in parallel on a single substrate protein?
Response: Our data demonstrate that Rbd2 and Ddi1 act in parallel since neither are dependent on each other for cleaving Vps10. We considered that if they acted on separate molecule loss of one should increase substrate availability for the other, resulting in elevated product levels. However, neither Rbd2 or Ddi1 deletion stimulated an increase in each other's cleavage products, indicating that both proteases acted on the same single molecule.

Consideration must also be given to the order of action. If Ddi1 acts first to remove the ubiquitinated C-terminus, this should prevent recognition of the substrate by Rbd2, unless Rbd2 can also recognize other features e.g. partially cleaved tails. Conversely, if Rbd2 acts first, the ubiquitinated C-terminus is still intact for Ddi1 processing and would not affect Ddi1 activity. Thus, we propose two models that explain our observations. 1) a flux-driven sequential model, in which Rbd2 outcompetes Ddi1 and proteasomes, cleaving the substrate first. Or 2) a parallel processing model, which is possible if Rbd2 could also recognize other features of Vps10, e.g. partially cleaved tails.

12. Is p97 required for the proteasome to engage the model substrate or does the proteasome act directly on the membrane?
Response: This is an intriguing question that we now address in Supplemental Figure 6E. We moved the *cdc48-2* (A547T, T803A) and the *cdc48-3* alleles (P257L, R387K) into our reporter strain system and found that at 37°C there was still degradation of the NeonGreen containing cytosolic tail upon SCF^{TIR1}-mediated ubiquitination. Thus, Cdc48 is not required, and we propose that the proteasome is acting directly on the membrane embedded substrate.

For clarity please refer to indole-3-acetic acid by IAA OR Aux in Figures and text.
> We now clarify in the methods section that the type of Auxin we use for these studies is exclusively IAA. And exclusively use Auxin in the figures and text unless explaining what IAA is.

Lines 242-252 duplicate the previous paragraph.
> Thank you for pointing out this error

Line 289 – was mCherry or RFP inserted? Please clarify.
> We have clarified in the text that mCherry, a red fluorescent protein (RFP) variant, was inserted. The text now states "mCherry (designated as RFP)" to indicate that mCherry is the specific RFP used in these experiments.

Line 348 – which additional mechanisms could underlie the degradation of the construct? Please elaborate.
> We now state 'These data indicated that Ddi1 is responsible for forming the clipped cytosolic fragment of Vps10-NG-AID, however, since full-length Vps10-NG-AID was not stabilized, additional degradation mechanisms act on Vps10-NG-AID.' This section is in the Results and explains the motivation to discover the additional degradation mechanisms, which we reveal is mediated by Rbd2.

Line 358 – should be Nrf1.
> Thank you. Corrected.

Figure 3B – please refer to the strain that was used to generate data in panel B in the figure legend.
> All strains used are explicitly called out in the supplementary strain table that describes the strains genotype and which figures and subpanels where it is used.

Line 379 – should be HA-tagged DDI1 variants.
> Yes, it should be and has been corrected.

Line 444 – Cdc48/p97 is not a Ub-adaptor protein. The AAA unfoldase cannot bind Ub on its own but requires adaptor proteins (UFD1/NPL4) to engage with ubiquitinated substrates. Cdc48/p97 works with a host of adaptors, some of which are the SHP box adaptors. Please clarify in the text.
> Yes, DOI: 10.1038/35087056 notwithstanding, Cdc48 uses a host of Ub-binding partners to reach its substrates. We no longer refer to Cdc48 itself as a Ub-adaptor protein.

Please highlight the vacuole/delimiting membrane, ER, and other cellular organelles where appropriate in fluorescence microscopy images for people not familiar with imaging studies in yeast.
> Localizations of fluorescent proteins are now pointed out in Fig. 1, 2, and 8.

The comprehensive literature review in the Supplement is a great resource. Possibly consider presenting in Table format in the main text.
> Thank you. We are not sure it fits physically there but will work with the editors accordingly.

Fielding, L. (2007). NMR methods for the determination of protein–ligand dissociation constants. *Progress in Nuclear Magnetic Resonance Spectroscopy*, *51*(4), 219-242. https://doi.org/https://doi.org/10.1016/j.pnmrs.2007.04.001

Saerens, D., Pellis, M., Loris, R., Pardon, E., Dumoulin, M., Matagne, A., Wyns, L., Muyldermans, S., & Conrath, K. (2005). Identification of a universal VHH framework to

graft non-canonical antigen-binding loops of camel single-domain antibodies. *J Mol Biol*, *352*(3), 597-607. https://doi.org/10.1016/j.jmb.2005.07.038

response to referees: **NCOMMS-25-30550B**

We have made the following changes in response to Reviewer 1, who pointed out some clumsy grammar.
Thanks for the opportunity to correct this.

Old: The resulting ΔI values were fit to a single-site binding model described in equation ..
New: The resulting ΔI values were used to fit the single-site binding model described in equation..

Old: Ub L71 was selected because it lies within the binding interface and exhibits both large chemical shift perturbations and peak intensity changes.
New: Ub L71 was selected because it lies within the binding interface and exhibits both larger chemical shift perturbations and peak intensity changes.