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Peer Review File

Structural dynamics of the midnolin-proteasome during ubiquitin-independent substrate turnover

Corresponding Author: Professor Ling Liang

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

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this paper ‘Structural dynamics of the midnolin–proteasome during ubiquitin-independent substrate turnover’, Zhu, Qin et al. investigate how midnolin (MIDN) mediates ubiquitin-independent proteasomal degradation. By combining cryo-electron microscopy (cryo-EM) with biochemical data, they reveal the mechanisms of the interaction between MIDN and the 26S proteasome, and proposing the role of the individual MIDN domains in substrate degradation. The work includes extensive structural work which offers various snapshots of the MIDN-proteasome interaction and of the substrate-protasome interaction. Overall, this paper contains valuable findings that will interest the proteasome research community. However, in light of the recently reported structures of the midnolin–proteasome complex (Nardone et al., 2025), the novelty of the work presented in the manuscript is limited. This wouln’t be per se a problem of the authors had actually solid evidence for the two mechanisms that they try to elucidate: (i) the role of the Catch domain in correctly placing the substrate for degradation and (ii) whether MIDN binds to the substrate prior to proteasome association. The experimental results are poorly described in a disordered way, jumping from one description to the next without a clear logic. At part sit is not even clear what experiments the authors have performer until one reads the methods parts. Moreover, several critical pieces of information are missing throughout the manuscript, and the work requires major revisions before it can be considered for publication.

Some more detailed comments below:
The abstract should be self standing and understandable, instead it includes many words of proteins and domains not explained at all (etc. midnolin, Catch).

The introduction contains many grammatical mistakes that even hinder the reading at parts.
1. In line 48, the authors stats that the identification of the MIDN pathway disrupts theestablished Ub-dependent pathway. To use the word disrupt is incorrect as the ubiquitin-independent path was already known. The new MIDN pathway adds up to the complexity of the system. We didn’t like the use of the word disrupt becasue it i san example of how language is used all along the text to boost the presented data at expenses of honest description of the scientific knowledge at the moment. Other examples of such usage are the words ‘unprecedented’ (line 77).
2. The naming of the domains changes multiple times in the text, sometime the C-terminus of MIDN is called αHelix-C, some others C-terminal α-helix. The authors should be more consistent in the use of the words.
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4. In line 102, the authors state: "These structures are mainly in substrate processing states that resemble the ED2 state (Fig. 1a and Extended Data Figs. 2, 3 ...)." However, it is unclear why Extended Data Figs. 2 and 3 are referenced here, as they do not demonstrate that the structures are primarily in the substrate processing state. A better comparison with the reported structures should be conducted to confirm the ED2 conformation of the observed complex. Additionally, the use of the term "mainly" is somewhat arbitrary; a more quantitative description would be beneficial. Overall, the paper would benefit from a more thorough comparison of the observed conformations of the 26S proteasome with previously published structures of different states of the complex. Citations should be always included e.g. in line 121 after the sentece ‘different from previous studies’.

5. In line 115, the authors state, "Here, we have successfully captured the structures of the natural endogenous substrate-bound 26S proteasome in action." Additionally, the discussion emphasizes that endogenous substrate-proteasome complexes used in the study, in contrast to other reported structures obtained with non-hydrolysable ATP analogs or RPN11 inhibitors, do not promote specific conformational states. However, it has to be noted that the proteasomes were halted using MG132, which could also skew the distribution of different states. It has been shown by Haselbach et al. (https://doi.org/10.1038/ncomms15578) that inhibitors acting on the 20S core particle can achieve a long-range allosteric effect that modulates the conformational landscape of the 19S particle. Authors should mention this when discussing the conformational landscape of the complex.
6.
7. In line 126-129 when discussing the C-terminal α-helix, the authors do not refer at all to the Nardone et al., 2025 paper where this feature is also described.
8. The results explained in the paragraph 'Uniform expression of midnolin is dependent on substrate' appears not very solid to us. It is unclear whether and how proteome analysis was performed in in hepatocellular carcinoma and glioblastoma multiforme cells and by looking at the graphs in Extended data Fig.6f the correlation is not so tight. That in vitro co-expression of a protein and its substrate stabilise the expression and folding is not surprising, it is in fact a very common phenomenon.
9. The paragraph regarding positioning of the substrate by the Ubl and Catch domains would benefit from a figure showing a cut-through of the map and snapshots of the different positions of the substrate depending on the positioning of the Catch domain. Moreover, why would a Cter substrate position of that type optimal for degradation?
10. In line 182, the authors mention, "Rigid-body docking of the predicted Catch–EGR1 model into the refined map yielded a strong fit, allowing unambiguous placement of the Catch domain (Fig. 3d)." To strengthen this claim, the authors should provide a close-up view of the model in relation to the map, supported by the Q score and the correlation coefficient between the model and the map.
11. In line 186, the authors wrote: "Collectively, our data capture two states: a high-resolution Ubl-bound state (RPN11 engagement) and a state in which both the Ubl and Catch–substrate modules are simultaneously engaged with the 26S proteasome." However, due to the poor clarity of the cryoEM data processing and numerous final maps shown in the data processing pipelines that are not well annotated or described in the manuscript, it is not evident to which maps the authors refer when saying the "two states".
12. The results described on pages 9-10 suggest that the MIND's Catch domain is crucial for stabilizing the complex. In the cryo-EM dataset of the proteasome reconstituted with a Catch-deficient protein, a significant fraction of the 26S particles was found in the MIND-free resting state. However, when processing the 26S-MIND FL dataset, the authors did not investigate the presence of the resting state, which weakens their conclusion.
Were there any other states, aside from the extended state (ED), observed in the endogenous 26S-MIND FL or MIDN-EGR1-human datasets? If so, what were the populations of these different states? Looking at the data processing pipeline of the 26S-MIND FL, a large proportion of the particles was rejected during the ab initio and 3D classification jobs. The ab initio maps were not shown, making it impossible to understand why those particles were removed and what they represent (whether they were low-quality data or different conformations).
In the second 3D classification, some of the maps (e.g., classes 12 and 13) appear to show a good amount of detail but were still rejected by the authors. It would be interesting to investigate whether other conformational states were discarded alongside those particles.
In conclusion, if the authors intend to use the presence of the resting state in the MIDNΔ(112-336)-proteasome dataset as evidence for the role of the Catch domain, they should conduct a more thorough analysis of the other datasets to check for the existence of the resting state in them.
13. It is not apparent which maps from Extended Data Fig.2 are those shown in Fig. 1 and in Extended Data Fig.4. To which map are the authors referring in the figure caption when mentioning ED2-like state? The annotations should be specific and consistent across the manuscript to ensure that the reader can easily associate the presented results with the correct maps.
14. Maps presented in Figures 1-4 appear to be slightly oversharpened. The specific sharpening parameters are not currently outlined in the Materials and Methods section. This information should be included, the degree of sharpening should be carefully verified and various sharpening methods could and should be used.
15. Authors revealed the Helix-C of midnolin interaction with RPN1 (PSMD2). It would be interesting to see a side-by-side comparison of the Helix-C interaction interface (at the residue level) with the other RPN1 T1/T2 interactions (like Usp14 or Ub). Moreover, view that then they use AlphaFold3 to predict the interaction between MIDN and proteasome, it would be interesting to know if this interaction was predicted and to what confidence level.
16. The use of AlphaFold and molecular dynamics is mentioned; however, the brief and vague description of the results makes it difficult to understand why these analyses were conducted in the first place and what insights they contribute to the overall narrative. The respective description in the methods section is also insufficient; Uniprot sequence IDs used for predictions should be specified along with the AlphaFold prediction confidence statistics. Moreover, in Extended Data Fig.9, the IDR2 and Ubl are described as dark orange.
17. In line 220, it states: "This demonstrates that the observed presence of 26S proteasomes without midnolin is not due to asymmetric binding—where midnolin engages one 19S regulatory particle of a 30S proteasome while the other remains unbound. This suggests that in the absence of substrate engagement, midnolin dissociated during size-exclusion chromatography, consistent with the dynamic binding-dissociation nature of midnolin-proteasome interactions we previously proposed." However, since a significant number of particles were discarded in other datasets (in which MIND contained the Catch domain) without careful examination of their conformational and compositional heterogeneity (specifically the presence or absence of MIND), it is challenging to unequivocally state whether the presence of MIND-free resting state complexes is unique to the cryo-EM sample prepared with the Catch-deficient MIND construct. Authors should verify the effect of the Catch domain deletion and the presence of the substrate on the stability of the 26S-MIND complex using biochemical and/or other biophysical assays. This could include techniques such as native PAGE with specific immunostaining or fluorescent tagging of the proteins of interest. In addition, authors should use RP2-CP and RP-CP

notation to differentiate 30S from 26S complexes.
18. In line 214, it states: ‘we found that the substrate in the processing state structurally resembles midnolin itself’ suggesting that MIDN gets degraded. Is there any biochemical evidence for it? In such case a citation of the relevant literature should be provided.
19. On page 11, there is no description of the various states relaying completely on the reader's good knowledge of the proteasome. Moreover no comparison is give side by side to other published structures. This makes the manuscritp difficult to read. Moreover, the states described as well as the whole Figure 6b is extremely reminescent of the paper by Arkinson et al., 2004 (https://doi.org/10.1101/2024.11.08.622731) and this paper is not even cited. We also find the proposed model of movement associated to Mg2+ release not very sound considering that the movement described is of 2.3Å so way below the overall resolution of the map (3Å at best).
The discussion part is again missing citations. More in details:
20. In line 307, the authors state: " Additionally, the IDR2 region of the Catch domain guides midnolin itself into the proteasome for degradation after substrate delivery." Do authors have any biochemical data confirming this?
21. In line 313, it states: ‘Our findings indicate that ATP hydrolysis plays a pivotal role in the displacement of the proteasome's AAA+ module, with Mg^{2+} dissociation also actively facilitating substrate translocation (Fig. 6b)’. This is quite misleading as it is based on observation of motion below the actual resolution limit of the data.
22. In line 322, it states: ‘These findings suggest that asymmetric ATP hydrolysis by the proteasome ATPase is a widespread phenomenon and may not be substantially related to substrates. The underlying reasons for this asymmetric hydrolysis merit further investigation’. This is not true, there are a wealth of data and discussions about the asymmetry of the proteasome and all other AAA+ unfoldases.
Other points (regarding the methodology):
23. The authors state that the 26S complex concentration was measured after the purification; however, there is no information about the concentration of the complex used for grid preparation.
24. There is currently no information provided regarding the software utilized for cryo-EM data processing and analysis. Similarly, Figure S5 seems to be prepared using ESPript. The authors should specify this and cite the relevant software utilized.
25. In the description of some of the cryo-EM data sets, the number of collected movies and particles in the initial stacks is missing. Authors should consider rearranging the sections that describe the processing of different samples to align with the order of the figures depicting the pipelines.
26. Authors use "two-dimensional" and "3D" interchangeably. I suggest using the simpler 2D/3D notation for clarity.
27. On page 22, what do the authors mean by "subjected to AAA+ ATPase domain-focused masking"? Are they referring to local refinement focused on the AAA region? The pipeline shown in Extended Figure 4 does not clarify the steps undertaken by the authors. One of the initial steps states "masked on AAA+ ATPase domain," and then four classes (#0, #5, #6, #19) from the previous (unmasked?) classification are presented. We suggest the authors redraw their data processing pipeline, showing all the maps for the same classification side by side. They should then indicate by separate outlines which classes were used for further data processing, similar to what they did in Figure S7.
28. On the same page, the authors state, "Notably, 63,758 particles selected through RPN1 region masking from the proteasome particle pool produced a high-resolution RPN1 subunit structure". However, the data processing pipeline (Extended Data Fig. 2) shows that the particles from class 0 and class 1 were pulled together, not "selected through RPN1 region masking."
29. On page 23, the authors mention "high-resolution 3D classification." However, the representative maps for these classes appear to be of lower resolution compared to those from other 3D classification jobs shown. How does this high-resolution classification differ from the other 3D classifications that the authors do not specify as "high-resolution"? Moreover, 3D classification was performer generating far two many classes based on numer of particles available (e.g. 160k particles into 20 classes -line538). It seems that the particles just got distributed equally in the various classes, which indicates that the 3D classification didn’t really work.
30. For symmetry expansion, particles should first be refined with C2 symmetry applied. Authors should specify the symmetry of their refinement for transparency and reproducibility of the analysis.
31. There is no description for the processing of "MIDN-EGR1-human 26S 1068 proteasome complex for the resolved Catch domain" shown in Extended Fig. 8.
32. The description of the ITC and SPR experiments reads more like a general overview of the protocol and lacks the specific details necessary to assess and reproduce the results. For the ITC experiments, the authors do not specify whether a blank titration was performed to determine the nonspecific heats of mixing, dilution, and any potential buffer mismatch. Therefore, in addition to clarifying this in the methods section, the authors should provide reviewers with the raw titration curves from the actual experiments, as well as the results from the blank experiments.
33. In the description of SPR measurements, there is no information about the concentration of the proteins used for the experiment or the amount of protein immobilized on the sensor. Were any negative controls included to test for nonspecific binding?
34. Authors should use black mesh or another more contrasting color combination, as it is almost impossible to glean the density details from Extended Data Fig. 4 in its current form.
35. NLS (same as all other abbreviations used) should be explained to facilitate understanding of the text
36. For the sake of accuracy, the color scalebar in Fig.2a should include units (e.g., kT/e)
Figures:
Figure 2 : the circle in figure2b is barely visible. How does this arrangement compare with the published structure? The measurements in the ITC panel (d,e) should have included full length MIDN and negative controls. Moreover more than 2 measurements should have been performed.
Figure 4b is based on image processing. How many particles were thrown away? And why not reporting these numbers for all the other maps as well?
Figure 5f is very confusing.

Figure 6b is almost copied from Arkinson et al., 2024 and yet the paper is not even cited.

The manuscript requires careful proofreading to correct numerous typos, grammatical errors and to improve the overall readability of the text.
We hope that these comments will help the authors to improve the manuscript.

Reviewer #2

(Remarks to the Author)
The manuscript under review provides interesting and novel insights into the mechanism by which midnolin targets proteins to the proteasome for degradation, and more broadly into the proteasome's mechanism of action.

Midnolin was recently discovered as a novel mediator of ubiquitin-independent proteasomal degradation. It was found to target products of immediate early genes for degradation by binding simultaneously to target proteins and the proteasome, shuttling the former for degradation without the need for ubiquitination. Initial studies suggested that proteasome binding occurs by an N-terminal domain and the C-terminal UBL domain, with the central Catch domain binding substrates.

The study under review here puts this model on solid footing by revealing a series of cryo-EM structures of the tertiary complex between substrate, midnolin, and the proteasome. I cannot assess the technical quality of the cryo-EM structure determination, but taken at face value, the structures provide valuable insights. The binding mode between midnolin and the proteasome is quite novel, including the detailed insights into the interaction between the N-terminal helix and Rpn1 and that of the C-terminal UBL domain with Rpn11. Pinning the N- and C-terminal domains of midnolin to the proteasome then allows midnolin's central catch domain to position bound substrate at the entrance to the degradation channel to be engaged by the proteasome.

The study caught the proteasome in a series of different conformations that are likely to represent different steps in the degradation cycle. Analysis of these structures suggests that Mg++ dissociation drives (together with ATP-hydrolysis) the proteasome through the conformational cycles that lead to substrate unfolding and translocation to the proteolytic sites. As far as I'm aware, the suggested role of Mg++ in the mechanism is novel and important.

There are a couple of conclusions in the manuscript that are not properly supported by the data presented, interesting as they are. I recommend that these parts of the manuscript be revised to indicate the speculative nature of the conclusions. For example, I think that the fact that midnolin is difficult to purify and aggregates when not co-expressed with a substrate can have many other interpretations - most simply that some scaffolding factor is missing at the right stoichiometry in the experimental setup. Similarly, the suggestion that the proteasome can digest midnolin by engaging the protein at its IDR2 is interesting and even plausible, but I propose that the autodegradation of midnolin as a regulatory mechanism is discussed as a hypothetical to be explored in the future.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)
Proteasome is the main degradation machine in eukaryotic cells. While ubiquitin-dependent degradation is well studied, the non-canonical degradation mechanism mediated by midnolin remains elusive. In this study, Zhu et al. investigates the molecular mechanisms of the midnolin in targeting substrates to the proteasome for degradation. The authors combine structural and biochemical analyses to characterize midnolin’s interactions with the proteasome, revealing how its αHelix-C and Ubl domains bind to proteasomal subunits RPN1 and RPN11 respectively, to direct substrates to the proteasome entry port via the Catch domain. The authors captured four continuous states of the midnolin-proteasome complex, revealing the functional dynamics of the proteasomal ATPase complex, and a novel role of Mg2+ dissociation in coordinated mechanochemical coupling. Overall, this study presents compelling structural evidence and valuable insights into the mechanism of midnolin in mediating non-ubiquitination-mediated degradation. However, the current study relies heavily on static structural observations, and it would be important to complement these with functional validation such as kinetic assays of substrate recognition and degradation, together with mutational analyses of key midnolin domains, as detailed below.

Questions:
1. Lines 100-101: There is grammar issue of the sentence.

2. It is interesting that co-expression of Catch domain and EGR1 stabilizes the Catch domain and facilitates their binding. However, this may reflect artifacts arising from using the isolated Catch domain. To clarify this, it would be important to perform the gel filtration assays with full-length midnolin with or without co-expression of EGR1.

The proposed working model that midnolin co-expresses with substrates to achieve rapid targeted degradation raises questions about substrate specificity. With elevated midnolin levels, how is selectivity maintained, and could this result in unintended degradation of non-target substrates?
A minor point: Extended Data Fig. 6g is not cited in the text.

3. In the introduction, the authors raise a question of whether midnolin first binds its substrates prior to proteasome association or constitutively engages the proteasome before recruiting substrates. This key question is not sufficiently addressed in the current work. The structural characterization of midnolin-substrate-26S proteasome complex and co-expression assays do not rule out the possibility that midnolin engages the proteasome before substrate loading.

4. The functional importance of different midnolin domains in proteasome targeting and degradation requires experimental validations. Specifically, while Fig.2 shows the dynamic binding of αHelix-C of midnolin to RPN1, how this interaction could be strengthened by the binding of Ubl domain to the RPN11, or antagonized by the potential interaction between Ubl and RPN1 remains unclear. Structure-based site-directed mutagenesis of these domains followed by functional readouts such as binding kinetics and degradation rates would help disentangle their functional contributions to proteasome targeting.

5. The Catch domain might allosterically regulate proteasome states but the current experiments and results in Fig. 4 do not separate this effect from substrate-induced activation. This issue could be addressed by perturbing the substrate recognition site of Catch domain. In addition, structural analysis of substrate-free midnolin-proteasome complexes within the existing datasets may provide useful insight.

6. Line 209: Extended Data Fig. 11 should be changed to Fig. 10.

7. The legend of Extended Data Fig.4 should be revised with further clarification.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In the revised version of the manuscript, the authors have made substantial efforts that have significantly improved the work. We are also glad that our suggestions have been positively used in the current manuscript.
Better annotations of the presented cryo-EM maps and a more logical arrangement of the results and supplementary material improved readability, facilitating interpretation. Additional biochemical analyses reveal the roles of the domains, while point mutations confirm their importance for binding and activation. Only few issues still need to be addressed before the piece can be recommended for publication.
In the abstract and discussion, the authors present the "capture-before-delivery" mechanism and state that the results support it. In reality, as the authors correctly noted in the rebuttal, the presented structural data represent only snapshots along the catalytic cycle and cannot unambiguously confirm the temporal sequence of the binding events. Therefore, the mechanism is only hypothetical and should be presented as such, this is also in line with the points raised by reviewer #4.
In Figure 1 and Figure 4, the authors present the EMReady treated maps. This is ok, but it should be spelt out in the figures captions and the unsharpened maps should be the one deposited to the PDB. Also, the statistics of the model refinements should be made against the unsharpened map. We also suggest that authors add a schematic diagram of the MIDN domain composition in linear form (similar to Figure 4a), with indications of the domains' start and end points in the sequence. This diagram would facilitate the interpretation of MIDN (337-468), MIDN (337-432), etc.
Another suggestion for figure improvement is to prepare the volume renders like in fig.4 d) (as well as in Figure 3b and Figure 4d), using the ChimeraX "volume zone" command with the "new Map" option, which will prevent "hole" artefacts in the extracted volume.
In some figure captions, the authors provide extensive details on the experimental setup of the proteasome activity assay. Such information should be described in the respective methods section, while keeping the figure caption concise and providing an informative description of the results shown in the graphs.
We are also bringing to the author's attention a few typos we noticed in the revised text:
On page 2, line 38: underlie > should be underlying
On page 5, line 106: previously > previous
On page 10, line 218: Fig. 3d > Fig. 3e
In the caption of Figure 2: panels are described as "top"/"bottom" > should be "left"/"right"
In the caption of Figure 2, line 1099: the authors use the word "equal" when comparing 300 nM and 600 nM affinities. More appropriate would be to say "same order of magnitude" or similar
In the caption of Figure 3, it's unclear what the authors mean by "representative maps" Authors should be precise and explicit on what maps they used. Instead, name the maps PS Ubl or PS Ubl_Catch if those are the final maps presented in Extended Data Figure 7.

Reviewer #2

(Remarks to the Author)
The authors addressed the concerns I raised fully in the revised manuscript. I have not further concerns.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)

Point-by-point Responses

Structural dynamics of the midnolin–proteasome during ubiquitin-independent substrate turnover" (NCOMMS-25-69834-T)

Point-by-point responses to the comments by Reviewer #1:

In this paper ‘Structural dynamics of the midnolin–proteasome during ubiquitin-independent substrate turnover’, Zhu, Qin et al. investigate how midnolin (MIDN) mediates ubiquitin-independent proteasomal degradation. By combining cryo-electron microscopy (cryo-EM) with biochemical data, they reveal the mechanisms of the interaction between MIDN and the 26S proteasome, and proposing the role of the individual MIDN domains in substrate degradation. The work includes extensive structural work which offers various snapshots of the MIDN-proteasome interaction and of the substrate-proteasome interaction. Overall, this paper contains valuable findings that will interest the proteasome research community. However, in light of the recently reported structures of the midnolin–proteasome complex (Nardone et al., 2025), the novelty of the work presented in the manuscript is limited. This wouln’t be per se a problem of the authors had actually solid evidence for the two mechanisms that they try to elucidate: (i) the role of the Catch domain in correctly placing the substrate for degradation and (ii) whether MIDN binds to the substrate prior to proteasome association. The experimental results are poorly described in a disordered way, jumping from one description to the next without a clear logic. At part sit is not even clear what experiments the authors have performed until one reads the methods parts. Moreover, several critical pieces of information are missing throughout the manuscript, and the work requires major revisions before it can be considered for publication.

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The introduction contains many grammatical mistakes that even hinder the reading at parts.

1. In line 48, the authors stats that the identification of the MIDN pathway disrupts the. established Ub-dependent pathway. To use the word disrupt is incorrect as the ubiquitin-independent path was already known. The new MIDN pathway adds up to the complexity of the system. We didn't like the use of the word disrupt because it is an example of how language is used all along the text to boost the presented data at expenses of honest description of the scientific knowledge at the moment. Other examples of such usage are the words 'unprecedented' (line 77).

Answer: Thank you for your thorough and professional review of our manuscript and for your invaluable suggestions on language usage. Your comments on the usage of "disrupt" and "unprecedented" were particularly insightful, and we fully agree. In the revised version we have changed "disrupts this established view" to the more neutral wording "expands this established view by revealing…".

We have now replaced the term "unprecedented" with the more specific and objective description "revealed mechanistic details".

2. The naming of the domains changes multiple times in the text, sometime the C-terminus of MIDN is called αHelix-C, some others C-terminal α-helix. The authors should be more consistent in the use of the words.

Answer: Thank you for carefully reviewing our manuscript and pointing this out. To maintain clarity and consistency throughout the text, we used "αHelix-C" as the name for this domain.

3. Cryo-EM structures are presented in a very disordered way, which makes the reading quite complicated. They are overall ok, but very little has been done with postprocessing to resolve better or at least visualise better the less-resolved MIDN parts. We suggest the authors to try different sharpening tools. As they are shown now the maps appear all oversharpened, we assume that only overall B-factor sharpening has been applied.

Answer: Thank you for your expert review of the cryo-EM structures and for the constructive suggestions. We agree that the presentation could be clearer and that the post-processing of the lower-resolution MIDN regions requires improvement. As recommended, we have moved beyond global B-factor sharpening and tested multiple

sharpening approaches. Specifically, we applied EMReady, a tool designed to enhance features in lower-resolution areas while minimizing over-sharpening artifacts. These adjustments have improved map interpretability.

A detailed description of all post-processing workflows has been provided in the revised "Methods" section to ensure reproducibility. We sincerely appreciate these valuable technical comments.

4. In line 102, the authors state: "These structures are mainly in substrate processing states that resemble the ED2 state (Fig. 1a and Extended Data Figs. 2, 3 ...)." However, it is unclear why Extended Data Figs. 2 and 3 are referenced here, as they do not demonstrate that the structures are primarily in the substrate processing state. A better comparison with the reported structures should be conducted to confirm the ED2 conformation of the observed complex. Additionally, the use of the term "mainly" is somewhat arbitrary; a more quantitative description would be beneficial. Overall, the paper would benefit from a more thorough comparison of the observed conformations of the 26S proteasome with previously published structures of different states of the complex. Citations should be always included e.g. in line 121 after the sentence 'different from previous studies'.

We thank the reviewer for these valuable comments on this section. We have changed the paragraph as follow.

Page5; Line 98-105

" After 3D classification, we determined four distinct structural states (designated MIDN-PS$_{RPT1}$, -PS$_{RPT5}$, -PS$_{RPT2}$, and -PS$_{RPT6}$ for subsequent discussion). Among these states, the RPN1 subunit exhibited varying degrees of resolution and occupied distinct positions relative to the AAA+ motor (Extended Data Fig. 2, 3 and Supplementary Table 1). The 3D classification yielded structural states that all represent substrate-processing conformations. This includes classes reconstructed from particles that were not assigned to a dominant state but still contributed to the overall conformational landscape. The highest resolution (2.87 Å) was achieved for the MIDN-PS$_{RPT1}$ state (Fig. 1a). "

We fully agree with the reviewer's recommendation to conduct a more comprehensive comparison with previously reported proteasomal conformations. This is indeed crucial

for better contextualizing the novelty of our findings. In the revised manuscript, we added a supplementary table that describes the diverse proteasome conformations documented in the literature (Structural landscape of the degrading 26S proteasome reveals conformation-specific binding of TXNL1, https://doi.org/10.1038/s41594-025-01695-2). This detailed comparison help clarify the conformational cycle of the AAA+ motor during midnolin-mediated, ubiquitin-independent degradation is in consistent with the established universal principles.

Supplementary Table 6 | Summary of key structural features of TXNL1-bound proteasome states

	PS_{RPT6} (9E8K)	PS_{RPT2} (9E8O)	PS_{RPT1} (9E8J)	PS_{RPT5} (9E8G)
Pore-loop staircase contacting substrate (from top to bottom)	RPT6 RPT3 RPT4 RPT5 RPT1	RPT2 RPT6 RPT3 RPT4 RPT5	RPT1 RPT2 RPT6 RPT3 RPT4	RPT5 RPT1 RPT2 RPT6 RPT3
RPT subunits disengaged from pore loop staircase	RPT2	RPT1	RPT5	RPT4
RPT subunits with ADP	RPT2 RPT1 RPT5 RPT4	RPT1 RPT5 RPT4 RPT3	RPT4 RPT3	RPT4 RPT3 RPT6
Exchange compared to previous state	N/A	RPT2	RPT1 RPT5	no
Hydrolysis compared to previous state	N/A	RPT3	no	RPT6

5. In line 115, the authors state, "Here, we have successfully captured the structures of the natural endogenous substrate-bound 26S proteasome in action." Additionally, the discussion emphasizes that endogenous substrate-proteasome complexes used in the study, in contrast to other reported structures obtained with non-hydrolysable ATP

analogs or RPN11 inhibitors, do not promote specific conformational states. However, it has to be noted that the proteasomes were halted using MG132, which could also skew the distribution of different states. It has been shown by Haselbach et al. (https://doi.org/10.1038/ncomms15578) that inhibitors acting on the 20S core particle can achieve a long-range allosteric effect that modulates the conformational landscape of the 19S particle. Authors should mention this when discussing the conformational landscape of the complex.

Answer: Thank you for raising this critical point. We agree that while hydrolyzable ATP was used to minimize bias, the essential use of MG132 to preserve the native complex during purification may have allosterically influenced the 19S conformational landscape, as demonstrated by Haselbach et al. (2018, Nature Communications)

We have revised the manuscript in two places: In the Results, we have added a clarifying note regarding MG132 usage when claiming capture of the "native" complex. In the Discussion, we have added a dedicated paragraph to transparently discuss this potential limitation and cite Haselbach et al. (2018, Nature Communications).

Page 6；Line 116-121

" In these reconstructions, we clearly resolved distinct densities corresponding to the inhibitor MG132. This enabled precise docking of the atomic model into the density map, which show MG132 specifically binds to the catalytic β subunits (β1, β2, β5) of the core particle (CP) (Extended Data Fig.4). We note that, as an active-site inhibitor, MG132 may allosterically influence the conformational landscape of the 19S regulatory particle[17], a consideration relevant to the interpretation of the observed states."

Page 15-16；Line 351-363

"Consistent with the well-established asymmetric mechanism of the proteasomal AAA+ motor[14,21], our structures capture consecutive spiral-staircase conformations (PS_{RPT6}, PS_{RPT2}, PS_{RPT1}, PS_{RPT5}) during midnolin-mediated substrate translocation. This confirms that the hand-over-hand translocation mechanism is operational in this ubiquitin-independent pathway. The specific distribution and progression of these states in our dataset provide a detailed view of the mechanochemical cycle in the context of midnolin engagement, although the general principle of asymmetry is

pervasively established across AAA+ ATPases. However, it has to be noted that the proteasomes were halted using MG132, which could also skew the distribution of different states. It has been shown by Haselbach et al.[17] that inhibitors acting on the 20S core particle can achieve a long-range allosteric effect that modulates the conformational landscape of the 19S particle. Future studies using alternative stabilization methods or time-resolved approaches will be valuable to elucidate the native conformational dynamics of the midnolin-proteasome complex in the absence of core particle inhibition."

7. In line 126-129 when discussing the C-terminal α-helix, the authors do not refer at all to the Nardone et al., 2025 paper where this feature is also described.

Answer: Thank you for pointing out this important citation oversight. In the section where we describe the interaction between the C-terminal α-helix of midnolin (αHelix-C) and RPN1, we should reference and discuss the relevant studies by Nardone et al. (Mol Cell, 2025) and Peddada et al. (PNAS, 2025), which also characterized this structural feature. We have now added these references in the revised manuscript.

Page 6；Line 128-130

"This binding is primarily driven by a hydrophobic core and extensive electrostatic complementarity (Fig. 2a, b), a finding independently corroborated by concurrent structural studies[12,13]."

8. The results explained in the paragraph 'Uniform expression of midnolin is dependent on substrate' appears not very solid to us. It is unclear whether and how proteome analysis was performed in in hepatocellular carcinoma and glioblastoma multiforme cells and by looking at the graphs in Extended data Fig.6f the correlation is not so tight. That in vitro co-expression of a protein and its substrate stabilise the expression and folding is not surprising, it is in fact a very common phenomenon.

Answer:

(1) On the comment that "co-expression stabilizing a protein is a common phenomenon":

We fully agree with the reviewer. In vitro, co-expressing an interaction partner (e.g., substrate EGR1) can significantly enhance the solubility and stability of an unstable

protein (e.g., the midnolin Catch domain), which is indeed a widespread practice in molecular biology and biochemistry. We cited this result not to claim novelty but to document a crucial experimental observation: the co-expression strategy was necessary to obtain a functional, stable midnolin-substrate complex suitable for subsequent biochemical and structural studies. This indirectly hints at the functional coupling between midnolin and its substrate.

Accordingly, we have revised the statement to: "For subsequent biochemical and structural studies, a co-expression strategy was required to obtain a functional, stable midnolin-substrate complex suitable for in vitro assays."

(2) Regarding the correlation analysis in Extended Data Fig. 6f and its data source:

The analysis mentioned in the figure legend was based on the public transcriptomic database GEPIA, which integrates RNA-seq data from projects like TCGA and GTEx. We used gene expression data (in TPM values) from hepatocellular carcinoma and glioblastoma samples within this database, not a proteomic analysis performed by ourselves. We clarified this point more explicitly in the revised figure legend and main text to prevent misunderstanding.

We understand and agree that the correlation shown ($R \sim 0.3$) is moderate, not strong. Our intent was not to present it as definitive causal evidence but as a supportive bioinformatic observation. It indicates a statistically significant positive trend ($p < 1e-5$) between MIDN and its substrate EGR1 mRNA levels in actual tumor samples, which is directionally consistent with our in vitro observation of "co-expression promoting stability/function." We have adjusted the text to be more precise, changing phrases like "supports our observation" to "is consistent with the trend observed in our biochemical assays"

Page 8-9;Line 184-190

" Additionally, the bioinformatic analysis (Extended Data Fig. 6f) was performed using the public transcriptomic database GEPIA[22], which integrates RNA-seq data from projects such as TCGA and GTEx. We directly extracted gene expression values (TPM) for hepatocellular carcinoma and glioblastoma multiforme samples. The results showed that MIDN mRNA levels are significantly and positively correlated with those of its substrate EGR1 in real tumor tissues ($p < 1e-5$), consistent with the trend observed in

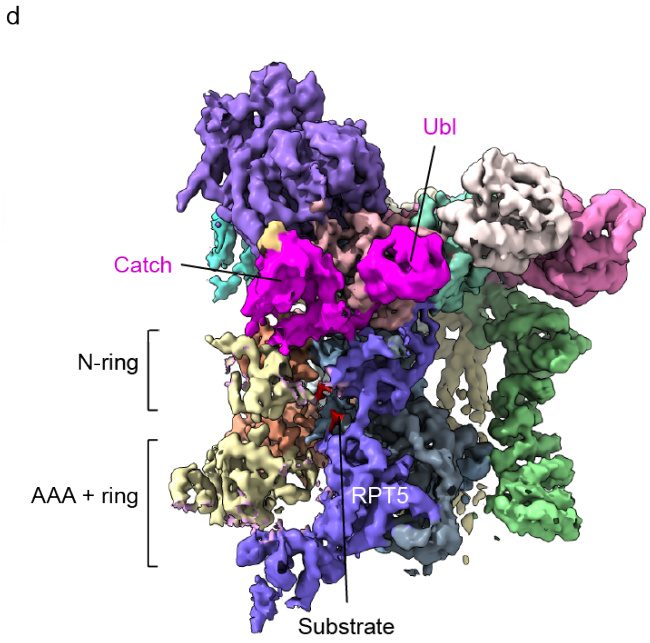
our biochemical assays. These findings indicate that midnolin may preferentially co-express with its substrates to achieve optimal activity. "

9. The paragraph regarding positioning of the substrate by the Ubl and Catch domains would benefit from a figure showing a cut-through of the map and snapshots of the different positions of the substrate depending on the positioning of the Catch domain. Moreover, why would a Cter substrate position of that type optimal for degradation?

Answer: Regarding the suggestion to add a cut-through view and substrate snapshots, we agree that this would be highly beneficial. While our current overall map shows the Catch domain and substrate density positioned above the entry port, it does not intuitively convey the substrate's precise spatial trajectory relative to the proteasomal entry port.

In the revised manuscript, we have addressed this by adding a new panel to Figure 3. This panel features a cut-through view of the cryo-EM map along the central channel axis. This visualization clearly illustrates how the density for the Catch domain–substrate complex is positioned directly above and aligned with the N-terminal (OB ring) entrance of the AAA+ ATPase ring, providing an unambiguous depiction of the substrate's path into the proteasome.

We suppose that the substrate is more susceptible to degradation from its C-terminus, primarily because the Catch domain precisely positions the substrate's C-terminus at the proteasome entry port. However, this model warrants further investigation.



Revised Figure 3d. A cut-through of the cryo-EM map of the midnolin/substrate density in the central channel, just reaching the ATPase ring.

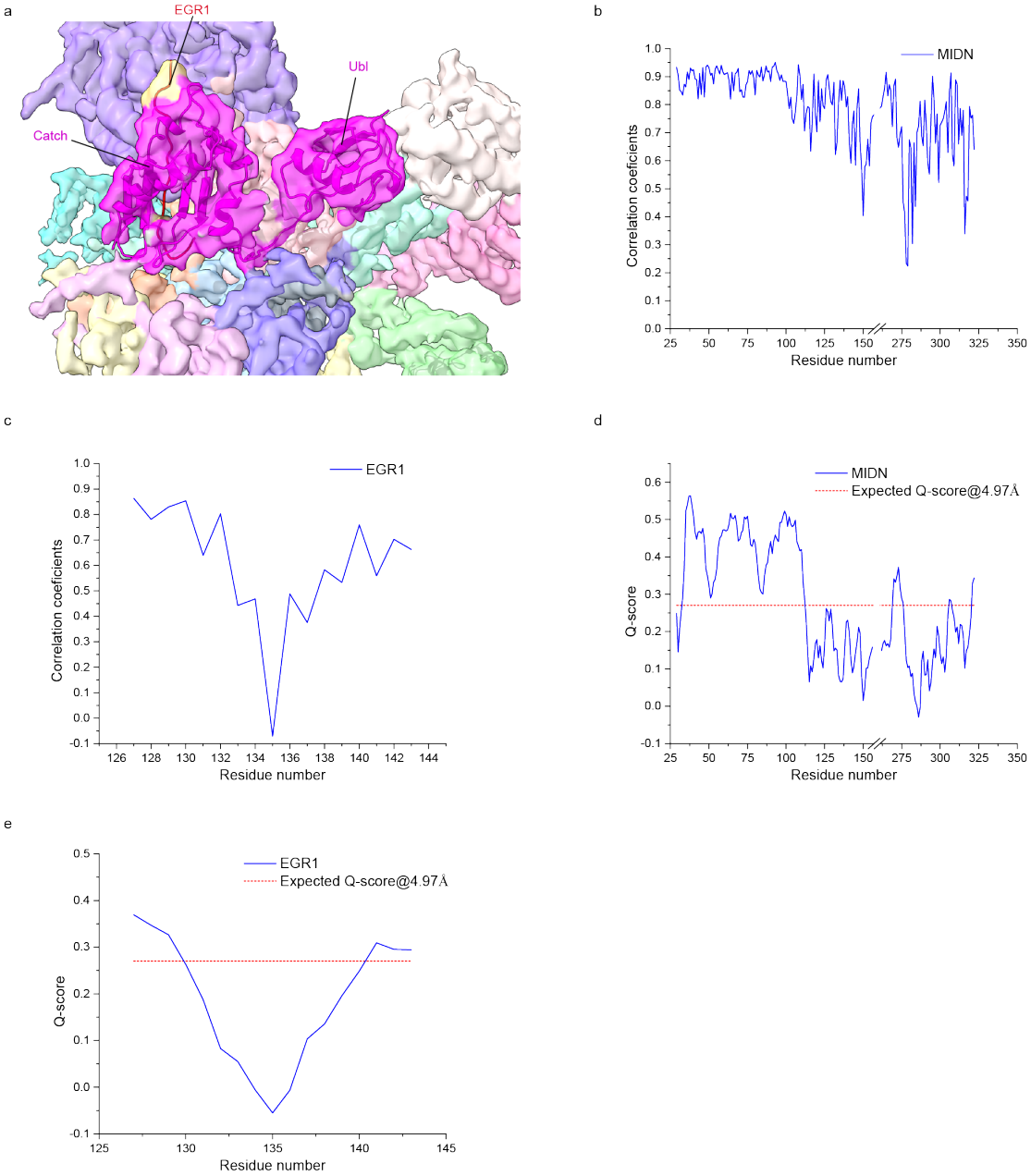
10. In line 182, the authors mention, "Rigid-body docking of the predicted Catch–EGR1 model into the refined map yielded a strong fit, allowing unambiguous placement of the Catch domain (Fig. 3d)." To strengthen this claim, the authors should provide a close-up view of the model in relation to the map, supported by the Q score and the correlation coefficient between the model and the map.

Answer: We thank the reviewer for this constructive suggestion to strengthen the validation of our model placement for the Catch–EGR1 module.

We have provided a new, detailed close-up view in Extended Data Fig. 8a, which clearly shows the fitted Catch–EGR1 model within the corresponding cryo-EM density. We now report the Q-score and the correlation coefficient (CC) calculated specifically for the Catch–EGR1 region within the refined map (Extended Data Fig. 8b-e). Importantly, beyond the rigid-body docking metrics, our refined map reveals continuous density connecting the Ubl and Catch domains. This unambiguous structural feature provides independent and direct validation for the correct spatial placement and trajectory of the Catch domain relative to the anchored Ubl domain, which is a key pillar supporting our model.

We note that the density for the substrate portion bound by the Catch domain is weaker and partially fragmented. This is consistent with the expected higher conformational dynamics of a substrate in the process of being engaged and translocated, and it does not detract from the unambiguous fit of the Catch domain itself to its well-defined density, as now supported by the quantitative metrics and the inter-domain connectivity described above.

We believe these additions provide the requested objective validation and significantly strengthen the claim regarding the unambiguous placement of the Catch domain.



Revised Extended Data Figure 8. Local quality of model fitting to cryo-EM density maps. (a) close-up view of the model in relation to the map for MIDN and EGR1. (b-c)

Fitted models were analyzed with PHENIX and their per-residue correlation coefficients (CC) are plotted, b for MIDN and c for EGR1. (d-e) Q-score plot of MIDN (d) and EGR1 (e) chains. The expected Q-score at 4.97 Å resolution is shown as red dashed lines. The 5-residue average of Q-scores is plotted here.

11. In line 186, the authors wrote: "Collectively, our data capture two states: a high-resolution Ubl-bound state (RPN11 engagement) and a state in which both the Ubl and Catch–substrate modules are simultaneously engaged with the 26S proteasome." However, due to the poor clarity of the cryoEM data processing and numerous final maps shown in the data processing pipelines that are not well annotated or described in the manuscript, it is not evident to which maps the authors refer when saying the "two states".

Answer: Thank you for this important point. We agree that the link between the two functional states and the corresponding cryo-EM maps was not sufficiently clear. To address this, we have systematically reorganized and annotated the data processing workflows (now presented in revised Extended Data Figure 7). This revision explicitly links each final map to its respective functional state, ensuring full traceability and improved clarity of our structural assignments.

12. The results described on pages 9-10 suggest that the MIND's Catch domain is crucial for stabilizing the complex. In the cryo-EM dataset of the proteasome reconstituted with a Catch-deficient protein, a significant fraction of the 26S particles was found in the MIND-free resting state. However, when processing the 26S-MIND FL dataset, the authors did not investigate the presence of the resting state, which weakens their conclusion.

Were there any other states, aside from the extended state (ED), observed in the endogenous 26S-MIND FL or MIDN-EGR1-human datasets? If so, what were the populations of these different states? Looking at the data processing pipeline of the 26S-MIND FL, a large proportion of the particles was rejected during the ab initio and 3D classification jobs. The ab initio maps were not shown, making it impossible to understand why those particles were removed and what they represent (whether they were low-quality data or different conformations).

In the second 3D classification, some of the maps (e.g., classes 12 and 13) appear to show a good amount of detail but were still rejected by the authors. It would be interesting to investigate whether other conformational states were discarded alongside those particles.

In conclusion, if the authors intend to use the presence of the resting state in the MIDNΔ(112-336)-proteasome dataset as evidence for the role of the Catch domain, they should conduct a more thorough analysis of the other datasets to check for the existence of the resting state in them.

Answer: Thank you for this critical comment. We agree that a comprehensive analysis of all datasets is essential to support the conclusion regarding the Catch domain's role. In response, we have now systematically re-analyzed the conformational landscapes of the full-length MIDN–proteasome datasets using the same classification strategy. The results confirm that the resting state (RS) was not populated in these native complexes, in contrast to its significant presence in the MIDNΔCatch/MIDNΔ(112–336) dataset. To ensure transparency, we have provided a detailed population analysis of all observed states across the full-length MIDN–proteasome datasets in the revised Results section.

Page11; Line 241-245

"Compared with full-length MIDN purified complexes, the MIDNΔ(112–336)–proteasome sample lacking the Catch domain exhibits a significantly higher proportion of particles in the substrate-free resting state (RS). This comparison indicates that the Catch domain, together with substrate binding, plays a pivotal role in stabilizing the MIDN–proteasome complex and maintaining it in an active, substrate-processing state."

13. It is not apparent which maps from Extended Data Fig.2 are those shown in Fig. 1 and in Extended Data Fig.4. To which map are the authors referring in the figure caption when mentioning ED2-like state? The annotations should be specific and consistent across the manuscript to ensure that the reader can easily associate the presented results with the correct maps.

Answer: We sincerely appreciate your important comments regarding figure labeling and terminology consistency. Your observations are accurate: the lack of clear correspondence among figures, main text, and figure legends, as well as the nonspecific

use of terms such as "ED2-like state," can indeed confuse readers and hinder comprehension of our findings. To prevent misinterpretation, we have replaced "ED2-like state" with "processing state." We fully agree that clear labeling and cross-referencing are fundamental to readability and scientific rigor. Following your suggestions, we have performed a comprehensive, systematic revision of all labels in the figures and text.

14. Maps presented in Figures 1-4 appear to be slightly oversharpened. The specific sharpening parameters are not currently outlined in the Materials and Methods section. This information should be included, the degree of sharpening should be carefully verified and various sharpening methods could and should be used.

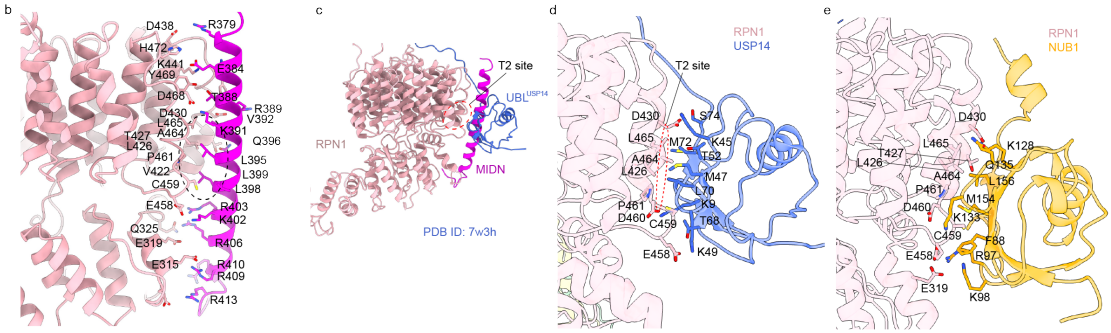
Answer: Thank you for your expert review of the cryo-EM structures and for the constructive suggestions. We agree that the presentation could be clearer and that the post-processing of the lower-resolution MIDN regions requires improvement. As recommended, we have moved beyond global B-factor sharpening and tested multiple sharpening approaches. Specifically, we applied EMReady, a tool designed to enhance features in lower-resolution areas while minimizing over-sharpening artifacts. These adjustments have improved map interpretability. A detailed description of all post-processing workflows has been provided in the revised "Methods" section to ensure reproducibility.

15. Authors revealed the Helix-C of midnolin interaction with RPN1 (PSMD2). It would be interesting to see a side-by-side comparison of the Helix-C interaction interface (at the residue level) with the other RPN1 T1/T2 interactions (like Usp14 or Ub). Moreover, view that then they use AlphaFold3 to predict the interaction between MIDN and proteasome, it would be interesting to know if this interaction was predicted and to what confidence level.

Answer: We appreciate the excellent suggestion to conduct a comparative analysis of the αHelix-C–RPN1 interaction interface. This comparison has effectively highlighted the similarities and distinctions between our identified binding mode and canonical T2-site binders such as USP14 and NUB1.

We have added a dedicated panel in the revised manuscript (in Fig. 2d, e). This new panel features a structural comparison among: (1) our resolved αHelix-C–RPN1

complex, (2) the previously reported USP14–RPN1 complex (PDB: 7W3H), and (3) the NUB1–RPN1 complex (PDB: 8USB). Our analysis reveals that all three complexes share a common hydrophobic core anchored by RPN1 residues L426 and L465. They are further stabilized by peripheral salt bridges and hydrogen bonds. A key distinction, however, lies in the fact that our αHelix-C engages RPN1 with a more extensive interaction interface and adopts a distinct linear conformation.



Revised Figure 2. (b) The interaction interface exhibits a tripartite pattern between αHelix-C of midnolin and RPN1. The circle part indicates a hydrophobic center. This central hydrophobic interface is flanked by two polar interaction networks. (c) Superimposition of the atomic models depicting the interaction interface between USP14(blue) and RPN1(pink) versus the interaction interface between RPN1 and αHelix-C of midnolin (magenta). The T2 site is indicated by a red dashed circle. (d) Side-chain interactions between the USP14 UBL domain (blue) and the RPN1 (pink) T2 site, derived from the USP14–RPN1 complex (PDB: 7W3H). The T2 site is indicated by a red dashed circle. (e) Side-chain interactions between the NUB1 UBL domain (yellow) and the RPN1 (pink) T2 site, derived from the NUB1–RPN1 complex (PDB: 8USB).

Furthermore, we note that AlphaFold3 models, independently predicted the αHelix-C–RPN1 interface. Crucially, the predicted binding mode, including the critical hydrophobic and polar interaction network, is in excellent agreement with the experimental structure we ultimately determined by cryo-EM.

16. The use of AlphaFold and molecular dynamics is mentioned; however, the brief and vague description of the results makes it difficult to understand why these analyses were conducted in the first place and what insights they contribute to the overall narrative. The respective description in the methods section is also insufficient; Uniprot sequence

IDs used for predictions should be specified along with the AlphaFold prediction confidence statistics. Moreover, in Extended Data Fig.9, the IDR2 and Ubl are described as dark orange.

Answer: We thank the reviewer for these essential and constructive comments regarding the computational analyses and figure details, which have significantly improved the clarity and rigor of our manuscript. We have addressed each point as follows.

We have revised the text to explicitly state that the AlphaFold3 prediction was used as an independent, prior validation tool. Specifically, the model for the midnolin-RPN1-RPN11-RPT1-6 complex, accurately predicted the key αHelix-C–RPN1 interaction interface. Considering the high accuracy of AlphaFold3 in predicting the structure of MIDN and proteasome, we generated the predicted midnolin-RPN1-RPN11-RPT1-6-substrate models to better visualize the potential substrate-binding mode.

Upon careful consideration of the reviewer's implied point—and in the interest of maintaining the highest rigor—we acknowledge that our MD simulations, conducted on a partial 19S complex, may not fully represent the physiological context. Therefore, we have removed the entire MD simulation section from the manuscript.

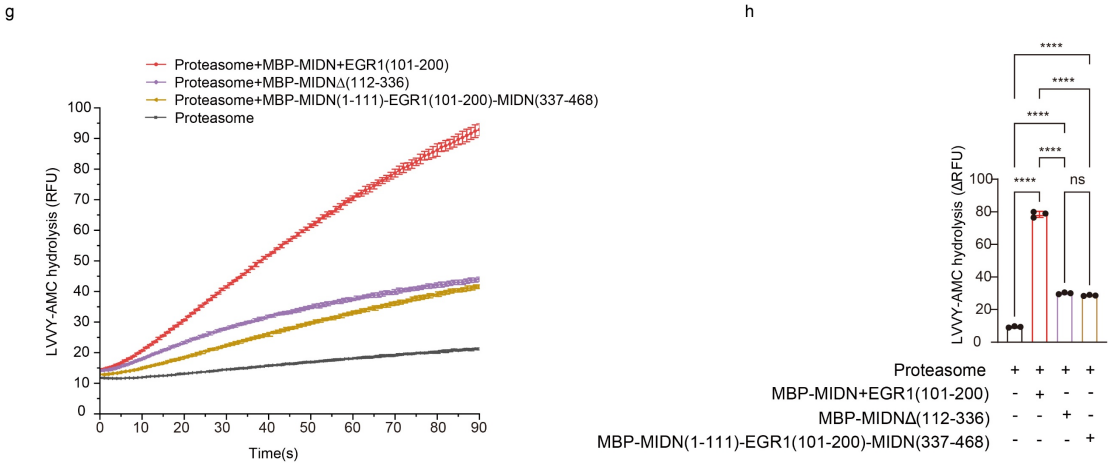
We have comprehensively revised the Methods section to include all requested details for the AlphaFold3 prediction. The UniProt sequence ID are provided: RPT1 (P35998), RPT2 (P62191), RPT6 (P62195), RPT3 (P43686), RPT4 (P62333), RPT5 (P17980), RPN11 (O00487), RPN1 (Q13200), MIDN (Q504T8), EGR1 (P18146), IRF4 (Q15306), FosB (P53539). Predicted structures of the complex and MIDN are colored by pLDDT in AlphaFold3 pattern.

We sincerely apologize for the error in color labeling. We have carefully corrected the legend for Extended Data Fig. 9. It now accurately describes the two distinct colors used: dark orange for the Ubl domain and orange red for IDR2.

17. In line 220, it states: "This demonstrates that the observed presence of 26S proteasomes without midnolin is not due to asymmetric binding—where midnolin engages one 19S regulatory particle of a 30S proteasome while the other remains unbound. This suggests that in the absence of substrate engagement, midnolin dissociated during size-exclusion chromatography, consistent with the dynamic binding-dissociation nature of midnolin-proteasome interactions we previously

proposed." However, since a significant number of particles were discarded in other datasets (in which MIND contained the Catch domain) without careful examination of their conformational and compositional heterogeneity (specifically the presence or absence of MIND), it is challenging to unequivocally state whether the presence of MIND-free resting state complexes is unique to the cryo-EM sample prepared with the Catch-deficient MIND construct. Authors should verify the effect of the Catch domain deletion and the presence of the substrate on the stability of the 26S-MIND complex using biochemical and/or other biophysical assays. This could include techniques such as native PAGE with specific immunostaining or fluorescent tagging of the proteins of interest. In addition, authors should use RP2-CP and RP-CP notation to differentiate 30S from 26S complexes.

Answer: Thank you for these important comments. We have now performed a unified, binding-state-focused analysis on the raw particle sets from all relevant datasets and standardized the data-processing flowcharts accordingly. To directly assess complex stability, we also added enzymatic-activity assays comparing Wild-type full-length midnolin [MBP-MIDN + EGR(101-200)] with its Catch-deletion counterpart [MIDN Δ(112-336)]; the full-length complex showed high activity, whereas the Catch-deletion mutant exhibited low activity, corroborating the particle statistics obtained by cryo-EM.



Revised Figure 4. (**g**) *In-vitro* peptidase activity was monitored over 90 min using the fluorogenic substrate Suc-LLVY-AMC (10 μM) for the 26S proteasome alone (black), or with Catch-deleted mutant [MBP-MIDNΔ(112-336)] (purple), with Catch-replaced midnolin [MBP-MIDN(1-111)-EGR1(101-200)-MIDN(337-468)], with wild-type MBP-MIDN + EGR1(101-200) (red). The y-axis indicates relative fluorescence units

(RFU). **(h)** Quantification of *in-vitro* peptidase activity results in (g). RFU changes between 90 min and 0 min are plotted. Bars represent means ± SD of three replicates (****, p <0.0001; n =3, p values were calculated using ANOVA with Tukey's post test.). ns means not significant.

We also appreciate your note on nomenclature. Throughout the revised manuscript we now use "RP_2-CP (30S)" for the double-capped proteasome and "RP-CP (26S) " for the single-capped proteasome to ensure professional clarity.

18. In line 214, it states: 'we found that the substrate in the processing state structurally resembles midnolin itself' suggesting that MIDN gets degraded. Is there any biochemical evidence for it? In such case a citation of the relevant literature should be provided.

Answer: Following your suggestion, we have revised the relevant statement and added appropriate citations to make this important conclusion more rigorous and credible. Your request for literature support is fully justified. Indeed, the seminal study that first reported the midnolin–proteasome pathway (Gu et al., Science 2023) already provided biochemical evidence that midnolin itself is degraded. For example, cycloheximide-chase assays in that paper showed that midnolin protein levels decline rapidly after substrate delivery, and this degradation depends on its αhelix-C and proteasome activity. Our biochemical assays confirm this observation, providing independent validation. Our structural data, which directly captures midnolin as a proteasome substrate, not only stands as independent evidence but also provides a mechanistic and visual explanation for the prior biochemical observation of midnolin's inherent instability and proteasome sensitivity. Together, these evidences establish a "self-sacrificing" degradation mode for midnolin. We have now inserted the following citation adjacent to the sentence that mentions midnolin's possible self-degradation.

Page 11; Line 248-249

"This structural observation is consistent with previous biochemical evidence demonstrating that midnolin undergoes proteasomal degradation after substrate delivery[3]."

To clarify the core function of the Catch domain, we engineered a "Catch-functional replacement" construct in which the known substrate fragment EGR1(101–200) was fused, via a flexible linker, between the Ubl domain and α-Helix-C of midnolin, completely replacing the native Catch domain. This chimera retains substrate-binding capacity (because the substrate is covalently "pre-bound") yet lacks the natural Catch domain and any specific spatial interface it might provide. In essence, we stripped the "substrate-binding" role from the wild-type Catch domain and supplied it in a simple, constitutive manner. If the Catch domain were solely responsible for substrate binding, the replacement construct should degrade the substrate as efficiently as the wild-type protein, since the substrate has already been "pre-delivered. " Conversely, if the Catch domain additionally performs a "positioning" or “optimized delivery” function—e.g., by contacting the proteasome entry region to guide the substrate terminus into an optimal location—then the replacement construct, although substrate-bound, should exhibit markedly lower degradation efficiency, resembling or only slightly exceeding that of the Catch-deletion mutant. The results of enzymatic assays revealed that the degradation activity of the full-length midnolin–substrate–proteasome complex is significantly higher than that of the Catch-deletion mutant and Catch-functional replacement, confirming the essential contribution of the Catch domain to the functional stability of the complex. Our enzymatic data demonstrate that the Catch domain executes a specific role beyond mere substrate binding, further supporting the hypothesis that it facilitates delivery of the substrate to the proteasomal entrance.

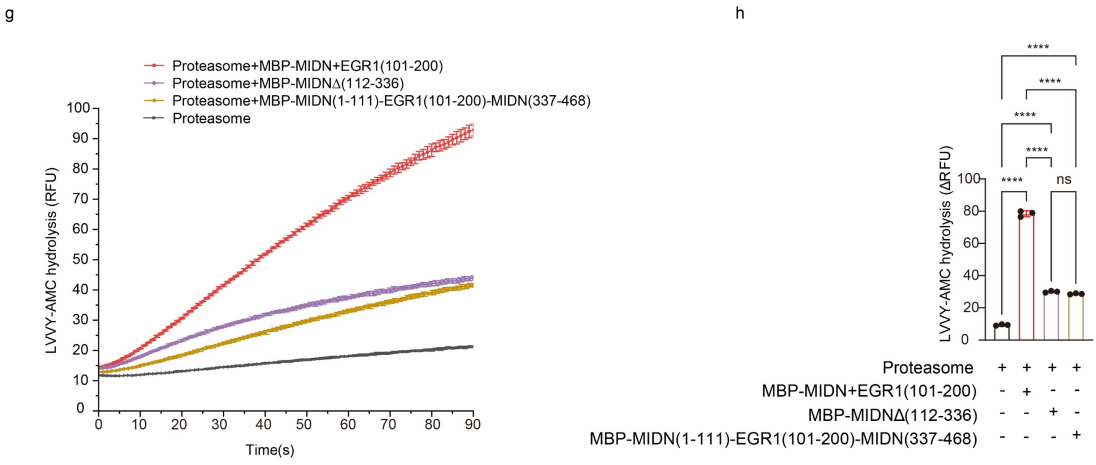


Figure 4(**g**) *In-vitro* peptidase activity was monitored over 90 min using the fluorogenic substrate Suc-LLVY-AMC (10 μM) for the 26S proteasome alone (black), or with Catch-deleted mutant [MBP-MIDNΔ(112-336)] (purple), with Catch-replaced midnolin [MBP-MIDN(1-111)-EGR1(101-200)-MIDN(337-468)], with wild-type MBP-MIDN + EGR1(101-200) (red). The y-axis indicates relative fluorescence units (RFU). **(h)** Quantification of *in-vitro* peptidase activity results in (g). RFU changes between 90 min and 0 min are plotted. Bars represent means ± SD of three replicates (****, $p < 0.0001$; n =3, p values were calculated using ANOVA with Tukey's post test.). ns means not significant.

Page11-12; Line 251-266

" To clarify the core function of the Catch domain, we engineered a "Catch-replaced midnolin" construct MBP-MIDN(1-111)-EGR1(101-200)-MIDN(337-468), where the native Catch domain is replaced by the known substrate fragment EGR1(101-200), covalently tethered via a flexible linker. This chimera retains constitutive substrate binding but lacks the native Catch interface. We then compared the stimulated proteasome activity of three proteins: wild-type full-length midnolin [MBP-MIDN + EGR1(101-200)], Catch-deleted mutant [MIDNΔ(112-336)], Catch-replaced midnolin. If the role of the Catch domain is to bind substrate, the Catch-replaced midnolin should stimulate proteasome activity as efficiently as wild-type because the substrate is already effectively "provided". Conversely, if the Catch domain possesses an additional "positioning" or "optimized delivery" function, then the Catch-replaced midnolin should show markedly lower stimulated efficiency, resembling or only slightly exceeding that of Catch-deletion mutant. The results show that the Catch-replaced midnolin, despite having the substrate pre-bound, showed a significantly lower degradation efficiency than to the wild type, much closer to the Catch-deleted mutant MIDNΔ(112-336). These results demonstrate that the Catch domain provides a specific function beyond simple substrate binding, consistent with its proposed role in actively positioning the substrate for optimal delivery to the proteasome."

References

[3] Gu, X. et al. The midnolin-proteasome pathway catches proteins for ubiquitination-independent degradation. Science 381, eadh5021, doi:10.1126/science.adh5021 (2023).

19. On page 11, there is no description of the various states relaying completely on the reader's good knowledge of the proteasome. Moreover no comparison is give side by side to other published structures. This makes the manuscritp difficult to read. Moreover, the states described as well as the whole Figure 6b is extremely reminescent of the paper by Arkinson et al., 2004 (https://doi.org/10.1101/2024.11.08.622731) and this paper is not even cited. We also find the proposed model of movement associated to Mg2+ release not very sound considering that the movement described is of 2.3Å so way below the overall resolution of the map (3Å at best).

The discussion part is again missing citations. More in details:

We agree that the original description relied too heavily on the reader's specialized knowledge. We have added the references, including the paper by Arkinson et al., 2025 (https://doi.org/10.1038/s41594-025-01695-2). In the revised manuscript, we have added a concise introductory paragraph as follow:

Page 12; Line 272-280

" Our current understanding of proteasomal translocation comes from cryo-EM studies of 26S proteasomes with substrates in processing states. These studies revealed that the AAA+ motor (RPT1–6) adopts right-handed spiral-staircase conformations, with typically 4–5 RPT subunits engaging the substrate via their pore-1 loops and 1–2 subunits (seam subunits) positioned off the substrate[22]. These observations led to the 'hand-over-hand' translocation model, wherein the sequential ATP hydrolysis cycle coordinates the movement of the seam subunit to drive stepwise substrate translocation. Our cryo-EM structures capture the midnolin-proteasome complex within this dynamic cycle, providing snapshots of specific substrate-engaged translocation intermediates.[14-16,23]. "

We have added the references, including the paper by Arkinson et al., 2025 (https://doi.org/10.1038/s41594-025-01695-2)

We agree with the reviewer that attributing the subtle (~2.3 Å) movement specifically to Mg^{2+} release is not robust given the ~3 Å global resolution. We have revised the sentence "Mg^{2+} dissociation actively drives substrate translocation through coordinated mechanochemical coupling" as follow:

Page 13; Line 293-296

" Collectively, these four sequential states provide a structural visualization of the hand-over-hand cycle[14-16] and indicate a potential regulatory role for Mg^{2+} release in coordinating the sequential rearrangements of the ATPase subunits."

20. In line 307, the authors state: "Additionally, the IDR2 region of the Catch domain guides midnolin itself into the proteasome for degradation after substrate delivery." Do authors have any biochemical data confirming this?

Our structural and biochemical data indicate that the presence of the N-terminal disordered region (IDR1) leads to the degradation of MIDN (Fig. 4c-g). Therefore, we corrected this sentence as "Additionally, the N-terminal disordered region (IDR1) region may guide midnolin itself into the proteasome for degradation after substrate delivery. "

21. In line 313, it states: 'Our findings indicate that ATP hydrolysis plays a pivotal role in the displacement of the proteasome's AAA+ module, with Mg^{2+} dissociation also actively facilitating substrate translocation (Fig. 6b)'. This is quite misleading as it is based on observation of motion below the actual resolution limit of the data.

We sincerely thank the reviewer for raising this critical point regarding the interpretation of our structural data. The reviewer is correct: attributing the observed subtle conformational shifts (~2.3 Å) near the global resolution limit (~3 Å) specifically to Mg^{2+} dissociation and describing it as "actively facilitating" translocation is statistically tenuous and may overstate the mechanistic evidence available from the current dataset. We apologize for this overinterpretation in our original wording.

We have revised the manuscript to ensure our conclusions are framed with appropriate caution as follows:

Page13; Line 288-296

" A notable transition occurs between MIDN-PS_{RPT1} and MIDN-PS_{RPT5}. In MIDN-PS_{RPT1}, RPT3 retains a bound Mg^{2+} ion, while in MIDN-PS_{RPT5}, this Mg^{2+} is released (Fig. 5d, e). Concomitantly, the entire AAA+ ring undergoes a ~2.4 Å downward rigid-body shift (Fig. 5f). The spatiotemporal correlation between Mg^{2+} release and ring movement suggests that Mg^{2+} dissociation from RPT3 may act as a coordinated trigger, working in concert with ATP hydrolysis to facilitate the stepwise substrate translocation

observed in our structural continuum (Movie S1). Collectively, these four sequential states provide a structural visualization of the hand-over-hand cycle[14-16] and indicate a potential regulatory role for Mg^{2+} release in coordinating the sequential rearrangements of the ATPase subunits."

22. In line 322, it states: 'These findings suggest that asymmetric ATP hydrolysis by the proteasome ATPase is a widespread phenomenon and may not be substantially related to substrates. The underlying reasons for this asymmetric hydrolysis merit further investigation'. This is not true, there are a wealth of data and discussions about the asymmetry of the proteasome and all other AAA+ unfoldases.

Answer: We thank the reviewer for this critical comment. You are absolutely correct that "there are a wealth of data and discussions about the asymmetry of the proteasome and all other AAA+ unfoldases. " Our original line 322—"These findings suggest that asymmetric ATP hydrolysis by the proteasome ATPase is a widespread phenomenon…"—was inappropriate because it wrongly implied that the universality of this asymmetry was first or primarily "discovered" or "suggested" by our study. In reality, the asymmetric, spiral-staircase mode of sequential ATP hydrolysis by the proteasomal AAA+ motor has been definitively established and is widely accepted as a fundamental principle (e.g., de la Peña et al., Science 2018; Dong et al., Nature 2019). Our work simply visualizes how this established mechanism operates within the specific context of midnolin-mediated, ubiquitin-independent degradation. Accordingly, we have revised the offending statement and added citations to the key studies that elucidated proteasomal asymmetry.

Page15-16; Line 351-357

" Consistent with the well-established asymmetric mechanism of the proteasomal AAA+ motor[14,21], our structures capture consecutive spiral-staircase conformations (PS_{RPT6}, PS_{RPT2}, PS_{RPT1}, PS_{RPT5}) during midnolin-mediated substrate translocation. This confirms that the hand-over-hand translocation mechanism is operational in this ubiquitin-independent pathway. The specific distribution and progression of these states in our dataset provide a detailed view of the mechanochemical cycle in the context of midnolin engagement, although the general principle of asymmetry is pervasively established across AAA+ ATPases. "

References

[14] Zhang, S. et al. USP14-regulated allostery of the human proteasome by time-resolved cryo-EM. Nature 605, 567-574, doi:10.1038/s41586-022-04671-8 (2022).

[30] Arkinson, C., Gee, C. L., Zhang, Z., Dong, K. C. & Martin, A. Structural landscape of the degrading 26S proteasome reveals conformation-specific binding of TXNL1. Nature structural & molecular biology 32, 2403-2415, doi:10.1038/s41594-025-01695-2 (2025).

Other points (regarding the methodology):

23. The authors state that the 26S complex concentration was measured after the purification; however, there is no information about the concentration of the complex used for grid preparation.

Answer: We have added the concentration of the complex used for cryo-grid preparation. Although the sample concentration was low, we obtained data of sufficient quality to determine the target structure by employing a two-round sample-blot strategy on graphene-oxide (GO) grids. For the in-vitro reconstituted complex (e.g., MIDN-EGR1-26S), we also added the concentration information.

Page27; Line 607-620

" The complex was concentrated to approximately 0.3 mg/mL in size-exclusion buffer, flash-frozen in liquid nitrogen, and stored at -80°C.

The E. coli co-expressed MBP-EGR1(101–200) and MBP-midnolin complex were mixed with the purified 26S proteasome at a 2:1 molar ratio, incubated on ice for 30 seconds , and immediately prepared for cryo-EM analysis. For cryo-EM grid preparation, the sample was thawed on ice and used at this concentration. The reconstituted complex(MIDN-EGR1-26S) was immediately used for cryo-EM grid preparation at a final concentration of approximately 4 mg/mL. 3 μL of the freshly concentrated sample was applied onto a freshly glow-discharged (at 20 mA for 70 seconds, negative charge) Quantifoil R1.2/1.3 300-mesh Au grid. Grids were blotted for 3 seconds under 100% humidity at 4°C using a Vitrobot (Thermo Fisher Scientific), then plunge-frozen in liquid ethane cooled by liquid nitrogen. The entire process from final concentration adjustment to plunge-freezing was completed within 10 minutes to

ensure complex stability and homogeneity. Frozen grids were stored in liquid nitrogen until data collection."

24. There is currently no information provided regarding the software utilized for cryo-EM data processing and analysis. Similarly, Figure S5 seems to be prepared using ESPript. The authors should specify this and cite the relevant software utilized.
Answer: We have conducted a comprehensive review to ensure that every procedure involving specialized software or online tools, ranging from cryo-EM data processing to "Isothermal titration calorimetry (ITC) ", and "Surface plasmon resonance (SPR) ", explicitly states the name and version of each software package or server used, and provides the corresponding citations.

" Multiple sequence alignment was performed using ESPript (Version 3.2)[49]."

25. In the description of some of the cryo-EM data sets, the number of collected movies and particles in the initial stacks is missing. Authors should consider rearranging the sections that describe the processing of different samples to align with the order of the figures depicting the pipelines.
Answer: We fully agree with your assessment and undertook a systematic revision of the relevant sections. Following your practical suggestion, we re-ordered the subsections in “Methods” dedicated to data processing and adjust all in-text citations accordingly, ensuring an intuitive, linear path from narrative to figure.

26. Authors use "two-dimensional" and "3D" interchangeably. I suggest using the simpler 2D/3D notation for clarity.
Answer: We have now used 2D/3D notation for clarity.

27. On page 22, what do the authors mean by "subjected to AAA+ ATPase domain-focused masking"? Are they referring to local refinement focused on the AAA region? The pipeline shown in Extended Figure 4 does not clarify the steps undertaken by the authors. One of the initial steps states "masked on AAA+ ATPase domain," and then four classes (#0, #5, #6, #19) from the previous (unmasked?) classification are presented. We suggest the authors redraw their data processing pipeline, showing all

the maps for the same classification side by side. They should then indicate by separate outlines which classes were used for further data processing, similar to what they did in Figure S7.

Answer: Thank you for this specific and highly constructive comment on our data-processing workflow. We apologize for the lack of clarity and thoroughly revised both the text and the figures according to your suggestions. "Subjected to AAA+ ATPase domain-focused masking" indeed refers to local refinement focused on the AAA+ region. We redrew the data processing pipeline and corrected this sentence as follow.

Page 29; Line 653-657

" Particles from Classes 0, 5, 6, and 19, which exhibited features of substrate-engaged states, were selected. To improve the resolution of the AAA+ motor, these particles underwent local refinement using a soft mask focused on the AAA+ ATPase domain, yielding two locally refined proteasome complex structures at 3.45Å (PS_{RPT1}, PDB: 9MBP, EMD-63766, 40,572 particles) and 3.66Å (PS_{RPT5}, PDB: 9MBQ, 23,186 particles), respectively. "

28. On the same page, the authors state, "Notably, 63,758 particles selected through RPN1 region masking from the proteasome particle pool produced a high-resolution RPN1 subunit structure". However, the data processing pipeline (Extended Data Fig. 2) shows that the particles from class 0 and class 1 were pulled together, not "selected through RPN1 region masking."

Answer: Thank you for this correction. The original description was indeed imprecise. We have revised the text to clarify this procedure: particles from classes 0 and 1 were selected, followed by RPN1-focused masking and local refinement. The flowchart in Extended Data Fig. 2 has been updated with explicit labels (e.g., "Particles selected for RPN1-focused analysis") to reflect this accurately.

Page 29;Line 658-668

" To resolve the interaction between midnolin's αHelix-C and RPN1 at high resolution, we selected particles from Classes 0 and 1, which represented substrate-engaged complexes. Notably, 63,758 particles selected through RPN1 region masking from the proteasome particle pool produced a high-resolution RPN1 subunit structure (PDB: 9MBO, EMD-63775, 3.07Å) after local refinement. For Classes 1-4 and 7-18

(combined 97,538 particles) underwent further 3D classification (20 classes), subsequent screening of 9,022 particles from Class 11 through 19S-focused masking generated a 4.48Å map (PS_{RPT6}, PDB: 9U4M, EMD-63850) of 19S regulatory particles. Additionally, 25,990 particles selected from Classes 0, 1, and 10 using 19S-specific masking were processed identically to obtain an improved 19S structure (PS_{RPT2}, PDB: 9U3L, EMD-63817) at 3.72Å resolution. The other classes (Classes:2-9 and 12-19) represented unassigned processing states. The final maps were further processed using EMReady[43]."

29. On page 23, the authors mention "high-resolution 3D classification." However, the representative maps for these classes appear to be of lower resolution compared to those from other 3D classification jobs shown. How does this high-resolution classification differ from the other 3D classifications that the authors do not specify as "high-resolution"? Moreover, 3D classification was performer generating far two many classes based on numer of particles available (e.g. 160k particles into 20 classes - line538). It seems that the particles just got distributed equally in the various classes, which indicates that the 3D classification didn't really work.

Answer: Thank you for pointing out this inaccuracy. The term "high-resolution 3D classification" referred to the use of a 3-Å filter resolution, not the resolution of the resulting maps. We have replaced this misleading term in the text (line 615) with the precise description: "3D classification after global refinement using a 3 Å filter resolution."(Page 29;Line 705-707)

Concerning the particle classification, our initial strategy of generating a larger number of classes (e.g., 20 from ~160k particles) was motivated by the goal of maximally resolving the diverse conformational states of the proteasome complex. While this approach distributed particles across multiple classes, it served as a necessary exploratory step to identify all potential states.

30. For symmetry expansion, particles should first be refined with C2 symmetry applied. Authors should specify the symmetry of their refinement for transparency and reproducibility of the analysis.

Answer: Thank you for highlighting this essential detail for reproducibility. We confirmed that the initial global refinement of the 26S proteasome complex was performed with C2 symmetry applied, prior to symmetry expansion, as is standard practice. This specification has been explicitly stated in the revised "Data processing of MIDN-EGR1-human 26S proteasome complex".

Page 31; Line699-705

" Prior to symmetry expansion, the selected particles were subjected to Non-uniform refinement with C2 symmetry imposed. The resulting alignment parameters were then used to perform symmetry expansion, doubling the particle count. The expanded particles were subsequently re-extracted, re-centered, and used for downstream focused classification and refinement steps. Since the 19S regulatory particle in the resulting 26S proteasome structure was not centered, symmetry expansion was applied to generate 610,432 particles. "

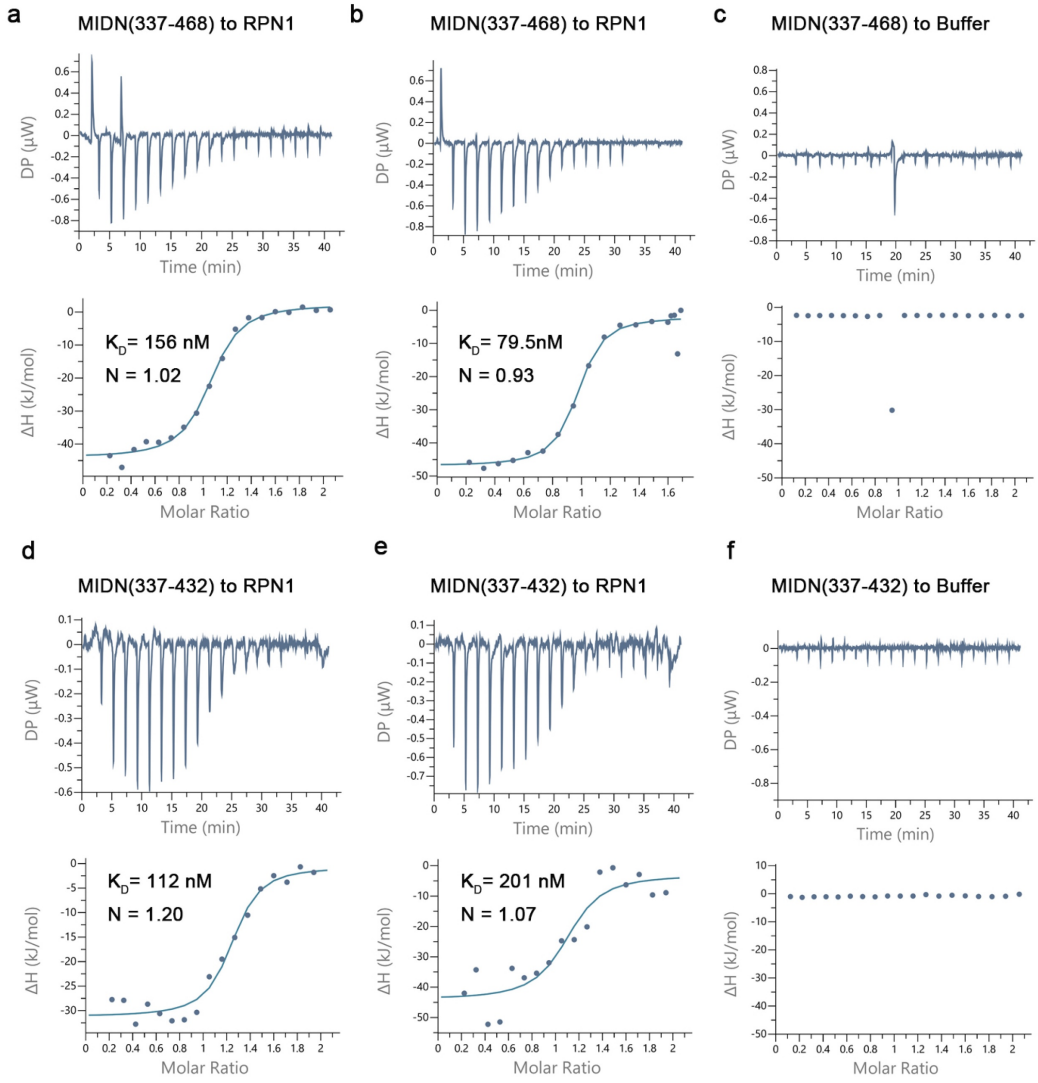
31. There is no description for the processing of "MIDN-EGR1-human 26S 1068 proteasome complex for the resolved Catch domain" shown in Extended Fig. 8.

Answer: We apologize for the incomplete description in the “Methods" section, where we failed to provide a dedicated and comprehensive account of the data-processing workflow corresponding to Extended Data Fig. 8 (Revised Extended Data Fig. 7, "Cryo-EM data processing workflow of MIDN-EGR1-human 26S proteasome complex for resolved Catch domain"). This omission hindered full methodological transparency and reproducibility. In the revised manuscript, we therefore added a new subsection immediately after the existing "Data processing of MIDN-EGR1-human 26S proteasome complex," titled “Data processing for the resolution of the Catch domain in the MIDN-EGR1-26S complex, " which clearly detailed every step outlined in Extended Data Fig. 8 (Revised Extended Data Fig. 7).

32. The description of the ITC and SPR experiments reads more like a general overview of the protocol and lacks the specific details necessary to assess and reproduce the results. For the ITC experiments, the authors do not specify whether a blank titration was performed to determine the nonspecific heats of mixing, dilution, and any potential buffer mismatch. Therefore, in addition to clarifying this in the methods section, the

authors should provide reviewers with the raw titration curves from the actual experiments, as well as the results from the blank experiments.

Answer: We apologize for the overly general description in the original manuscript. We have revised the main text as the reviewer suggested.

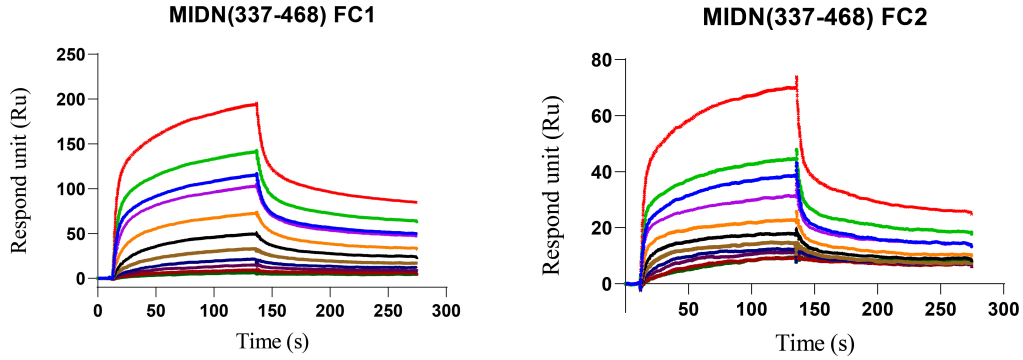


ITC measurements of binding affinities between midnolin (337-468) or midnolin (337-432) and RPN1. The binding constants (K_D values) and stoichiometries (N) are indicated. (a-b) Titration curves of MIDN(337 468) into RPN1. (c) Negative control titration of MIDN(337-468) into buffer. (d-e) Titration curves of MIDN(337-432) into RPN1. (f) Negative control titration of MIDN(337-432) into buffer.

33. In the description of SPR measurements, there is no information about the concentration of the proteins used for the experiment or the amount of protein

immobilized on the sensor. Were any negative controls included to test for nonspecific binding?

Answer: Thank you for raising these essential points. We have now fully detailed the SPR methodology in the revised manuscript to ensure reproducibility. We added the follow information in the "Methods" section: the concentrations of both the analyte and ligand used, the specific immobilization level of the ligand on the sensor chip (in response units), and the use of a blank reference flow cell as a negative control, with all binding signals double-referenced against it for specificity.



To correct for non-specific binding signals, an independent reference flow cell(FC2) containing no immobilized ligand was employed, and the response signals of the analyte in all active channels were double-referenced by subtracting the signal from this blank control channel before kinetic analysis.

34. Authors should use black mesh or another more contrasting color combination, as it is almost impossible to glean the density details from Extended Data Fig. 4 in its current form.

Answer: Thank you for your valuable feedback on the visual clarity of our figures. We fully agree with your observation that the low-contrast grid colors in Extended Data Fig. 4 make it difficult to discern density details, thereby compromising the structural information the figure is intended to convey. In response, we have revised Extended Data Fig. 4 to enhance contrast so that the key structural densities are now clearly visible.

35. NLS (same as all other abbreviations used) should be explained to facilitate understanding of the text

Answer: Thank you for your valuable feedback on the readability of the manuscript. We have performed a thorough review of the entire text to ensure that the above rule is applied consistently and that no abbreviation is used without being defined first.

36. For the sake of accuracy, the color scalebar in Fig.2a should include units (e.g., kT/e)

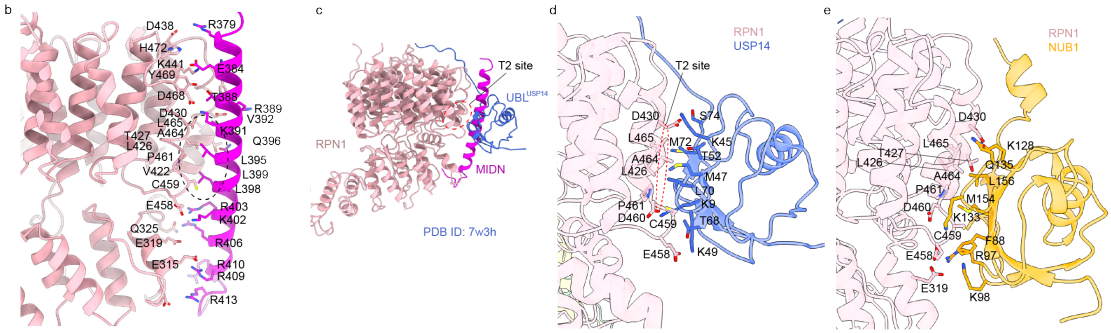
Answer: Thank you for pointing out this issue. We have added the standard unit "kT/e" "to the color scale bar of the electrostatic potential surface in Figure 2a.

Figures:

Figure 2 : the circle in figure2b is barely visible. How does this arrangement compare with the published structure? The measurements in the ITC panel (d,e) should have included full length MIDN and negative controls. Moreover, more than 2 measurements should have been performed.

Answer: To better visualize, we have boldened the circle in Figure 2b.

We compared our identified binding mode with canonical T2-site binders such as USP14 and NUB1. This new panel features a structural comparison among: (1) our resolved αHelix-C–RPN1 complex, (2) the previously reported USP14–RPN1 complex (PDB: 7W3H), and (3) the NUB1–RPN1 complex (PDB: 8USB). Our analysis reveals that all three complexes share a common hydrophobic core anchored by RPN1 residues L426 and L465. They are further stabilized by peripheral salt bridges and hydrogen bonds. A key distinction, however, lies in the fact that our αHelix-C engages RPN1 with a more extensive interaction interface and adopts a distinct linear conformation.



Revised Figure 2. (b) The interaction interface exhibits a tripartite pattern between αHelix-C of midnolin and RPN1. The circle part indicates a hydrophobic center. This central hydrophobic interface is flanked by two polar interaction networks. (c) Superimposition of the atomic models depicting the interaction interface between

USP14(blue) and RPN1(pink) versus the interaction interface between RPN1 and αHelix-C of midnolin (magenta). The T2 site is indicated by a red dashed circle. (d) Side-chain interactions between the USP14 UBL domain (blue) and the RPN1 (pink) T2 site, derived from the USP14–RPN1 complex (PDB: 7W3H). The T2 site is indicated by a red dashed circle. (e) Side-chain interactions between the NUB1 UBL domain (yellow) and the RPN1 (pink) T2 site, derived from the NUB1–RPN1 complex (PDB: 8USB).

We attempted to perform ITC experiments using purified full-length proteins. However, due to the high protein requirements of ITC and the low yield of the full-length protein, we were unable to obtain sufficient protein for the ITC measurements. As an alternative, we conducted surface plasmon resonance (SPR) assays to measure the binding of MIDNΔ(112-336) to the 26S proteasome. We employed this truncated form, which lacks the Catch domain, to avoid potential interference from the substrate with binding affinity. The measured binding affinity (162 nM) was essentially consistent with that between the isolated αHelix-C and RPN1 (130 nM), indicating that the interaction of MIDN with the proteasome is primarily mediated by αHelix-C. On the other hand, we have included negative controls and performed triplicate experiments. The binding constant (K_D) values are reported as "mean ± standard deviation" in both the main text and the figures to provide statistical reliability.

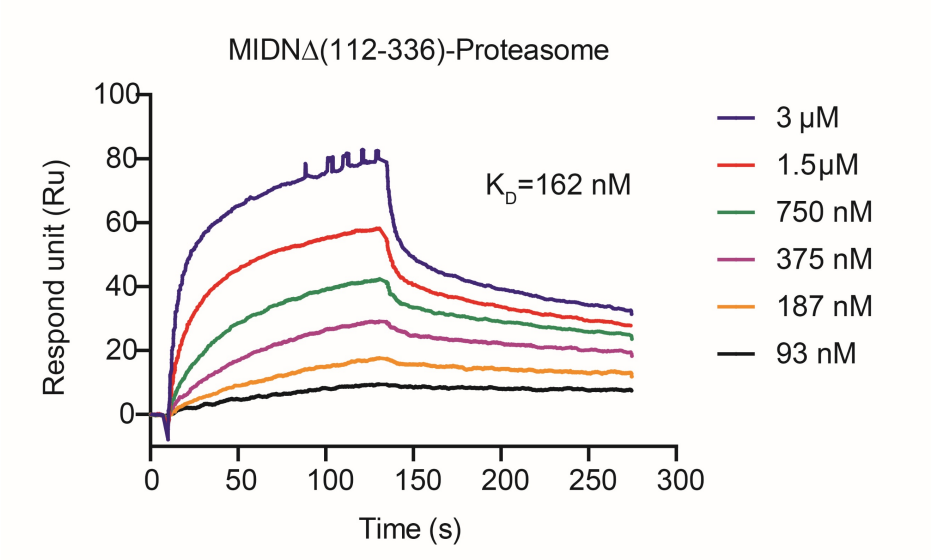


Figure 4b is based on image processing. How many particles were thrown away? And why not reporting these numbers for all the other maps as well?

Answer: Thank you for this important question regarding particle accounting and data transparency. We have systematically reported particle counts for the datasets in the revised manuscript.

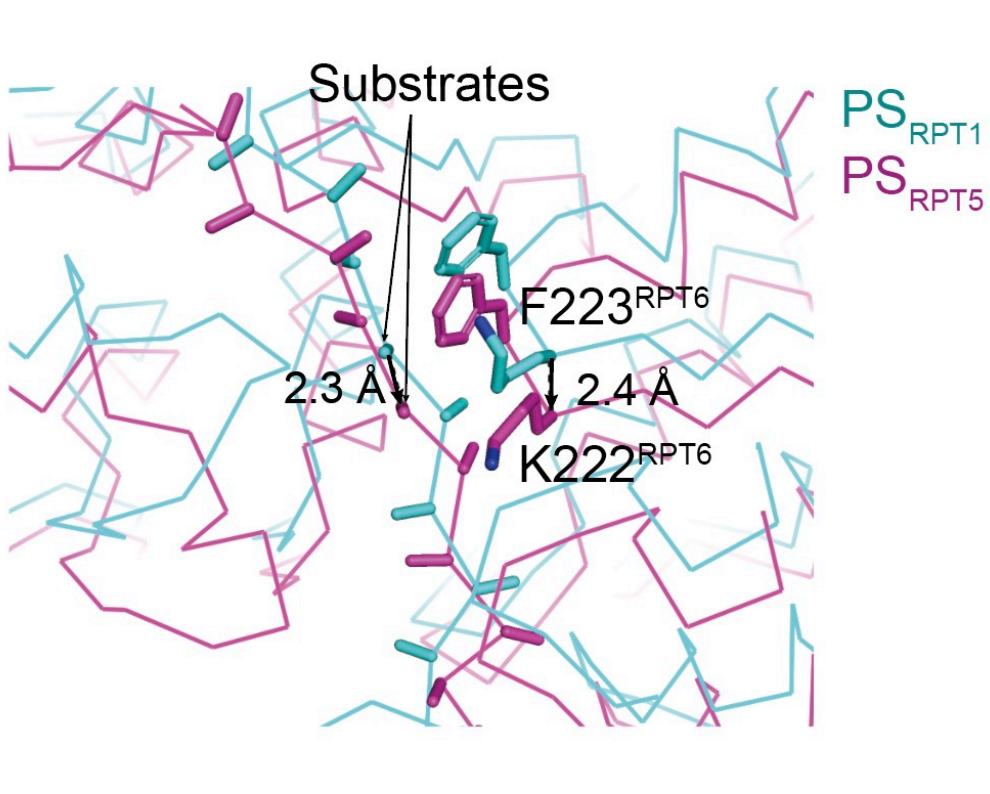
For Figure 4b, the population percentages were derived from a 3D classification of the MIDNΔ(112-336)-26S dataset. The total particle pool for this analysis comprised 84,995 particles. Following classification, 54,459 particles were assigned to the substrate-engaged Processing State, and 29,705 particles to the Resting State. The negligible remainder (~800 particles) corresponded to poorly aligned or ambiguous classes, which were excluded from high-resolution refinement.

Notably, parallel analysis of the full-length MIDN–proteasome datasets consistently yielded only substrate-engaged states, with no detectable population of the Resting State. This contrast directly underscores the stabilizing role of the Catch domain.

We have updated the processing workfolw to clearly annotate particle counts at each key step. We appreciate your suggestion to enhance the rigor of our data reporting.

Figure 5f is very confusing.

We thank the reviewer for this critical feedback. We agree the original Figure 5f was visually complex. In response, we have simplified the figure to provide a direct, side-by-side comparison of the two key conformational states. The revised version uses distinct colors (cyan for PS_{RPT1} and magenta for PS_{RPT5}) to separately depict these states, focusing on the RPT6 subunit to represent the AAA+ ring's motion. This new visualization clearly demonstrates the coupled movement: the downward shift of the Rpt6 pore-1 loop is precisely aligned with the concurrent downward translocation of the substrate. The revised figure removes extraneous detail to deliver a clear message that the ATPase cycle drives stepwise substrate translocation through coordinated pore-loop motions.



Revised Figure 5f. Structural superimposition shows the movement of the pore-1 loop between PS_{RPT1} and PS_{RPT5}. Specifically, Lys222, Phe223 in RPT6 and their coordinated substrate residue shift downwards by 2.4 Å and 2.3 Å, respectively.

Figure 6b is almost copied from Arkinson et al., 2024 and yet the paper is not even cited.

Answer: We sincerely apologize for the oversight in not citing Arkinson et al., 2024, as the core model for the AAA+ motor cycle in Figure 6b is indeed adapted from their work. We now place a clear and primary citation to Arkinson et al. at the very beginning of the paragraph in the main text that describes Figure 6b and its mechanism.

Reference

[22]Arkinson, C., Gee, C. L., Zhang, Z., Dong, K. C. & Martin, A. Structural landscape of the degrading 26S proteasome reveals conformation-specific binding of TXNL1. Nature structural & molecular biology 32, 2403-2415, doi:10.1038/s41594-025-01695-2 (2025).

The manuscript requires careful proofreading to correct numerous typos, grammatical errors and to improve the overall readability of the text.

We hope that these comments will help the authors to improve the manuscript.

Answer: Thank you for your important comments on our manuscript. We fully agree that the original text contains typographical errors, grammatical issues, and some descriptions that are not fluent enough, which indeed affect the overall clarity and professionalism of the text. We apologize for this and have conducted a thorough language proofreading and polishing of the entire text. Thank you again for your careful reading and valuable suggestions.

Reviewer #2 (Remarks to the Author):

The manuscript under review provides interesting and novel insights into the mechanism by which midnolin targets proteins to the proteasome for degradation, and more broadly into the proteasome's mechanism of action.

Midnolin was recently discovered as a novel mediator of ubiquitin-independent proteasomal degradation. It was found to target products of immediate early genes for degradation by binding simultaneously to target proteins and the proteasome, shuttling the former for degradation without the need for ubiquitination. Initial studies suggested that proteasome binding occurs by an N-terminal domain and the C-terminal UBL domain, with the central Catch domain binding substrates.

The study under review here puts this model on solid footing by revealing a series of cryo-EM structures of the tertiary complex between substrate, midnolin, and the proteasome. I cannot assess the technical quality of the cryo-EM structure determination, but taken at face value, the structures provide valuable insights. The binding mode between midnolin and the proteasome is quite novel, including the detailed insights into the interaction between the N-terminal helix and Rpn1 and that of the C-terminal UBL domain with Rpn11. Pinning the N- and C-terminal domains of midnolin to the proteasome then allows midnolin's central catch domain to position bound substrate at the entrance to the degradation channel to be engaged by the proteasome.

The study caught the proteasome in a series of different conformations that are likely to represent different steps in the degradation cycle. Analysis of these structures suggests that Mg++ dissociation drives (together with ATP-hydrolysis) the proteasome through the conformational cycles that lead to substrate unfolding and translocation to the proteolytic sites. As far as I'm aware, the suggested role of Mg++ in the mechanism is novel and important.

There are a couple of conclusions in the manuscript that are not properly supported by the data presented, interesting as they are. I recommend that these parts of the manuscript be revised to indicate the speculative nature of the conclusions. For example, I think that the fact that midnolin is difficult to purify and aggregates when not co-expressed with a substrate can have many other interpretations - most simply that some scaffolding factor is missing at the right stoichiometry in the experimental setup. Similarly, the suggestion that the proteasome can digest midnolin by engaging the protein at its IDR2 is interesting and even plausible, but I propose that the autodegradation of midnolin as a regulatory mechanism is discussed as a hypothetical to be explored in the future.

Answer: We sincerely thank the reviewer for the positive assessment of our work and for recognizing the novelty of the mechanistic insights. We fully agree that certain conclusions should be presented more cautiously to better distinguish between experimental observations and mechanistic inferences.

Regarding the observation that midnolin is difficult to purify and tends to aggregate without co-expressed substrate: We revised the text to clarify that this finding primarily relates to the practical requirements for sample preparation in our experimental system. We presented it as an intriguing observation whose in vivo relevance and underlying mechanism (including potential explanations such as the absence of a scaffold factor) remain subjects for future investigation, rather than a definitive conclusion.

Regarding the hypothesis that "the proteasome may degrade midnolin via IDR1" and its potential regulatory role: Our structural and biochemical data indicate that the presence of the N-terminal disordered region (IDR1) can lead to the degradation of MIDN (Fig. 4c-g). Therefore, we corrected this sentence as "Additionally, the N-terminal disordered region (IDR1) region may guide midnolin itself into the proteasome for degradation after substrate delivery."

We are grateful for these insightful suggestions.

Reviewer #3 (Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Answer: Thank you for reviewing our manuscript and for your valuable comments. We greatly appreciate Nature Communications' initiative to formally involve early-career researchers in peer review, which strengthens the scientific community. Your participation as a co-reviewer brought an insightful perspective to this process, and we are sincerely grateful.

Reviewer #4 (Remarks to the Author)

Proteasome is the main degradation machine in eukaryotic cells. While ubiquitin-dependent degradation is well studied, the non-canonical degradation mechanism mediated by midnolin remains elusive. In this study, Zhu et al. investigates the molecular mechanisms of the midnolin in targeting substrates to the proteasome for degradation. The authors combine structural and biochemical analyses to characterize midnolin's interactions with the proteasome, revealing how its αHelix-C and Ubl domains bind to proteasomal subunits RPN1 and RPN11 respectively, to direct substrates to the proteasome entry port via the Catch domain. The authors captured four continuous states of the midnolin-proteasome complex, revealing the functional dynamics of the proteasomal ATPase complex, and a novel role of Mg2+ dissociation in coordinated mechanochemical coupling. Overall, this study presents compelling structural evidence and valuable insights into the mechanism of midnolin in mediating non-ubiquitination-mediated degradation. However, the current study relies heavily on static structural observations, and it would be important to complement these with functional validation such as kinetic assays of substrate recognition and degradation, together with mutational analyses of key midnolin domains, as detailed below.

Questions:

1. Lines 100-101: There is grammar issue of the sentence.

Answer: Thank you for pointing out the grammatical issue. We have restructured the sentence following your suggestion to ensure grammatical correctness and logical clarity. We appreciate your help in improving the language quality of our manuscript.

Page 5; Line 98-105

" After 3D classification, we determined four distinct structural states (designated MIDN-PS$_{RPT1}$, -PS$_{RPT5}$, -PS$_{RPT2}$, and -PS$_{RPT6}$ for subsequent discussion). Among these states, the RPN1 subunit exhibited varying degrees of resolution and occupied distinct positions relative to the AAA+ motor (Extended Data Fig. 2, 3 and Supplementary Table 1). The 3D classification yielded structural states that all represent substrate-processing conformations. This includes classes reconstructed from particles that were not assigned to a dominant state but still contributed to the overall conformational landscape. The highest resolution (2.87 Å) was achieved for the MIDN-PS$_{RPT1}$ state (Fig. 1a)."

2. It is interesting that co-expression of Catch domain and EGR1 stabilizes the Catch domain and facilitates their binding. However, this may reflect artifacts arising from using the isolated Catch domain. To clarify this, it would be important to perform the gel filtration assays with full-length midnolin with or without co-expression of EGR1.

The proposed working model that midnolin co-expresses with substrates to achieve rapid targeted degradation raises questions about substrate specificity. With elevated midnolin levels, how is selectivity maintained, and could this result in unintended degradation of non-target substrates?

A minor point: Extended Data Fig. 6g is not cited in the text.

Answer: Thank you for these important comments.

We agree that using the isolated Catch domain may not fully represent the full-length protein. To address this, we performed the suggested control experiment. We purified full-length midnolin expressed alone or co-expression with EGR1. As expected, the complex shows a single, symmetric peak, whereas midnolin alone showed significant

oligomerization/aggregation, confirming that substrate binding is essential for stabilizing the full-length midnolin.

We agree that selectivity is a critical aspect of our model. In the revised Discussion, we will emphasize that specificity is primarily ensured by the high-affinity, selective binding of the Catch domain to specific β-strand degrons in substrates like EGR1 (Nardone et al., Mol Cell, 2025, Gu et al., Science 2023). We proposed that spatiotemporal coupling, through co-expression and localization, further minimizes off-target degradation, while acknowledging that other regulatory layers may exist and warrant future study.

We have cited Extended Data Fig. 6g in the text.

Page 9; Line 193-196

" To elucidate the molecular mechanism of midnolin-mediated substrate delivery to the proteasome entry port, we purified human proteasomes via an mCherry-hexahistidine-twin-strep-tagged RPN11 (Extended Data Fig. 6g) and incubated them with purified midnolin-EGR1(101-200) complex before plunge-freezing. "

References

[3] Gu, X. et al. The midnolin-proteasome pathway catches proteins for ubiquitination-independent degradation. Science 381, eadh5021, doi:10.1126/science.adh5021 (2023).

[13] Nardone, C. et al. Structural basis for the midnolin-proteasome pathway and its role in suppressing myeloma. Mol Cell 85, 2597-2609 e2511, doi:10.1016/j.molcel.2025.05.030 (2025).

3. In the introduction, the authors raise a question of whether midnolin first binds its substrates prior to proteasome association or constitutively engages the proteasome before recruiting substrates. This key question is not sufficiently addressed in the current work. The structural characterization of midnolin-substrate-26S proteasome complex and co-expression assays do not rule out the possibility that midnolin engages the proteasome before substrate loading.

Answer: We thank you for raising this profound and pivotal question. Following your suggestion, we expanded the “Discussion” in the revised manuscript to address the issue more deeply and cautiously, using our data to argue preferentially for one model

while explicitly acknowledging that our current structural and biochemical results cannot temporally resolve which of the two pathways outlined in the Introduction operates first. The captured complex is a single "snapshot" along the degradation trajectory, not a time-resolved record of assembly; consequently, we cannot provide definitive proof. Nevertheless, integrating our new findings allows us to present a logically and energetically more favorable scenario: midnolin first binds its substrate and then is the binary complex recruited to the proteasome.

Page 14-15; Line 319-332

" Our findings support a sequential “capture-before-delivery” mechanism for midnolin-mediated proteasomal degradation. In this model, substrate binding to the Catch domain is a prerequisite step that stabilizes midnolin, particularly the Catch domain, into a soluble and functional conformation. This substrate-occupied complex constitutes a stable “ready-to-degrade” module, primed for proteasomal engagement. This capture-first mechanism, in which specificity is determined by the initial engagement of the substrate with midnolin, functionally parallels ubiquitination. This mechanism acts as a "midnolin tag" that marks the substrate for subsequent proteasomal delivery and degradation. On the other hand, in the absence of substrates, the Catch domain of midnolin exhibits a deficiency in a central β-sheet structure, which predisposes it to instability and subsequent aggregation. Although midnolin can express independently, its capacity to degrade substrates is probably rather limited on its own. Interestingly, it has been reported that midnolin is required for the DNA-binding activity of EGR1[29], which is consistent with our observation that midnolin co-express with substrates. Whether the DNA-binding activity of other substrates is also dependent on midnolin deserves further investigation."

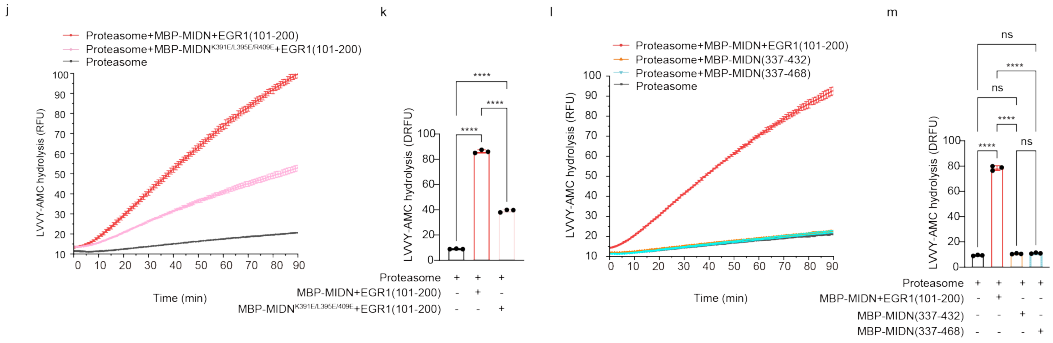
References

[29] Chiba, A. et al. Midnolin, a Genetic Risk Factor for Parkinson's Disease, Promotes Neurite Outgrowth Accompanied by Early Growth Response 1 Activation in PC12 Cells. Mol Cell Biol 44, 516-527, doi:10.1080/10985549.2024.2399358 (2024).

4. The functional importance of different midnolin domains in proteasome targeting and degradation requires experimental validations. Specifically, while Fig.2 shows the

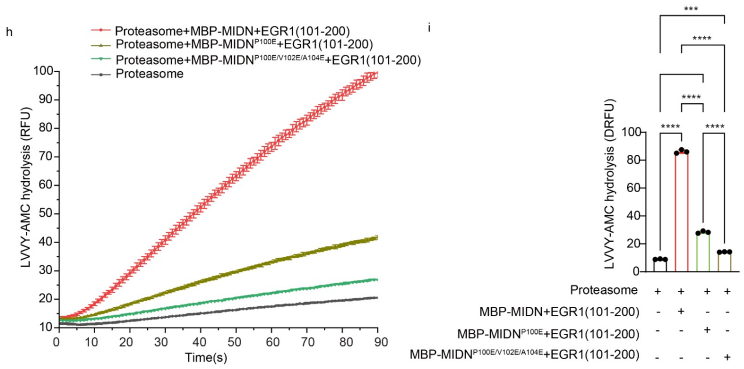
dynamic binding of αHelix-C of midnolin to RPN1, how this interaction could be strengthened by the binding of Ubl domain to the RPN11, or antagonized by the potential interaction between Ubl and RPN1 remains unclear. Structure-based site-directed mutagenesis of these domains followed by functional readouts such as binding kinetics and degradation rates would help disentangle their functional contributions to proteasome targeting.

Answer: Thank you very much for this crucial suggestion. Although our structural work has revealed how each midnolin domain engages proteasome subunits (Ubl–RPN11 and ahelix-C–RPN1), it remains unclear whether these interactions act independently, cooperatively, or regulate one another. Without functional validation, our mechanistic understanding would stop at static correlations. We fully agree that structure-guided point mutations coupled to functional assays are the "gold standard" for dissecting the contribution of each domain. To answer your question directly and rigorously, we designed and performed point-mutation experiments that quantify how each domain contributes to degradation efficiency. Our biochemical data demonstrate that mutations in either the Ubl or the aHelix-C domain significantly impair midnolin's activity. These results indicate that the full-length, intact midnolin protein is required to achieve maximal substrate degradation efficiency.



Revised Figure 2. **(j)** *In-vitro* peptidase activity was monitored over 90 min using the fluorogenic substrate Suc-LLVY-AMC (10 μM) for the 26S proteasome alone (black), or with MBP-MIDN$^{K391E/L395E/R409E}$ (pink), with wild-type MBP-MIDN (red). The y-axis indicates relative fluorescence units (RFU). **(k)** Quantification of *in-vitro* peptidase activity results in (j). RFU changes between 90 min and 0 min are plotted. **(l)** *In-vitro* peptidase activity was monitored over 90 min using the fluorogenic substrate Suc-LLVY-AMC (10 μM) for the 26S proteasome alone (black), or with MBP-MIDN(337-

432) (orange), with MBP-MIDN(337-468) (blue), with wild-type MBP-MIDN + EGR1(101-200) (red). The y-axis indicates relative fluorescence units (RFU). **(m)** Quantification of *in-vitro* peptidase activity results in (l). RFU changes between 90 min and 0 min are plotted. Bars represent means ± SD of three replicates (****, p <0.0001; n =3, p values were calculated using ANOVA with Tukey's post test.) (k and m). ns means not significant.



Revised Figure 3. (**h**) *In-vitro* peptidase activity was monitored over 90 min using the fluorogenic substrate Suc-LLVY-AMC (10 µM) for the 26S proteasome alone (black), or with MBP-MIDN^{P100E} + EGR1(101-200) (dark green), with MBP-MIDN$^{P100E/V102E/A104E}$ + EGR1(101-200) (light green), with wild-type MBP-MIDN + EGR1(101-200) (red). The y-axis indicates relative fluorescence units (RFU). (**i**) Quantification of *in-vitro* peptidase activity results in (h). RFU changes between 90 min and 0 min are plotted. Bars represent means ± SD of three replicates (****, p <0.0001; ***, p <0.001;n =3, p values were calculated using ANOVA with Tukey's post test.)

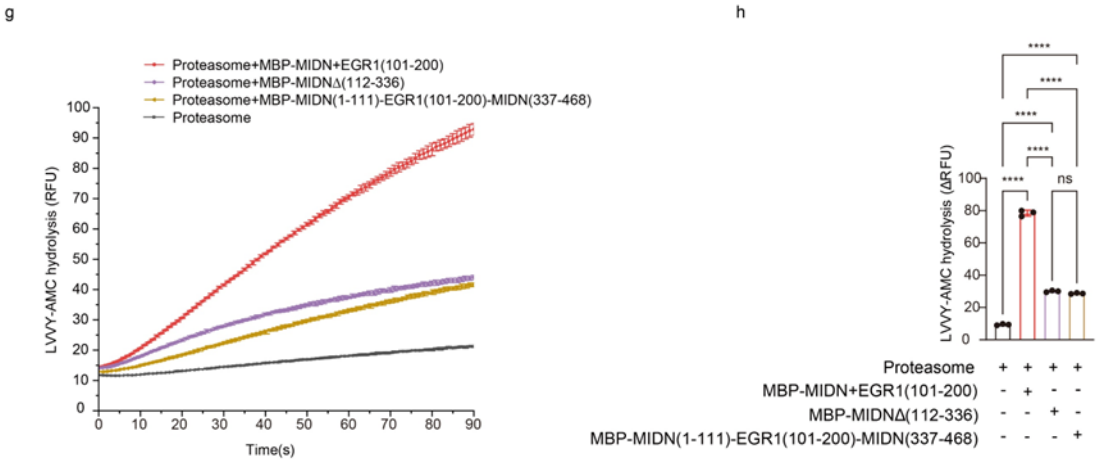
5. The Catch domain might allosterically regulate proteasome states but the current experiments and results in Fig. 4 do not separate this effect from substrate-induced activation. This issue could be addressed by perturbing the substrate recognition site of Catch domain. In addition, structural analysis of substrate-free midnolin-proteasome complexes within the existing datasets may provide useful insight.

Answer: We thank the reviewer for this insightful point. We agree that the current data cannot distinguish whether the effect observed in Fig. 4 stems from the loss of substrate binding or a specific positioning function of the Catch domain.

To directly address this, we performed a decisive domain-swap experiment. We engineered a "Catch-replaced midnolin" construct [MIDN-EGR1(fusion)], where the

native Catch domain is replaced by the known substrate fragment EGR1(101-200), covalently tethered via a flexible linker. This chimera retains constitutive substrate binding but lacks the native Catch interface.

We then compared the degradation efficiencies of three proteins: Wild-type full-length midnolin [MBP-MIDN + EGR1(101-200)], Catch-deletion mutant [MIDNΔ(112-336)], Catch-replaced midnolin [MBP-MIDN(1-111)-EGR1(101-200)-MIDN(337-468)]. If the role of the Catch domain is to bind substrate, the Catch-replaced midnolin should degrade substrate as efficiently as wild-type because the substrate is already effectively "provided." Conversely, if the Catch domain possesses an additional "positioning" or "optimized delivery" function, then the Catch-replaced midnolin should show markedly lower degradation efficiency, resembling or only slightly exceeding that of Catch-deletion mutant. The biochemical results show that the Catch-replaced midnolin, despite having the substrate pre-bound, showed a significantly lower degradation efficiency than the full-length, much closer to the Catch-deleted mutant MIDNΔ(112-336). These results demonstrate that the Catch domain provides a specific function beyond simple substrate binding, consistent with its proposed role in actively positioning the substrate for optimal delivery to the proteasome.



Revised Figure 4. (**g**) *In-vitro* peptidase activity was monitored over 90 min using the fluorogenic substrate Suc-LLVY-AMC (10 μM) for the 26S proteasome alone (black), or with Catch-deleted mutant [MBP-MIDNΔ(112-336)] (purple), with Catch-replaced midnolin [MBP-MIDN(1-111)-EGR1(101-200)-MIDN(337-468)], with wild-type MBP-MIDN + EGR1(101-200) (red). The y-axis indicates relative fluorescence units

(RFU). **(h)** Quantification of *in-vitro* peptidase activity results in (g). RFU changes between 90 min and 0 min are plotted. Bars represent means ± SD of three replicates (****, p <0.0001; n =3, p values were calculated using ANOVA with Tukey's post test.). ns means not significant.

6. Line 209: Extended Data Fig. 11 should be changed to Fig. 10.

Answer: Thank you for pointing out this detail. The "Extended Data Fig. 11" cited in line 209 has been changed to "Extended Data Fig. 10".

7. The legend of Extended Data Fig.4 should be revised with further clarification.

Answer: We have revised the figure legend accordingly.

Extended Data Fig. 4 | Cryo-EM densities of MG132 bound to the proteasome.

(a) Overall surface representation of the proteasome (grey), shown in cross-section, with MG132 (purple) bound to three β-subunits of the core particle (CP) in the MIDN-PS_{RPT5} state. (b) Top view of the CP with seven subunits depicted in cartoon representation (grey). In this view from the MIDN-PS_{RPT5} state, MG132 molecules bound to the three β-subunits (β1, β2, β5) of the distal ring are colored purple, while those bound to the equivalent subunits of the proximal (symmetry-related) ring are colored blue. The β1 (PSMB6), β2 (PSMB7), and β5 (PSMB5) subunits are annotated with their respective catalytic activities: aspartyl protease-like, neutral protease, and carboxypeptidase activities. (c) Cryo-EM density map (grey mesh) of MG132 (purple) bound to three β-subunits of the CP (dark goldenrod) in the MIDN-PS_{RPT5} state. (d) Cryo-EM density map (grey mesh) of MG132 (purple) bound to three β-subunits of the CP (dark goldenrod) in the MIDN-PS_{RPT1} state.

Point-by-point Responses

Structural dynamics of the midnolin–proteasome during ubiquitin-independent substrate turnover" (NCOMMS-25-69834A)

Point-by-point responses to the comments by Reviewer #1:

In the revised version of the manuscript, the authors have made substantial efforts that have significantly improved the work. We are also glad that our suggestions have been positively used in the current manuscript. Better annotations of the presented cryo-EM maps and a more logical arrangement of the results and supplementary material improved readability, facilitating interpretation. Additional biochemical analyses reveal the roles of the domains, while point mutations confirm their importance for binding and activation. Only few issues still need to be addressed before the piece can be recommended for publication.

1. In the abstract and discussion, the authors present the "capture-before-delivery" mechanism and state that the results support it. In reality, as the authors correctly noted in the rebuttal, the presented structural data represent only snapshots along the catalytic cycle and cannot unambiguously confirm the temporal sequence of the binding events. Therefore, the mechanism is only hypothetical and should be presented as such, this is also in line with the points raised by reviewer #4.

Response: We are very grateful to the reviewer for the positive comments on our revision. We fully agree with the reviewer's point. Therefore, we have revised the wording of this model in both the Abstract and Discussion sections, now presenting it clearly as a hypothesis derived from the existing data.

In the Abstract, we have deleted the sentence "Biochemically, we demonstrate that midnolin captures substrates before delivering them to proteasomes through a sequential “capture-and-deliver” mechanism."

In the Discussion section, we revised "Our findings support a sequential 'capture-before-delivery' mechanism..." to "Our findings lead us to propose a sequential 'capture-before-delivery' model for midnolin-mediated proteasomal degradation.".

2. In Figure 1 and Figure 4, the authors present the EMReady treated maps. This is ok, but it should be spelt out in the figures captions and the unsharpened maps should be

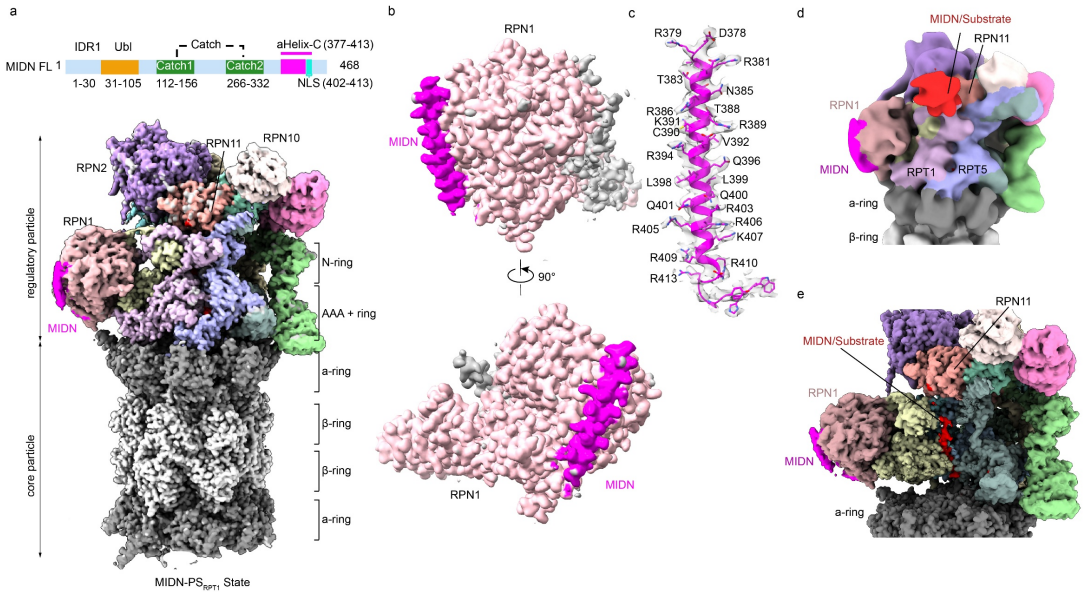
the one deposited to the PDB. Also, the statistics of the model refinements should be made against the unsharpened map.

Response: We thank the reviewer for the professional suggestions. We have made the requested clarifications and revisions accordingly.

In the figure legends for Figure 1 and Figure 4, we have stated that the displayed density maps were post-processed using EMReady for optimal visualization. At the same time, we emphasize that all maps deposited to the PDB and EMDB and used for the statistics of model refinements are the unsharpened maps. We have added relevant statements in the Data processing section and the Model building and refinement section of the Methods.

3. We also suggest that authors add a schematic diagram of the MIDN domain composition in linear form (similar to Figure 4a), with indications of the domains' start and end points in the sequence. This diagram would facilitate the interpretation of MIDN (337-468), MIDN (337-432), etc.

Response: This is an excellent suggestion that significantly improves readers' understanding of the different truncation constructs. We have added a linear schematic diagram of the midnolin domain organization in revised Figure 1a.



Revised Figure 1. Cryo-EM structures of the midnolin-26S proteasome complex in substrate-processing states.

(**a**) Schematic representation of the midnolin (Full length, FL) and representative cryo-EM map of the midnolin-26S proteasome (a processing state, MIDN-PSRPT1), showing the midnolin αHelix-C (magenta) interacting with the RPN1(PSMD2) of 19S regulatory particle. In the Figures, MIDN is short for midnolin. (**b**) Local refinement of RPN1 (pink) highlights the interaction with the midnolin αHelix-C (magenta). (**c**) Atomic model of midnolin αHelix-C and corresponding Cryo-EM map. (**d**) A low-pass filtered map (15 Å) shows midnolin/substrate density (red) at the N-ring entrance of 26S proteasome. (**e**) Cryo-EM map of the midnolin/substrate density in the central channel, reaching through the N-ring, the ATPase ring, and into the degradation chamber of the 20S core particle. Density maps in **a**, **b** and **e** were post-processed with EMReady for optimal visualization.

4. Another suggestion for figure improvement is to prepare the volume renders like in fig.4 d) (as well as in Figure 3b and Figure 4d), using the ChimeraX "volume zone" command with the "new Map" option, which will prevent "hole" artefacts in the extracted volume.

Response: Thank you for your professional review. This suggestion is particularly helpful, and we had been looking for a way to generate cleaner volume renders without hole artifacts. Following your advice, we have regenerated Figures 1e, 3b, 3d, and 4d. The revised figures now display the extracted volumes without holes, significantly improving their clarity and visual quality.

5. In some figure captions, the authors provide extensive details on the experimental setup of the proteasome activity assay. Such information should be described in the respective methods section, while keeping the figure caption concise and providing an informative description of the results shown in the graphs.

Response: We agree with the reviewer's point. We have reviewed and streamlined all figure legends involving proteasome activity assay experiments (Figures 2j, 2l, 3h, and 4g). These legends now provide only brief descriptions of the experimental conditions and direct readers to the "Proteasome stimulation activity assay" section in the Methods for detailed protocols.

6. We are also bringing to the author's attention a few typos we noticed in the revised text:

On page 2, line 38: underlie > should be underlying

On page 5, line 106: previously > previous

On page 10, line 218: Fig. 3d > Fig. 3e

In the caption of Figure 2: panels are described as "top"/"bottom" > should be "left"/"right"

In the caption of Figure 2, line 1099: the authors use the word "equal" when comparing 300 nM and 600 nM affinities. More appropriate would be to say "same order of magnitude" or similar

In the caption of Figure 3, it's unclear what the authors mean by "representative maps" Authors should be precise and explicit on what maps they used. Instead, name the maps PS Ubl or PS Ubl_Catch if those are the final maps presented in Extended Data Figure 7.

Response: We are very grateful to the reviewer for pointing these typos and unclear description out. We have made the corrections accordingly.

Point-by-point responses to the comments by Reviewer #2:

The authors addressed the concerns I raised fully in the revised manuscript. I have no further concerns.

Response: We thank the reviewer for the time and positive feedback.

Point-by-point responses to the comments by Reviewer #3:

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response: We thank the reviewer for the time and positive feedback.

Point-by-point responses to the comments by Reviewer #4:

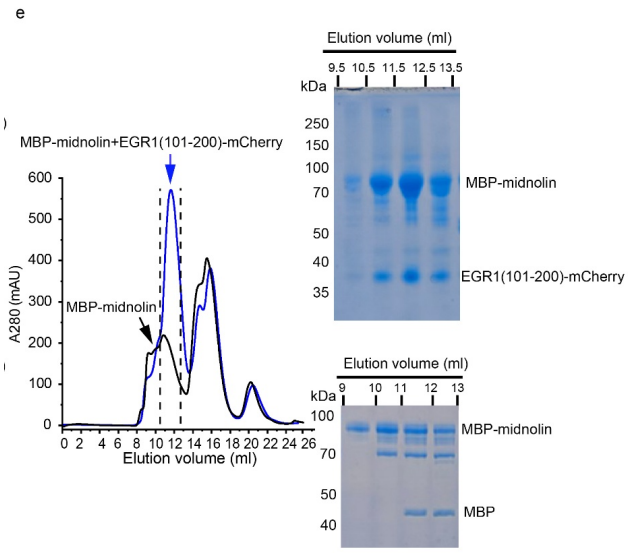
The authors have addressed most of my concerns except several points:

2. The authors state that “As expected, the complex shows a single, symmetric peak, whereas midnolin alone showed significant oligomerization/aggregation, confirming

that substrate binding is essential for stabilizing the full-length midnolin." However, I didn't find additional supporting data aside from Extended Data Fig. 6e, which only showed the co-purification results.

In addition, I didn't see a discussion about substrate selectivity in the revised Discussion.

Response: We are sorry for missing this data. We have revised Extended Data Fig. 6e, which shows obvious oligomerization/aggregation of full-length midnolin alone. We have revised this manuscript as "As expected, the complex shows a single, symmetric peak, whereas midnolin alone shows obvious oligomerization/aggregation, confirming that substrate binding is essential for stabilizing the full-length midnolin (Extended Data Fig. 6e).".



Revised Extended Data Fig. 6e. Overlay of size-exclusion chromatography profiles for MBP-midnolin purified alone (left, black line) and the co-expressed MBP-midnolin and EGR1(101-200)-mCherry complex (left, blue line). Coomassie brilliant blue staining analysis of the purified samples revealed that MBP-midnolin co-expressed with the substrate EGR1(101-200)-mCherry exhibited a homogeneous and monodisperse state (right top), whereas MBP-midnolin expressed alone contained high-order oligomeric states (right bottom).

The discussion regarding substrate selectivity is indeed crucial. We have added a paragraph addressing substrate selectivity in the Discussion section.

"The Catch domain of midnolin specifically recognizes a β-strand degron in IEG proteins by forming an FG zipper[3,13]. Our structural data show that the Catch domain

is positioned directly above the proteasome entry port, suggesting that it may ensure efficient delivery and degradation of only its captured substrates through a spatial selection mechanism, thereby preventing off-target degradation of non-specific proteins. Furthermore, the possible co-expression mechanism of midnolin with its substrates may contribute to an additional regulation, ensuring that this pathway is activated only when needed."

4. The functional assays are nicely performed. However, the conclusion in Line 228-230 is misleading. Based on the results from the "Catch-replaced midnolin" experiment (which is really a smart design), substrate binding does not necessarily equate to proteasome activation. A similar distinction may apply to the Ubl-RPN11 interaction, so the results in Fig. 3h only confirmed that this interaction is essential for stimulating proteasomal activities, but do not directly support its role in substrate delivery.
Therefore, I recommend a minor revision.
Response: Thank you for your positive comments and constructive suggestions. We have revised this sentence as "This finding directly confirms that these hydrophobic residues are essential for the Ubl domain's function in stimulating proteasomal activities."