nature portfolio

Peer Review File

Visualization of the Cdc48 AAA+ ATPase protein unfolding pathway

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
Evaluation of the manuscript “Visualization of the Cdc48 AAA+ ATPase protein unfolding pathway” by Ian Cooney et al.
The paper describes several structures of the AAA ATPase Cdc48 from Saccharomyces cerevisiae during substrate processing. The initial cryo-EM structure was obtained after a pull-down of the Cdc48 adaptor Shp1 in the presence of the nonhydrolyzable analog ADP.BeFx and crosslinking. This led to a symmetric hexameric ring structure and a staircase-like structure characteristic for AAA+ ATPases during substrate processing. These structures of Cdc48 are similar to what was published previously from yeast and p97 from humans by the authors and other groups. In the current manuscript, the staircase-like structure was further dissected by 3D variability analysis leading to nine maps that cover large parts of the ATPase and substrate processing cycle of Cdc48. In this respect, the manuscript provides additional information compared to previous published structures from Cdc48 and p97. In addition, the manuscript shows a low-resolution structure of Cdc48 in complex with Glc7-Sds22 associated with the A subunit N-domain and the UBX domain of Shp1 associated with the B-subunit. A similar structure of the p97-PP1-SDS22 complex was previously presented by a different group. The dissection of the various stages of Cdc48 in course of substrate processing provides information interesting to the AAA-community and could warrant the publication of the manuscript in Nature Communications. However, there are some major points that have to be addressed by the authors before the manuscript should be considered for publishing.
Main points:
The authors are extremely sloppy in the use of defined terms and also in their citations. The lead authors are asked here to carefully proofread the manuscript to make sure that what is attributed to the cited reference is really written there and vice versa that papers that are not from the Hill/Shen group are credited accordingly.
The authors use the term Cdc48 synonymous to p97 and VCP. However, Cdc48 is the designation of the yeast (Saccharomyces cerevisiae) protein, while p97/VCP is the designation for the metazoan ortholog which has a broader and very likely also different spectrum of activities. Therefore, the authors can not simply use Cdc48 as synonym for p97/VCP. Mutations in Cdc48 therefore do not “cause multisystem proteinopathy associated with the degeneration of the brain, bones, and muscles” (lane 33 to 34) because the unicellular organism yeast, does not have such organs. The authors have to stick with the correct designation of the proteins and state that mutations in the human orthologue p97/VCP cause these defects. Similarly, it is wrong when the authors state that “specific inhibitors for Cdc48 have been evaluated in clinical trials”. These inhibitors were developed for p97/VCP.

It is also wrong when the authors state throughout this manuscript and also in their previous papers that the D1 and D2 domains are comprised of a small and large ATPase domain. While few authors use the term small and large subdomain, the AAA-community usually uses the term (all) α and α/β subdomain to describe the structure of the AAA-domain. In any case, at least the term subdomain has to be included in all instances.
Similarly, the authors use the term "cassette" to describe the AAA-domain. This is also avoided by most members of the AAA-community to prevent confusion with the ABC transporters family, where cassette is integrative part of the designation.
Moreover, it is wrong when the authors state (lane 42/43) "…that bind the unfolded substrate in a groove through the central hexameric pore" Ref 9–12. The authors here cite two of their own papers and Pan et al., 2022 and Twomey et al., 2019, that use the term groove in a completely different way that is not related with the central pore. Twomey et al., 2019 use the term for a groove in Npl4, while Pan et al., uses it only for an inhibitor binding site or other interactions within the protein, but not for the central pore. This is just one of many examples for the sloppy citations in this manuscript. Beside this, the term groove is often used for the groove within the bipartite N-domain in AAA-ATPases representing the interaction site with adaptor proteins.
Minor issues:
It has to be stated also in the results section that the structure was determined from a crosslinked complex.
The resolution of the consensus map is sometimes stated as 2.8Å and sometimes as 2.9Å. The authors should unify this.
lane 134/135 "that D2 plays the dominant role in imparting the translocation force that drives unfolding by gripping onto substrate more tightly" Ref39,40. I am not aware that what the authors state here was explicitly mentioned in the Bodnar and Esaki papers.
184 to 186: "…chains, although in all active sites displaying ADP•BeFx density, the Walker B glutamate main chain and nucleotide superimposed closely with other well-defined structures of AAA+ ATPase-ATP complexes" Ref 9,11. The authors only cite their own work (Cooney and Xu). Are their structures really the only well-defined "AAA+ ATPase-ATP complexes"? Here it would be interesting to align the glutamate from other AAA-ATPases (also from other groups) and to see how similar the positioning relative to the nucleotide is.
279/280: "The best scoring model showed a specific region of Shp1 (residues 125-138) that fit precisely as a rigid-body within the extra density". This analysis is not really convincing and should be backed up by crosslinking MS.
Methods section: what is a "lysate-to-grid preparation"? Please explain in more detail!
Video 3: the video jumps back to the beginning immediately when the last frame is shown. The authors should include a duplicate of the last frame, to increase the time it is shown. This would make it easier to see the last stages of the movement. The authors should also consider to highlight the (beginning of) N-D1 linker. This would help to see

how the movement of the D1 domain might affect the positioning of the N-domain. The possible movement of the N-domain during the transition of the F-protomer to the top position would also be interesting to discuss. Especially since the α/β subdomain makes a significant rotation towards the substrate chain.
Finally, the conclusion also needs significant rework:
the authors discuss phosphate release. I am not aware, where the authors get the evidence for the phosphate release from their structures.
293 to 295: "…. being linked by inter-subunit contacts and up to three subunits observed moving along the translocation pathway simultaneously". There is no evidence in the structures of this manuscript (and for other AAA-ATPases) that three subunits move simultaneously along the translocation pathway. The authors should clarify what they mean here.
299 to 301 "Our findings provide a foundation to investigate conservation of the translocation mechanism in other AAA+ ATPases and further investigate function and interactions of Shp1, and can inform efforts to develop novel therapeutics such as inhibitors of Cdc48/p97". This is a very strong statement for the presented data. Hand-over-hand translocation is now well established for various AAA-ATPases and the interaction of Shp1 with the N-domain was shown previously, except the proposed interaction with the D1 domain, which is at this stage not very convincing. How this paper might contribute to the development of novel therapeutics is also not clear.
Taken together, the authors should also thoroughly work on the conclusion to recap what they really show in this manuscript.

Reviewer #2:
Remarks to the Author:
In this study the authors set out to understand the unfolding dynamics of the Cdc48 AAA+ ATPase protein. They purify Cdc48 from yeast with Shp1 and visualize a variety of these complexes with substrates to build a model for the processing of these substrates. While this may be of interest the work show is rather limited in scope (4 figures) and does not add much to our knowledge of how truly Cdc48 functions. The way in which the complexes were purified (from yeast) seems particularly troublesome as the authors do not take into account the large number of PTMs this protein has (84 as per the GPM database) which may influence the structure. Overall this work seems more suited to a more specialized journal.

Reviewer #3:
Remarks to the Author:

Cooney and colleagues present an ensemble of cryo-EM structures of the Cdc48-Shp1 complex that is translocating a substrate. This ensemble, determined using extensive 3D variability analysis, represents a continuum of translocation steps that occur throughout the mechanism of substrate threading. Detailed evaluation of the inter-subunit interactions and nucleotide identities allow for an explanation of how ATP hydrolysis affects these nucleotide pockets for the hand-over-hand translocation mechanism.

The manuscript also describes the interactions between Cdc48 and its cofactors Shp1 and PP1. The cofactors were identified and modeled using a combination of proteomics and ColabFold, which was necessary to rigid body fit them into the low-resolution density of the map.

The cryo-EM and image analysis was done to a high standard and well documented.

Major points:

The challenge of comparing an ensemble of models of a homohexameric complex is that it can be unclear the order in which they occur in the translocation mechanism. The current ordering is logical, based on the progression of ATP hydrolysis and nucleotide release of the individual pockets that appear to drive progression of sequential, hand-over-hand translocation.

The issue is that the direction of the movement of subunit E and F is upward, and the rotation of the large and small domains of their nucleotide pockets is creating a compaction. Is this what we should expect with the transition from ATP to ADP to apo? How does this compare to previous AAA+ substrate bound structures that compare the monomers within the spiral staircase as they make these same transitions?

This sequential ordering between your maps, and the connection between the rotation of the small and large domains with hydrolysis and nucleotide release, needs to be clarified. The current main text and figures do not do a sufficient job making this connection. In Figure 1 we see the trajectory of subunit F and E, but the rotations we see in Figure 2 are for D and E. How is subunit F rotating? What about the changes in the inter-subunit interactions? Please expand on how these all occur in the text and modify your figures to better demonstrate the movements associated with hydrolysis and translocation. You begin to get into possible explanations in lines 201-208, which may be an appropriate place to expand.

Minor points:

The measurements shown for the DE interface between arginine fingers and the F3 of BeFx in Supplemental Figure 9 help demonstrate the nucleotide assignments for those pockets. Please label them with ATP/ADP/Apo according to how you have assigned them. Please make a similar figure for the EF interface that can similarly demonstrate why you assigned Apo, as the densities shown in Suppl. Fig 8 are not sufficient.
In lines 280-283 authors suggest that Shp1 density is present at B, C and D, but could potentially be found on the other three subunits. Does this make sense based on the stoichiometry at which Shp1 binds to Cdc48? Doesn't Shp1 usually exist as a trimer? Is this an artifact of the averaging in single particle cryo-EM?

In Figure 4A in the top-down view we can see the extra density over the central channel- is this the same density as in Figure 4B shown in purple? It is hard to tell what we are looking at between the A, B and C views. Better labeling of subunits and consistent coloring between panels would clarify this.

Surprisingly little is described about what the Shp1/PP1 structure contributes to this story. What is the significance of seeing the Sph1 UBX and PP1 associated with an NTD? What does this mean for substrate hand off?

We thank the reviewers for their evaluation of our manuscript. Their comments are pasted below verbatim, and our point-by-point responses are included below in blue font. All edits to the revised manuscript are included in a "Track Changes" mode of the document. A version of the manuscript without markups is also provided in PDF format. Please note that the line numbers used in our responses below refer to the version without markups.

Evaluation of the manuscript "Visualization of the Cdc48 AAA+ ATPase protein unfolding pathway" by Ian Cooney et al.
The paper describes several structures of the AAA ATPase Cdc48 from Saccharomyces cerevisiae during substrate processing. The initial cryo-EM structure was obtained after a pull-down of the Cdc48 adaptor Shp1 in the presence of the nonhydrolyzable analog ADP.BeFx and crosslinking. This led to a symmetric hexameric ring structure and a staircase-like structure characteristic for AAA+ ATPases during substrate processing. These structures of Cdc48 are similar to what was published previously from yeast and p97 from humans by the authors and other groups. In the current manuscript, the staircase-like structure was further dissected by 3D variability analysis leading to nine maps that cover large parts of the ATPase and substrate processing cycle of Cdc48. In this respect, the manuscript provides additional information compared to previous published structures from Cdc48 and p97. In addition, the manuscript shows a low-resolution structure of Cdc48 in complex with Glc7-Sds22 associated with the A subunit N-domain and the UBX domain of Shp1 associated with the B-subunit. A similar structure of the p97-PP1-SDS22 complex was previously presented by a different group.
The dissection of the various stages of Cdc48 in course of substrate processing provides information interesting to the AAA-community and could warrant the publication of the manuscript in Nature Communications. However, there are some major points that have to be addressed by the authors before the manuscript should be considered for publishing.

The manuscript has been substantially revised. Major edits were made to improve clarity in protein name designations, citation references, and other descriptions as summarized below.

Main points:
The authors are extremely sloppy in the use of defined terms and also in their citations. The lead authors are asked here to carefully proofread the manuscript to make sure that what is attributed to the cited reference is really written there and vice versa that papers that are not from the Hill/Shen group are credited accordingly.

The manuscript has been completely revised to use standard terms and include appropriate citations.

The authors use the term Cdc48 synonymous to p97 and VCP. However, Cdc48 is the designation of the yeast (Saccharomyces cerevisiae) protein, while p97/VCP is the designation for the metazoan ortholog which has a broader and very likely also different spectrum of activities. Therefore, the authors can not simply use Cdc48 as synonym for p97/VCP. Mutations in Cdc48 therefore do not "cause multisystem proteinopathy associated with the degeneration of the brain, bones, and muscles" (lane 33 to 34) because the unicellular organism yeast, does not have such organs. The authors have to stick with the correct designation of the proteins and state that mutations in the human orthologue p97/VCP cause these defects. Similarly, it is wrong when the authors state that "specific inhibitors for Cdc48 have been evaluated in clinical trials". These inhibitors were developed for p97/VCP.

We have edited the manuscript thoroughly to distinguish between yeast Cdc48 and its human/mammalian ortholog (VCP/p97).

It is also wrong when the authors state throughout this manuscript and also in their previous papers that the D1 and D2 domains are comprised of a small and large ATPase domain. While few authors use the term small and large subdomain, the AAA-community usually uses the term (all) α and α/β subdomain to describe the structure of the AAA-domain. In any case, at least the term subdomain has to be included in all instances.
Similarly, the authors use the term “cassette” to describe the AAA-domain. This is also avoided by most members of the AAA-community to prevent confusion with the ABC transporters family, where cassette is integrative part of the designation.

We recognize the range of terminologies used when referring to different portions of AAA+ domains. To improve clarity, we have replaced “cassette” with “AAA+ ATPase domain” and revised our introduction to the large and small subdomains as follows in the main text (lines 40-42): “The structures of D1 and D2 each comprise canonical AAA+ large α/β and small α subdomains that share sequence and structural similarity (hereafter simply referred to as “large” and “small” subdomains, respectively).”

Moreover, it is wrong when the authors state (lane 42/43) “…that bind the unfolded substrate in a groove through the central hexameric pore” Ref 9–12. The authors here cite two of their own papers and Pan et al., 2022 and Twomey et al., 2019, that use the term groove in a completely different way that is not related with the central pore. Twomey et al., 2019 use the term for a groove in Npl4, while Pan et al., uses it only for an inhibitor binding site or other interactions within the protein, but not for the central pore. This is just one of many examples for the sloppy citations in this manuscript. Beside this, the term groove is often used for the groove within the bipartite N-domain in AAA-ATPases representing the interaction site with adaptor proteins.

We agree with the reviewer that the usage of “groove” may carry ambiguous interpretations. Our usages of “groove” have now been re-worded as “substrate binding pocket”.

Minor issues:
It has to be stated also in the results section that the structure was determined from a crosslinked complex.

It is now stated in the Results section that the complexes were stabilized through chemical crosslinking prior to cryo-EM specimen preparation (lines 83-85): “Native Cdc48-Shp1 complexes were purified by co-immunoprecipitation of FLAG-tagged Shp1 from budding yeast (*S. cerevisiae*) lysates and then stabilized through chemical crosslinking prior to cryo-EM specimen preparation, as described previously[9].”

The resolution of the consensus map is sometimes stated as 2.8Å and sometimes as 2.9Å. The authors should unify this.

We thank the reviewer for catching this inconsistency. All instances of “2.8 Å” have been revised to “2.9 Å”.

lane 134/135 “that D2 plays the dominant role in imparting the translocation force that drives unfolding by gripping onto substrate more tightly” Ref39,40. I am not aware that what the authors state here was explicitly mentioned in the Bodnar and Esaki papers.

All references have been reworked throughout the paper. The references have been removed from this specific sentence. We added a new sentence to properly cite the insights provided by the Esaki paper in terms of the non-canonical residues in pore loop-1 of D1 (lines 155-157): “Yet, the apparent weaker binding in D1 appears to be functionally important because mutations of M288 to canonical large hydrophobic residues causes a lethal phenotype in yeast.”

184 to 186: “…chains, although in all active sites displaying ADP•BeFx density, the Walker B glutamate main chain and nucleotide superimposed closely with other well-defined structures of AAA+ ATPase-ATP complexes” Ref 9,11. The authors only cite their own work (Cooney and Xu). Are their structures really the only well-defined “AAA+ ATPase-ATP complexes”?

We thank the reviewer for catching this oversight. We now include a new supplemental figure that shows the overlay and individual models of 14 structures of ATP or ATP-like bound binding pockets (Supplemental Fig. 8). We also acknowledge that insights into AAA+ ATPase unfolding mechanism have been derived from multiple structures (lines 60-63; references 9-12 & 18-37).

Here it would be interesting to align the glutamate from other AAA-ATPases (also from other groups) and to see how similar the positioning relative to the nucleotide is.

Our revised manuscript now includes an overlay of 14 different ATP-bound structures that shows their consistent positioning of Walker A and Walker B motifs (glutamates, or glutamines when E→Q mutations were used) in Supplemental Figure 8.

279/280: “The best scoring model showed a specific region of Shp1 (residues 125-138) that fit precisely as a rigid-body within the extra density”. This analysis is not really convincing and should be backed up by crosslinking MS.

Crosslinking mass spectrometry would go beyond the scope of the current manuscript. We believe it is notable that a structure prediction program modeled a segment of Shp1 that fits the density as convincingly as well-resolved portions of Cdc48. We do, however, take the point that this observation is not validated by a complementary method, and have therefore adjusted the wording to explicitly emphasize that this is a hypothesis suggested from the structure (lines 324-325).

Methods section: what is a “lysate-to-grid preparation”? Please explain in more detail!

The complex isolation portion within the Methods section has been expanded and now includes more details about “lysate-to-grid” preparation and a reference to the Lysate-to-grid protocol paper (lines 432-435; Cooney et al. *Bio-protocol* 2023).

Video 3: the video jumps back to the beginning immediately when the last frame is shown. The authors should include a duplicate of the last frame, to increase the time it is shown. This would make it easier to see the last stages of the movement.

Movie 3 has been modified such that it ends on the last stages of Cdc48 movement. Its playback speed has also been greatly slowed to help it be easier to follow.

The authors should also consider to highlight the (beginning of) N-D1 linker. This would help to see how the movement of the D1 domain might affect the positioning of the N-domain. The possible movement of the N-domain during the transition of the F-protomer to the top position would also be interesting to discuss. Especially since the α/β subdomain makes a significant rotation towards the substrate chain.

We thank the reviewer for raising this interesting question about N-domain positions. Our structural analysis reveals that the N-domain is constitutively in the so-called "up" position. Despite extensive 3DVA and cryoDRGN analysis, we do not observe meaningful positional variability in N-domain or N-D1 linker regions, and a sentence has been added in the main text to describe this observation (lines 108-110): "Across all classes, the N-domains were resolved in the "up" state elevated above the D1 ring and did not display conformational variability relative to the D1 domain, although their weak density is indicative of high mobility." We note for the reviewer that this analysis is what led to our recovery of the PP1-bound class.

Finally, the conclusion also needs significant rework:
the authors discuss phosphate release. I am not aware, where the authors get the evidence for the phosphate release from their structures.

We do not have direct structural evidence for phosphate release beyond the established fact that Cdc48 is an ATPase and we can see states whose density corresponds to ATP or ADP. Our key point is that ADP density is only observed when the subunit interface is open, which leads to our hypothesis that phosphate release happens as the interface opens but not when the interface is closed. We have made minor adjustments to the text to be clear that this is a hypothetical inference rather than a direct observation (lines 227-236, new Supplemental Figures 9-11).

293 to 295: "…. being linked by inter-subunit contacts and up to three subunits observed moving along the translocation pathway simultaneously". There is no evidence in the structures of this manuscript (and for other AAA-ATPases) that three subunits move simultaneously along the translocation pathway. The authors should clarify what they mean here.

We do see simultaneous movement among 3 subunits (D, E, and F). This is an important point of the paper, and we have revised the text to improve clarity. We also hope the revised Movie 3 also shows this more clearly (referenced in lines 189-191 and 341-343). The following sentence was added to reinforce this point (lines 191-193): "Most of the motion occurs within subunits E and F, and the inter-subunit interaction between the large subdomain of E and the small subdomain of D causes concomitant motion within subunit D."

299 to 301 "Our findings provide a foundation to investigate conservation of the translocation mechanism in other AAA+ ATPases and further investigate function and interactions of Shp1, and can inform efforts to develop novel therapeutics such as inhibitors of Cdc48/p97". This is a very strong statement for the presented data. Hand-over-hand translocation is now well established for various AAA-ATPases and the interaction of Shp1 with the N-domain was shown previously, except the proposed interaction with the D1 domain, which is at this stage not very convincing. How this paper might contribute to the development of novel therapeutics is also not clear.

We have revised this text and, specifically, have removed discussion of therapeutics development.

Taken together, the authors should also thoroughly work on the conclusion to recap what they really show in this manuscript.

Thank you for this suggestion. The conclusion section has been carefully revised.

Reviewer #2 (Remarks to the Author):

In this study the authors set out to understand the unfolding dynamics of the Cdc48 AAA+ ATPase protein. They purify Cdc48 from yeast with Shp1 and visualize a variety of these complexes with substrates to build a model for the processing of these substrates. While this may be of interest the work show is rather limited in scope (4 figures) and does not add much to our knowledge of how truly Cdc48 functions. The way in which the complexes were purified (from yeast) seems particularly troublesome as the authors do not take into account the large number of PTMs this protein has (84 as per the GPM database) which may influence the structure. Overall this work seems more suited to a more specialized journal.

The revised manuscript has been expanded slightly in scope. There are now 5 figures in the main manuscript and 16 figures in the supplemental information file.

Regarding the comment about PTMs. We agree that our rapid lysate to grid approach means that purified proteins may contain a mixture of posttranslational modifications. However, we emphasize that is a feature rather than a bug. Conventional approaches of extensively purifying recombinant proteins offers the advantage of working with very well defined systems. However, our approach has the advantage of working with complexes that are more likely to represent the true physiological states. Both approaches have advantages and limitations, and are valid.

Reviewer #3 (Remarks to the Author):

Cooney and colleagues present an ensemble of cryo-EM structures of the Cdc48-Shp1 complex that is translocating a substrate. This ensemble, determined using extensive 3D variability analysis, represents a continuum of translocation steps that occur throughout the mechanism of substrate threading. Detailed evaluation of the inter-subunit interactions and nucleotide identities allow for an explanation of how ATP hydrolysis affects these nucleotide pockets for the hand-over-hand translocation mechanism.

The manuscript also describes the interactions between Cdc48 and its cofactors Shp1 and PP1. The cofactors were identified and modeled using a combination of proteomics and ColabFold, which was necessary to rigid body fit them into the low-resolution density of the map.

The cryo-EM and image analysis was done to a high standard and well documented.

Major points:

The challenge of comparing an ensemble of models of a homohexameric complex is that it can be unclear the order in which they occur in the translocation mechanism. The current ordering is logical, based on the progression of ATP hydrolysis and nucleotide release of the individual pockets that appear to drive progression of sequential, hand-over-hand translocation.

The issue is that the direction of the movement of subunit E and F is upward, and the rotation of the large and small domains of their nucleotide pockets is creating a compaction. Is this what we should expect with the transition from ATP to ADP to apo? How does this compare to previous AAA+ substrate bound structures that compare the monomers within the spiral staircase as they make these same transitions?

We thank the reviewer for this clarifying question. Indeed, the ordering of our classes was based on a sequential trajectory of subunit motion that also correlated with nucleotide state. Our revised manuscript emphasizes that the upward movement of subunits E and F creates an **expansion** between subunit interfaces (not a compaction). This is important because, as noted by the reviewer, expanded interfaces are an expected part of the nucleotide hydrolysis cycle. To further clarify this, we have created new Supplemental Figures 9-11 to show the progression of subunit motion and nucleotide state assignments at the DE, EF, and FA interfaces across all 9 classes.

The proposed mechanism is consistent with other AAA+ structures at the subunit level, as noted in the opening paragraph of the Conclusion section of the revised manuscript. We then elaborate that our new insights include recognition of the coordinated movement among three consecutive subunits.

This sequential ordering between your maps, and the connection between the rotation of the small and large domains with hydrolysis and nucleotide release, needs to be clarified. The current main text and figures do not do a sufficient job making this connection. In Figure 1 we see the trajectory of subunit F and E, but the rotations we see in Figure 2 are for D and E. How is subunit F rotating? What about the changes in the inter-subunit interactions? Please expand on how these all occur in the text and modify your figures to better demonstrate the movements associated with hydrolysis and translocation. You begin to get into possible explanations in lines 201-208, which may be an appropriate place to expand.

The revised manuscript includes a new Fig. 1 that shows the remarkable motion observed within subunits E and F (panels B and C). The trajectory of subunits E and F pore loops are shown in Figure 2 (previously Fig. 1), while Figure 4 defines the apparent nucleotide state for all classes along the translocation trajectory and also shows (schematically) how this is correlated with changes in subunit interfaces. The changing interfaces and nucleotide state are now shown in supplemental figures 9-12. The inter-subunit interface between subunits F and A narrows from the initial apo state of class 1 to the final ATP-bound state of class 9 (Supplemental Figure 11).

Minor points:

The measurements shown for the DE interface between arginine fingers and the F3 of BeFx in Supplemental Figure 9 help demonstrate the nucleotide assignments for those pockets. Please label them with ATP/ADP/Apo according to how you have assigned them. Please make a similar figure for the EF interface that can similarly demonstrate why you assigned Apo, as the densities shown in Suppl. Fig 8 are not sufficient.

The figure has been updated with the appropriate labels. We have also replaced the previous Supplemental Figure 8 with a new supplemental figure (Suppl. Fig. 10) to show nucleotide density at subunit E from a clearer perspective across all classes. Note that this new figure does not show subunit F due to its wide separation from subunit E (>10 Å).

In lines 280-283 authors suggest that Shp1 density is present at B, C and D, but could potentially be found on the other three subunits. Does this make sense based on the stoichiometry at which Shp1 binds to Cdc48? Doesn't Shp1 usually exist as a trimer? Is this an artifact of the averaging in single particle cryo-EM?

Early studies of human Shp1 (p47) indicate that its SEP domain enables trimerization and that a p47 trimer interacts with a p97/VCP hexamer (Kondo et al., *Nature* 1997; Neuron et al., *EMBO J.* 2006; Zhang et al. *PNAS* 2015). A more recent study demonstrated that p47-p97 complexes display variable levels of p47 occupancy per p97 hexamer (Conicella et al. *PNAS* 2020). Thus, the stoichiometry of Shp1/p47 binding to Cdc48/p97 is not settled. Further, although yeast Shp1 also contains a SEP domain, we are not aware of studies that have formally tested whether it interacts with Cdc48 as a trimer. We therefore prefer to refrain from making claims about Shp1 stoichiometry based on our structure.

Regardless of stoichiometry, we agree that Shp1-D1B density over subunits E, F, and A is likely being averaged out and emphasize this possibility in the revised manuscript (lines 310-313): "Densities at subunits B and D are consistent with the model built at subunit C, and it seems probable that equivalent structures are present at subunits A, E, and F but are not apparent due to the poorer local resolution for those subunits or being otherwise averaged out from low occupancy density."

In Figure 4A in the top-down view we can see the extra density over the central channel- is this the same density as in Figure 4B shown in purple? It is hard to tell what we are looking at between the A, B and C views. Better labeling of subunits and consistent coloring between panels would clarify this.

Yes, the densities above the central pore in panel A and B likely represent the same unstructured substrate and/or adaptor density. We have updated the figure (now Figure 5) to include better labeling and consistent coloring between the panels.

Surprisingly little is described about what the Shp1/PP1 structure contributes to this story. What is the significance of seeing the Sph1 UBX and PP1 associated with an NTD? What does this mean for substrate hand off?

We have now expanded the text to elaborate on the significance of our new Shp1 structure in the context of Cdc48 binding and substrate engagement (lines 313-325). Our interpretations are supported by structures of human complexes that show divergent modes of VCP interactions among various domains. We also include a new supplemental figure that shows the multiple sequence alignment among Shp1 (yeast), p37 (human), and p47 (human), along with the annotated domains within Shp1 (Supplemental Fig. 16).

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The manuscript improved significantly compared to the previous version. The authors addressed most of my queries. Only Video 3 is still jumping back immediately, but this is a minor issue. I recommend publication by Nature Communications.

Helmut Bergler

Reviewer #2:
Remarks to the Author:
The authors have put substantial effort into revising their manuscript and I believe have not only addressed my concerns but those of the other reviewers too. Overall this work is much improved from the original submitted version and will be of interest to readers of the journal and the general community. Overall, I am happy to recommend this study for publication.

Andrew Truman

Reviewer #3:
Remarks to the Author:
Cooney et al have addressed all of my comments and I am happy with the revised manuscript. I appreciate all of the clarifying statements and improvement of figures.

I recommend this manuscript for publication.