Interactions of ubiquitin and CHMP5 with the V domain of HD-PTP reveals role for regulation of Vps4 ATPase

Natalya Pashkova, Liping Yu, Nicholas Schnicker, Chun-Che Tseng, Lokesh Gakhar, David Katzmann, and Robert Piper

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RE: Manuscript #E21-04-0219
TITLE: Interactions of ubiquitin and CHMP5 with the V domain of HD-PTP reveals role for regulation of Vps4 ATPase

Dear Dr. Piper,

Thank you very much for submitting your manuscript to Molecular Biology of the Cell. Two reviewers, who are both experts in the field, have seen your paper, and I have now received their reports. As you will see, both reviewers raise some issues that need to be addressed - in particular reviewer two who is somewhat more critical. Since more work is needed to address these points, the paper will have to be re-reviewed.

Looking forward to receiving your revised manuscript.

Sincerely,

Jean Gruenberg
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Piper,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

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When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you haveopted out of publishing the review history.

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Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

Natalya Pashkova and colleagues describe in first part of their manuscript how the V domain of HD-PTP interacts with ubiquitin. They employ a combination structural approaches (SAXS, PRE, NMR), modeling and biochemical binding experiments and identify two distinct ubiquitin binding sites that are in close vicinity. They suggest a model in which two ubiquitin molecules, or possibility two K63 linked ubiquitin molecules, interact with these ubiquitin binding site in the V domain (the overall configuration reminded me on a puck on a hockey stick).

In the second part of the manuscript, they demonstrate that that the binding of ubiquitin to the V domain of HD-PTP stimulates Vps4 ATPase activity in vitro.

In the third part, they used a clever two hybrid screen to demonstrate that mutations in the ubiquitin binding sites in the V domain of HD-PTP do not affect dramatically the 'interactome' of HD-PTP. In this screen, they identified the ESCRT-III subunit Chmp5 as an interactor. They confirmed the interaction between the V domain of HD-PTP and Chmp5 in vitro. Finally, the used budding yeast as a model system to demonstrate that vps60 contributes to MVB sorting.

Overall, this work provides new insight into how the ESCRT machinery functions. In particular, it points to an important role for HD-PTP (and other V domain containing proteins) in coordinating the sorting of ubiquitinated membrane proteins with the membrane remodeling activity of the ESCRT machinery.

I only have few requests and suggestions that might help to improve to manuscript:

1. Pulldown experiments in Figure 3B: the lane containing the negative control appears to be cut off in some instances. Please fix that.
2. Figure 3B,C: Different V mutants were used for GST-Ub pulldown and NMR titration experiments. Also, it would be perhaps useful to compare the binding of the different mutants directly. Therefore,

they should be loaded on the same gel.
3. In my version of the manuscript, Figure 3D, E was missing (or wrong figure call-outs in the text). Please fix that.
4. Figure 5C - the text in the manuscript does not correspond to the figure. In the text site 1 mutant (L371A, E374A and E375A) was described for the additional introduction of site 2 mutations. In the figure a different site 1 mutant (L371A, Y372A, E374A) is used.
5. Figure 5C: To directly compare the effect on ubiquitin binding of the site 2 mutants, they should be loaded on the same gel, together with a WT V domain and the site 1mutant (please see point 4), as controls (these controls appear important since there is some 'unspecific' binding to GST).
6. It would be interesting to see if there are differences in the binding of the V domain to mono- or di-ubiquitin or ubiquitin chains.
7. Besides Chmp5, did the DEEPN analysis identify any other ESCRT subunits?
8. At the moment the yeast experiments in Figure 9B appear to be disconnected from the rest of paper. Would it make sense to examine how mutations in the V domain of Bro1 affect MVB sorting in vps60 mutants? These experiments could then provide link to the rest of the manuscript.

Reviewer #2 (Remarks to the Author):

Piper and colleagues report that the HD-PTP V-domain binds ubiquitin, Chmp5 and the Vps4 MIT domain thereby coupling HD-PTP to the Vps4 ATPase activity regulation.
Major findings are:
The authors use recombinant HD-PTP V and paramagnetic relaxation enhancement experiments (PRE) to localize two Ub binding site. The sites, which were confirmed by mutagenesis and Ub pull down experiments. Furthermore, binding affinities were determined by NMR titration experiments using wild type V and mutants. Binding of Ub to HD-PTP V was modeled based on the UB-V-Bro1 structure, which produced a model in agreement with the PRE data and spin labels at position 368. The second UB site is close to the first one. BLI experiments with wt HD-PTP V and mutants of site 1 and site 2 showed that the mutations abolished micromolar binding. Some millimolar binding is still observed, indicating that the 5 mutations did not abolish binding completely. This is surprising since the overall affinity of UB binding is already low. Does the modelling explain this weak interaction or are these non-specific interactions?
NMR was then used to map the interaction of Ub with site 1 by recording HSQC spectra. Site 1 was determined to be the major high affinity binding site. Ub binding modelling suggested binding of K63-linked di-ubiquitin to the N-terminal helix thereby occupying both Ub sites.
Mutations within the Ub binding sites do not affect interaction with UBAP1. A yeast 2-hybrids screen was further employed to detect potential differences in interactions with wt versus mutant HD-PTP V. Among the interacting proteins, CHMP5 was identified to interact with both wt and mutant HD-PTP V. Pull-downs confirmed binding to the helical core domain including the Lip5 binding site. Similarly, yeast V-Bro1 bound the Chmp45 ortholog Vps60.
HD-PTP V binds to the Vps4 MIT domain. The Vps4 ATPase activity was slightly enhanced in the presence of HD-PTP V and Ub. F678D mutation abrogated binding to CHMP5 and Vps4 MIT.
Ste3-GFP sorting into the vacuole was tested using vps27deltaCHC (clathrin binding site of Vps27) and delta-vps60 mutants alone and in combination, which showed major defects for the double mutants indicating that Vps60 functions together with Bro1 in MVB sorting.
In summary the manuscript contains a detailed structural description of HD-PTP V interaction with Ub, thereby providing novel important insight into HD-PTP linking its activity to the regulation of the Vps4 ATPase activity via CHMP5 and the Vps4 MIT domain. The manuscript is thus of high interest to the ESCRT field.

Points that need to be addressed:
1. The authors state that HD-PTP V binds Ub attached to cargo. What is the evidence?
2. The Vps4 ATPase activity is enhanced in the presence of HD-PTP V with an additional small increase by adding Ub. However, the UB binding site mutated version of V shows the same increase as wt, indicating that Ub binding should not play any role in regulating the ATPase activity.
3. Vps4 becomes active once it is assembled into a spiral hexamer. Does the V domain promote assembly of Vps4 hexamers thereby increasing its ATPase activity?
4. Figure 8D: The intensity of the pull down bands is very similar for wt HD-PTP and its mutant form with respect to the interaction with CHMP5 and Vps4B_MIT… Are these small differences significant? This needs to be supported by other biophysical methods such as BLI or SPR in order to map the binding site to the F678D mutation.
5. Figure 9: The model of Vps4 is shown as a dodecamer. Numerous structural studies indicate that the active form of Vps4 is a spiral hexamer.
6. The major function of Vps4 is remodeling and final disassembly of ESCRT-III filaments. This is achieved by its MIT domain interaction with MIMs present in ESCRT-III proteins. How can this be reconciled with the proposal of this manuscript that suggests that the Vps4 ATPase activity is regulated by the MIT domain - HD-PTP V interaction?
7. Figure 7E: CHMP5 is a member of the ESCRT-III protein family. Its structure will thus likely resemble other ESCRT-III members independent of the high sequence variability. It should be modelled based on existing closed conformation structures of ESCRT-III such as CHMP3 and/or IST1 or the CHMP1 model.
8. Are the CHMP5 fusion proteins used in the pull downs monodisperse after purification?

Thanks to both reviewers for the careful, helpful, and encouraging comments.
We have addressed the concerns enumerated below and added some additional data to enhance the symmetry of the story. Our comments are designated by ">"

Review 1 comments

1. Pulldown experiments in Figure 3B: the lane containing the negative control appears to be cut off in some instances. Please fix that.
> Fixed

2. Figure 3B,C: Different V mutants were used for GST-Ub pulldown and NMR titration experiments. Also, it would be perhaps useful to compare the binding of the different mutants directly. Therefore, they should be loaded on the same gel.
> We used the GST pulldown assays to survey different regions and mutations. These experiments were followed with quantitative binding studies using different techniques to back up the qualitative GST pulldown data. The GST pulldowns were analyzed together in the same experimental run. Multiple gels had to be used to capture all the samples and the signals were captured on film. We stacked the images for easy comparison. We used cell lysates from bacteria induced to produce the mutant V domains. We have found anecdotally that the mix of bacterial proteins serves as a convenient protein milieu to block non-specific binding. This does produce slightly different levels of each V domain. Nonetheless, the GST pulldowns as they are provide interpretable data indicating which mutants have specific Ub-binding and which don't.

3. In my version of the manuscript, Figure 3D, E was missing (or wrong figure call-outs in the text). Please fix that.
> Fixed

4. Figure 5C - the text in the manuscript does not correspond to the figure. In the text site 1 mutant (L371A, E374A and E375A) was described for the additional introduction of site 2 mutations. In the figure a different site 1 mutant (L371A, Y372A, E374A) is used.
> Fixed. Thanks for observing this.

5. Figure 5C: To directly compare the effect on ubiquitin binding of the site 2 mutants, they should be loaded on the same gel, together with a WT V domain and the site 1mutant (please see point 4), as controls (these controls appear important since there is some 'unspecific' binding to GST).
> We agree. Measuring binding in Site1Δ mutants is difficult by GST pulldown even with Ub concatemers that increase avidity of binding. We now show binding experiments using the peak broadening in HSQC spectra of ^{15}N-Ub in the presence of different HDPTP V domain mutants that also have Site1Δ. This assay is used throughout the manuscript and is used in this figure to show they type of survey information we used to hone in on the mutations that incapacitate Site2.

6. It would be interesting to see if there are differences in the binding of the V domain to mono- or di-ubiquitin or ubiquitin chains.
> The affinity of the V domains is already pretty high for mono-Ub, so it does not look like a typical di-Ub specific Ub-binding protein with two weak binding sites that only work effectively when binding two linked Ubs simultaneously. We did comment on how two Ubs laid across the tandem binding sites might accommodate a di-Ub, but we thought we would not gain too much functional insight from measuring differences in mono vs K63 di-Ub, especially since separating avidity for a chain with several mono-Ubs (which works well to boost capture of mono-Ub

binding proteins) vs affinity for a K63 linked di-Ub per se would be beyond the scope of what would be useful here.

7. Besides Chmp5, did the DEEPN analysis identify any other ESCRT subunits?
> We did not see other ESCRT subunits. The interactors and their subregions that were captured in the DEEPN screen are included in the supplemental data. Keep in mind that we only performed the screen on the V domain, and several of the other ESCRTs that interact with HD-PTP do so via other domains.

8. At the moment the yeast experiments in Figure 9B appear to be disconnected from the rest of paper. Would it make sense to examine how mutations in the V domain of Bro1 affect MVB sorting in vps60 mutants? These experiments could then provide link to the rest of the manuscript.
> We see the reviewers point. We are not sure how examining mutations in the V domain of Bro1 combined with *vps60*Δ mutations would elucidate more, but we did take steps to enhance this aspect of the story by including Vta1. The model that the *vps60*Δ experiment helps support was built on the idea that Bro1 constitutes a processing pathway for Ub-cargo that is functionally distinct from ESCRT-0,I,II and that Vps60p plays a role along this Bro1-dependent route. Vps60 also binds Vta1, which actually has a modest effect on MVB sorting when knocked out of the parental strain we used here as has been previously documents. Thus, we extended the figure to provide evidence that both Vps60 and Vta1 operate along the 'Bro1-dependent' pathway based on the synthetic defect that their respective deletion causes when combined with ESCRT-0 alleles that shunt cargo more into the 'Bro1 pathway'. These results are now in Figure 9 and help expand this portion of the paper to make it more significant and relate it back to the interactions of Vps60/CHMP5.

Review 2 comments

1. The authors state that HD-PTP V binds Ub attached to cargo. What is the evidence?
> We do not state that. In the Discussion, we say that Ub-binding could be for cargo or to recognize other ubiquitinated components and cite papers showing ubiquitination of ESCRT subunits. We do picture this in the model, as that is the simplest idea and consistent to the loss of Ub-cargo sorting when Ub-is routed through the Bro1-mediated pathway when Bro1 lacks its Ub-biding site within the V domain.

2. The Vps4 ATPase activity is enhanced in the presence of HD-PTP V with an additional small increase by adding Ub. However, the Ub binding site mutated version of V shows the same increase as WT, indicating that Ub binding should not play any role in regulating the ATPase activity.
> The difference should only be seen when Ub is present in the reaction, not when it is absent. We believe the results in 8C are the expected result to support that Ub does regulate Vps4 activity through the Ub-binding site in the V domain.
The figure shows that in the absence of Ub, bot WT and the ΔUb V domains stimulate Vps4 activity. This is reassuring - the mutant V has the ability to bind and stimulate Vps4 showing nothing is structurally wrong with it. It is when you add Ub that there is a difference. Addition of Ub to the WT V further stimulates activity - a Ub-mediated hyper-stimulation. Ub has no affect on the system when the HDPTP Ub-binding domains are gone.

3. Vps4 becomes active once it is assembled into a spiral hexamer. Does the V domain promote assembly of Vps4 hexamers thereby increasing its ATPase activity?

> The mechanism for V-dependent stimulation of Vps4 ATPase activity is described in a recent preceding paper, on which that topic was the focus. This paper was in review during the review of the current manuscript but is now referenced:
doi.org/10.1083/jcb.202102070

4. Figure 8D: The intensity of the pull down bands is very similar for wt HD-PTP and its mutant form with respect to the interaction with CHMP5 and Vps4B_MIT... Are these small differences significant? This needs to be supported by other biophysical methods such as BLI or SPR in order to map the binding site to the F678D mutation.
> We agree. We did not initially appreciate the importance of this observation as the reviewer points out. We have removed these data from the paper. We performed additional experiments based on the idea that such dependence of the YPxL-binding site would be conserved. We performed MIT binding experiments using the *S. cerevisiae* Rim20 V domaiin. That V domain binds the strongest by GST pulldown assay and is quite specific for the S.c. Vps4 MIT domain (Fig. 8B). The corresponding F>D mutant in this V domain had no effect on MIT binding. We also examined CHMP5 binding using additional approaches. One was the yeast 2 hybrid assay, which was the way we discovered CHMP5 binds HDPTP V. Here though, both WT and the FD mutant supported a positive yeast 2 hybrid reaction. We also examined the effect of the FD mutant in Bro1 V on Vps60 binding and discerned little effect by GST pulldown. What would be best is to understand the structural basis how the V domain engages CHMP5, MIT and other partners like YPxL, but without having that, we did not think we can comment one way or another on how the V-domain binds these different components. Competition experiments were equivocal, likely because the low affinity of these interactions makes it so that 100% occupancy of the V domain by one partner is unfeasible to achieve.

5. Figure 9: The model of Vps4 is shown as a dodecamer. Numerous structural studies indicate that the active form of Vps4 is a spiral hexamer.
> Thankyou for pointing out this flaw The model has been changed and simplified to emphasize the binding details most relevant to this manuscript.

6. The major function of Vps4 is remodeling and final disassembly of ESCRT-III filaments. This is achieved by its MIT domain interaction with MIMs present in ESCRT-III proteins. How can this be reconciled with the proposal of this manuscript that suggests that the Vps4 ATPase activity is regulated by the MIT domain - HD-PTP V interaction?
> This is best discussed in the aforementioned JCB paper. Using mutations in MIT we know that the interfaces on the MIT used for MIM1/2 interactions are distinct from that used to engage the V domain. So simultaneous binding might be possible.

7. Figure 7E: CHMP5 is a member of the ESCRT-III protein family. Its structure will thus likely resemble other ESCRT-III members independent of the high sequence variability. It should be modelled based on existing closed conformation structures of ESCRT-III such as CHMP3 and/or IST1 or the CHMP1 model.
> We agree. We now use the model calculated by AlphaFold2, which is also conveniently available to everyone.

8. Are the CHMP5 fusion proteins used in the pull downs monodisperse after purification?
> The ones with the 'core' alpha helical domain show polydispersity. These are the full-length CHMP5, the version lacking the C-terminal Brox-binding site, and a version lacking the C-terminal Lip5 and Brox binding sites. The former two bind the V-domain, the latter does not. By Dynamic Light Scattering they all have similar polydispersity at the same concentration: 18.6%, 57.1%, 36.6%, respectively.

RE: Manuscript #E21-04-0219R
TITLE: "Interactions of ubiquitin and CHMP5 with the V domain of HD-PTP reveals role for regulation of Vps4 ATPase"

Dear Robert,

Thank you very much for the revised manuscript. Both reviewers feel that you have successfully addressed most of their concerns. As you will see, however, reviewer two made a couple of minor points that you may need to address before the paper can be formally accepted.

Looking forward to receiving the final corrected version of your manuscript,

Best regards,

Jean Gruenberg

Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Piper,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

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Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

The authors have address all major points. The new data is convincing and supports the original conclusion. I have no furhter points of concerns and the manucsript should be published.

Reviewer #2 (Remarks to the Author):

The authors have responded to most of my concerns. Two, however, still remain.
Point 7, Figure 7E: The model calculated by Alphafold is likely wrong, it does neither present the closed conformation as determined for CHMP3 and IST1 nor the open polymer conformation (CHMP1B, Snf7 etc..). So the bestway to model CHMP5 would be on the closed conformation - CHMP3 or Ist1.

Point 8: The fact that CHMP5 preparations used in the pull downs are polydisperse should be mentioned in the manuscript, since CHMP aggregation/polymerization might have affected the pull downs.

RE: Manuscript #E21-04-0219RR
TITLE: "Interactions of ubiquitin and CHMP5 with the V domain of HD-PTP reveals role for regulation of Vps4 ATPase"

Dear Robert,

Thank you very much for this new version of your manuscript. I am very happy to let you know that your manuscript is now formally accepted for publication in Molecular Biology of the Cell.

Congratulations for a very nice paper!

Best regards,

Jean Gruenberg
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Piper:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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