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VHS domains of ESCRT-0 cooperate in high-avidity binding to polyubiquitinated cargo

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 30 October 2009

Thank you for submitting your manuscript to the EMBO Journal. Your manuscript has now been seen by four referees and their comments to the authors are provided below. While referee #3 is not persuaded that the advance provided over previous work is sufficient to consider publication here, the other three referees are more supportive and find the structural analysis and the support provided for that multiple UBDs cooperate in poly-ubiquitin binding interesting. Referee #1 is satisfied with the manuscript as it is, but referees #2 and 4 also clearly state that significant revisions would be needed in order to consider publication in the EMBO Journal. In particular, further insight into ESCRT-0 ubiqutin recognition and a more detailed analysis of the cooperative ubiquitin binding is needed. Given all the available input, I would like to ask you to submit a suitable revised manuscript should you be able to address the concerns raised in full. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor

The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The manuscript describes the structure of a STAM-VHS/ubiquitin complex. The authors have done a remarkably thorough job of characterising a large number of VHS domains for ubiquitin binding, and they have come to the remarkable conclusing that ubiquitin binding is probably the most conserved functional feature of these domains. The authors have also gone on to charcterise binding of mono and polyubiquitin binding to ESCRT-0. The ESCRT-0 complex has five Ub binding elements in it. They have succinctly shown that mutation of one or two Ub-recognising elements in this complex do not compromise the function of the complex in sorting to vacuoles. However, mutating three or more leads to a class E phenotype. This proides an elegant basis for understanding how Ub recognition really works in the ESCRT pathway. The observation that the complex has a strong preference for K63-linked polyubiquitination is consistent with a variety of circumstantial observations, but the manuscript of Ren and Hurley nicley demonstrates this point. The manuscript strikes a good balance between observations and heathy speculation. It will be of great interest. The work is technically well done, and should be published with no revisions.

Referee #2 (Remarks to the Author):

Referee report for Ren and Hurley ' VHS domains of ESCRT-0 cooperate in high-avidity binding to and sorting of polyubiquitinated cargo'

This manuscript provides a molecular basis for the observed ubiquitin binding capacity of VHS domains, by providing the first crystal structure of the STAM VHS domain-ubiquitin complex. The novelty of the findings has been dented somewhat by the recent NMR analysis of the STAM VHS domain with ubiquitin, which essentially comes to similar conclusions. Still, the presented structure is a valuable addition to the list of structurally unrelated ubiquitin receptors. The authors generalize their findings by analyzing the ubiquitin binding properties of other VHS domains. Furthermore this report shows that two VHS domains in ESCRT-0 cooperate with the remaining three UBDs in this complex in avid interactions with K63-linked ubiquitin chains. This is analyzed by biophysical measurements, and by in vivo reconstitution of yeast ESCRT-0 variants. Overall, this is a nice paper. However, the conclusions and the models suggested for ESCRT-0 ubiquitin recognition need further experimental work to convince this referee.

Major points

a) Do VHS domains contain a second ubiquitin interaction region? The authors conclude that this is unlikely, they argue that diUb will by default result in a two-fold increase compared to monoUb binding, which seems to be an oversimplification. What are the authors implying here?
The position of Ub Lys63- in the complex structure (should be indicated!) suggests that a distal moiety would be in close proximity to the C-terminal helices 7/8 of the STAM VHS. What are the surface features of this region? Is the surface conserved, maybe even hydrophobic? A weak secondary interaction may explain the observed increase in binding. Some UBDs contact linkage residues directly, and such determinants would likely reside on the VHS domain. This should be discussed, and potentially experimentally addressed. The fact that K48 is directly contacted may also explain why the VHS does not bind K48 chains.

b) It is striking that the VHS-UIM construct provides the same affinity for K63 Ub4 compared to the complete ESCRT-0 complex. This suggests that the important region is the STAM VHS-UIM (page 7), but this needs to be characterized further.
Curiously, the authors dismiss a direct mechanism allowing K63 preference. The molecular ruler mechanism to distinguish chain types has not so much to do with 'expected' affinities (<1uM), but with fold difference between chain types. The addition of the UIM increases the binding to K63-diUb (from 2fold increase in VHS, to 5fold in VHS+UIM), but K48 diUb does not bind to VHS+UIM, suggesting that the UIM provides the preference for K63. Unfortunately, K48-binding

data is incomplete. Data on K48-binding to VHS alone needs to be provided and compared. However, even the K63 vs NC data is intriguing:
K63 VHS: Ub 220uM → 110 → 33uM Ub4
NC VHS: 220 → 100 → 48 (ie equivalent with K63)
BUT:
K63 VHS-UIM: 320 → 76 → 17
NC VHS-UIM: 320 → 90 → 80
This shows that UIM only increases the affinity for K63 but not NC chains in Ub4. From the structure, it seems that while K63 is in proximity to the VHS (forcing additional weak interactions?), a N-terminal extension would not be.
Similarly, also the ESCRT-0 complex appears to distinguish between these chains
K63: 920→140→18
NC: 920→350→140
K48: 920→370→(ND)

Again, this data suggests that the 10x difference in tetramer binding results from elements directly recognizing K63 chains. Overall, the authors might miss an interesting phenomenon in ESCRT-0. A "loose" or "indirect readout" binding mode is not sufficiently explained and not supported by the current data.

In order to convince this referee that a molecular ruler or other specificity mechanism is not involved, these things have to be addressed in addition to (a):
- is the linker between VHS and UIM conserved (in length)?
- can the linker be shortened/removed/extended without affecting K63-Ub2/Ub4 binding?
- do cells with Hse1 [deltaVHS/deltaUIM] together with wt vps27 have a stronger phenotype than Hse1 [deltaVHS] alone?
AFAICS, all data are consistent with the STAM VHS1+UIM providing selective interaction with K63 chains.

Minor points

c) The description of ubiquitin chain conformation in the discussion is somewhat unusual. "NC-Ub is thought to be flexible, thought not as extended as K63-Ub". I thought that both chains crystallized in the same crystal form, eg conformation. Komander et al reported equivalent conformations. "while K48-Ub is relatively compact due to the proximity of Lys48 and the C-terminus of Ub" K48 chains have a different conformation as they interact through their Ile44 region, which has nothing to do with K48. Actually, this sentence does not make much sense. K48-chains can also be open and dynamic though.

d) It would be a valuable addition, to provide a cartoon with the general outline of the human and yeast ESCRT-0 / ESCRT machinery. The complex-forming proteins could alternatively be indicated in Fig 2.

e) The VHS alignment has lost its formatting, and conservation should be shown rather than only important residues (which can be annotated additionally). Furthermore, it should be extended to the entire domain, in the light of above comments (a).

f) The y-axis description of Fig 4 is very confusing. How can 240% saturation be achieved? Where do these curves (are expected to) reach a plateau? The binding data is not intuitive and should be transformed into a more meaningful format (maybe as in Sims and Cohen, NSMB 2009)?

g) Similarly, why does NCUb4 stay at 100% while K63 Ub4 increases beyond 100% in fig4c?

h) Would it not make much more sense to immobilize the Ub chains and analyze ESCRT-0 assembly on such scaffold? This seems more similar to a physiological situation?

Referee #3 (Remarks to the Author):

This manuscript describes a crystal structure of the VHS domain of STAM, in complex with

ubiquitin. The authors proceed to map the interaction site, conclude that the key residues are also present in the VHS domains of most other VHS-domain containing proteins, and provide evidence that ubiquitin binding is a function common to most VHS domains. Functional experiments in yeast indicate that sorting of cargo proteins to the vacuole depends on cooperativity between the multiple ubiquitin binding domains (UBDs) encoded by the ESCRT-0 components Vps27 and Hse1 (yeast STAM orthologue).

I find no fault with the experimental data, however, given the prior literature, the manuscript fails to provide significant new insights into the general subject of ubiquitin binding to the proteins that mediate endosomal sorting, nor does it generate new ideas on how sorting might be coordinated by the various ESCRT complexes. A solution structure for the VHS domain of STAM and coordinates for its ubiquitin interaction surface were published earlier this year (Hong et al., FEBS 2009), which is in good agreement with the data presented here. A VHS-FYVE domain structure of Hrs has also been reported previously. Ubiquitin binding to the VHS domain of STAM has first been shown in 2003 (Mizuno 2003).

A structure of the VHS domain in tandem with a UIM domain may provide interesting information on how ubiquitin chains of different linkages may be recognized - it is also still unclear whether the VHS and UIM domains of one ESCRT component would bind to a poly-ubiquitin chain attached to cargo. Alternatively, cooperativity between multiple UBDs may provide a means to enhance sorting of multiple cargo molecules by a single ESCRT-0 moiety. Cooperativity between multiple components of the ESCRT-0 complex in sorting proteins to the vacuole has also been reported previously by the Piper lab (Bilodeau, Nat Cell Biol 2002). It is good to see that the authors do refer to this prior literature, however I am unconvinced that the information contained in this manuscript is of sufficient interest and of broad scientific significance for the wider readership of EMBO J, nor does it provide major new insights to the specific question asked. I therefore suggest that the authors seek publication of what is clearly a nice set of data in a different journal.

Referee #4 (Remarks to the Author):

The process of Multivesicular Body sorting is a critical step within the endocytic system enabling the lysosomal degradation of membrane proteins. As such, entry into this pathway is tightly regulated to ensure that only bona fide cargoes are sorted for degradation. Ubiquitination of the cargoes has been identified as the predominantly signal for inclusion into this pathway, and the presence of ubiquitin binding motifs within the MVB sorting machinery (ESCRTs) has suggested a mechanism for cargo recognition. However, the complexity of ubiquitin binding domains within the ESCRTs has also raised a question as to how cargo recognition is accomplished.To address this issue, Ren and Hurley have explored the recognition of ubiquitin by ESCRT-0.

The crystal structure the VHS domain of STAM1 in complex with ubiquitin is reported. This structure identifies residues within alpha 2 and alpha 4 of STAM1 that mediate association with the I44 surface of ubiquitin, consistent with and extending upon the NMR characterization reported by Hong et al. (2009). [Interestingly, NMR studies by the Shekhtman group have suggested a second surface of ubiquitin recognized by the STAM2 VHS domain, although this disparity is not addressed.] Mutational analysis of the STAM1 VHS domain supports the mode of association apparent in the crystal structure. The conservation of residues critical for STAM1 VHS-ubiquitin association suggests that ubiquitin binding may be a general feature of VHS domains, and this model is supported by quantitative ubiquitin binding studies with a wide range of VHS domains (although significantly reduced binding is observed in some cases). These studies support the model that VHS domains can generally mediate association with the I44 surface of ubiquitin. While there is overlap with previous publications, this conserved mode of interaction is a significant extension. Ren and Hurley then turn to the bigger question of ESCRT-0 recognition of ubiquitinated cargo via various ubiquitin binding domains. In a particularly intriguing set of experiments, quantitative assessment of ubiquitin chain binding was performed in the context of the STAM1 VHS domain alone, the STAM1 VHS-UIM peptide, and the ESCRT-0 (STAM1-Hrs) complex as well as the UIMs of Hrs (and Vps27). While these studies indicate a level of cooperativity for binding to K63-linked ubiquitin chains, these experiments are under-developed. The use of ESCRT-0 in these studies along with the individual ubiquitin binding domains (and subsets thereof, such as STAM1 VHS-UIM) affords an opportunity for dissecting ubiquitin binding to a level not previously possible

and may permit additional insights into how these domains function in the context of the complex. The authors explain that the analysis of full ESCRT-0 binding to ubiquitin represents a summation of the individual ubiquitin-UBD associations. However, it seems that the tools to dissect this complicated interaction are now available and worthwhile to more fully exploit. For instance, does the ESCRT-0 association with free ubiquitin represent unbiased associations with each of the domains (as suggested) or is accessibility to specific domains limited in the context of the complex? This may in part be addressed through modeling the summation of the individual affinities as well as through analyzing the effects of mutations on ubiquitin-ESCRT-0 binding (as attempted indirectly in the in vivo analysis). Additionally, the specificity of VHS-UIM combinations for K63 linked ubiquitin (as opposed to any tandem combination of ubiquitin binding domains represented within ESCRTs) should be addressed. While the authors have presented compelling evidence for cooperative ubiquitin binding, a more detailed analysis of this association would provide more insight.

To explore the in vivo relevance of cooperative binding, Ren and Hurley examined yeast with various Vps27 or Hse1 ubiquitin binding mutants for defects in MVB sorting (using GFP-CPS). However, this analysis was not compelling. The authors utilize a qualitative assessment (limiting membrane versus lumenal distribution) to explore a process where ubiquitin recognition has been subtly altered. However, the extent to which affinity has been reduced in each context has not been addressed (as mentioned above) nor has a means to quantitatively assess the level of MVB sorting activity. Moreover, the presence of Class E compartments suggest a complete disruption in MVB sorting rather than a specific block in cargo recognition, as the authors are attempting to address. While it is possible that reducing ubiquitin binding by ESCRT-0 may compromise endosomal structure, it is difficult to conclude that the defects observed are specifically related to defects in cooperative recognition of K63-linked polyubiquitinated cargo. It is equally plausible that the combined defects have compromised additional aspects of ESCRT-0 function, and these possibilities (ESCRT-0 stability, ESCRT-0 recruitment, Rsp5 recruitement, etc.) must be addressed to reach the conclusion specifically related to cargo recognition. Moreover, it is unclear if K63-linked polyubiquitination is required in yeast for MVB sorting as analysis by Lauwers et al. (2009) and Bilodeau et al. (2003) utilize different methods to reach distinct conclusions. In short, this in vivo analysis of the contribution of cooperative ubiquitin binding within ESCRT-0 is under-developed.

In summary, Ren and Hurley are addressing a very intriguing question of cooperative recognition of poly-ubiquitinated proteins (whether that be cargo or machinery) in MVB sorting using tools they are uniquely positioned to employ. The successful completion of these studies will be tremendously insightful and of great interest to the broad readership of EMBO. However, the incomplete nature of the current studies prevents recommendation for its publication at this time. Addressing the issues described above will prove useful in generating a revised manuscript with substantial impact.

1st Revision - authors' response 07 December 2009

Referee #1

We are very grateful for the enthusiasm and kind comments about the technical quality and importance of the work.

Referee #2

We appreciate that the referee finds the work valuable and we have adopted his/her suggestion to carry out further experimental work. The additional experiments with K48-Ub4 have led to quite different results than obtained with K48-Ub2, and show that ESCRT-0 binding polyubiquitin avidly but with little selectivity. This has led to significant revisions in the discussion. We appreciate that the referee has spurred us to make these important findings. The primary message of the paper was and remains that the VHS domains of ESCRT-0 are biologically important contributors to avid Ub binding.

This manuscript provides a molecular basis for the observed ubiquitin binding capacity of VHS domains, by providing the first crystal structure of the STAM VHS domain-ubiquitin complex. The novelty of the findings has been dented somewhat by the recent NMR analysis of the STAM VHS domain with ubiquitin, which essentially comes to similar conclusions. Still, the presented structure is a valuable addition to the list of structurally unrelated ubiquitin receptors. The authors generalize their findings by analyzing the ubiquitin binding properties of other VHS domains. Furthermore this report shows that two VHS domains in ESCRT-0 cooperate with the remaining three UBDs in this complex in avid interactions with K63-linked ubiquitin chains. This is analyzed by biophysical measurements, and by in vivo reconstitution of yeast ESCRT-0 variants. Overall, this is a nice paper. However, the conclusions and the models suggested for ESCRT-0 ubiquitin recognition need further experimental work to convince this referee.

Major points

a) Do VHS domains contain a second ubiquitin interaction region? The authors conclude that this is unlikely, they argue that diUb will by default result in a two-fold increase compared to monoUb binding, which seems to be an oversimplification. What are the authors implying here?

If one considers binding of an ideal diUb chain, in which the two Ub moieties have neither cooperative nor anticooperative interactions, to an immobilized monovalent UBD, the number of binding modes is two, twice that for monoUb. The partition function is greater by a value of two, the free energy is more favorable by ñRtlog2, and Kd decreases by a factor of two. This comment caused us to consider that detailed questions of the dependence of affinity on chain-length are a distraction from the main message, however, so we have deleted the data on diUb binding and deleted the text that led to this comment from the reviewer.

The position of Ub Lys63- in the complex structure (should be indicated!)

I44, K48, and K63 are now shown in Fig. 1A.

Ssuggests that a distal moiety would be in close proximity to the C-terminal helices 7/8 of the STAM VHS.

We have modeled this. The distal moiety of K63 di Ub is ~30 Å away from the C-terminal helices, but there is no apparent obstacle to a structural change that would bring them closer, consistent with an affinity gain slightly larger than the factor of 2 from entropy. However, the K63 linkage is not close enough to contact the C-terminal helices, so little or no preference for K63 would be expected from a single VHS domain, consistent with nearly lack of linkage specificity in binding to the tetraubiquitin chains.

What are the surface features of this region? Is the surface conserved, maybe even hydrophobic? A weak secondary interaction may explain the observed increase in binding. Some UBDs contact linkage residues directly, and such determinants would likely reside on the VHS domain.

This possibility seemed reasonable to us based on the data in the initial submission, but seems less likely now given that the data on K48 vs K63 tetraubiquitin binding shows such small differences. The C-terminal half of the VHS domain is less conserved than the N-terminal half and lacks conserved hydrophobic patches.

This should be discussed, and potentially experimentally addressed. The fact that K48 is directly contacted may also explain why the VHS does not bind K48 chains.

This was a good point in light of the diUb binding data; however, the new tetraUb binding data show only a two-fold preference for K63 over K48.

b) It is striking that the VHS-UIM construct provides the same affinity for K63 Ub4 compared to the complete ESCRT-0 complex. This suggests that the important region is the STAM VHS-UIM (page 7), but this needs to be characterized further.
Curiously, the authors dismiss a direct mechanism allowing K63 preference. The molecular ruler mechanism to distinguish chain types has not so much to do with 'expected' affinities (<1uM), but

with fold difference between chain types. The addition of the UIM increases the binding to K63-diUb (from 2fold increase in VHS, to 5fold in VHS+UIM), but K48 diUb does not bind to VHS+UIM, suggesting that the UIM provides the preference for K63. Unfortunately, K48-binding data is incomplete. Data on K48-binding to VHS alone needs to be provided and compared.

A complete set of K48-Ub4 binding analyses, including binding to the STAM VHS alone has been carried out and is shown in the revised Fig. 4 and Table II.

Again, this data suggests that the 10x difference in tetramer binding results from elements directly recognizing K63 chains. Overall, the authors might miss an interesting phenomenon in ESCRT-0. A "loose" or "indirect readout" binding mode is not sufficiently explained and not supported by the current data.

In light of the new data and these comments, this portion of the discussion has been completely rewritten, with no mention of the phrases "loose" or "indirect readout".

In order to convince this referee that a molecular ruler or other specificity mechanism is not involved, these things have to be addressed in addition to (a):
- is the linker between VHS and UIM conserved (in length)?

The linker varies from 19 residues in S. cerevisiae Hse1 to 30 in human STAM1 (good question, though). This amount of variability, and the length (contrast to the 7 residues in interUIM linker in RAP80) of the linker, does not seem compatible with a molecular ruler mechanism. The presence of a long and variable linker seems very consistent with what we now know is a mostly non-specific chain recognition mechanism.

- can the linker be shortened/removed/extended without affecting K63-Ub2/Ub4 binding?

See our response above.

- do cells with Hse1 [deltaVHS/deltaUIM] together with wt vps27 have a stronger phenotype than Hse1 [deltaVHS] alone?

The Hse1 Δ VHS phenotype is about equal to hse1 Δ; since the loss of the VHS is already equivalent to loss of the entire gene, a stronger effect is not possible. This observation is consistent with a critical role for the Hse1 VHS domain in function.

AFAICS, all data are consistent with the STAM VHS1+UIM providing selective interaction with K63 chains.

Yes, we strongly agree that the VHS+UIM are making an important contribution avid chain binding, which we note. We also add that the VHS+UIM also bind K48-Ub4 rather well based on the new data.

Minor points
c) The description of ubiquitin chain conformation in the discussion is somewhat unusual. "NC-Ub is thought to be flexible, thought not as extended as K63-Ub". I thought that both chains crystallized in the same crystal form, eg conformation. Komander et al reported equivalent conformations. "while K48-Ub is relatively compact due to the proximity of Lys48 and the C-terminus of Ub" K48 chains have a different conformation as they interact through their Ile44 region, which has nothing to do with K48. Actually, this sentence does not make much sense. K48-chains can also be open and dynamic though.

The referee makes some good points. In light of these comments and the new data, this section of the discussion has been deleted.

d) It would be a valuable addition, to provide a cartoon with the general outline of the human and yeast ESCRT-0 / ESCRT machinery. The complex-forming proteins could alternatively be indicated in Fig 2.

This is an excellent idea, and a cartoon of the Ub binding domains as organized in the human and yeast heterodimeric complexes, and as we think bind to poly and mono Ub cargo, have been added as Fig. 4D.

e) The VHS alignment has lost its formatting, and conservation should be shown rather than only important residues (which can be annotated additionally). Furthermore, it should be extended to the entire domain, in the light of above comments (a).

This seems to have happened during the pdf conversion of the previous submission and has been corrected. In the interest of saving space the poorly conserved C-terminal half, which is not demonstrated to have a role in Ub binding, has been made into a new Figure S1. Fig. 2A and S1 both have conserved non-binding residues shaded per the suggestion.

f) The y-axis description of Fig 4 is very confusing. How can 240% saturation be achieved? Where do these curves (are expected to) reach a plateau? The binding data is not intuitive and should be transformed into a more meaningful format (maybe as in Sims and Cohen, NSMB 2009)?

This was explained in the methods, but now is further explained in the figure legend to avoid confusion. There is a saturable and nonsaturable component to the polyUb binding curves. The nonsaturable component is most pronounced for the full ESCRT-0 complex, which is not surprising for a 150 kDa complex with large unstructured regions. The data are scaled to that saturation of the saturable component is set as 100%. It is not possible to normalize based on the non-saturable component.

g) Similarly, why does NCUb4 stay at 100% while K63 Ub4 increases beyond 100% in fig4c?

See above; NCUb4 data have been removed in any case.

h) Would it not make much more sense to immobilize the Ub chains and analyze ESCRT-0 assembly on such scaffold? This seems more similar to a physiological situation?

This would require production of 100s of liters of insect cells to produce the necessary amounts of ESCRT-0, followed by its concentration many-fold higher than its solubility limit. Please bear in mind this type of study is far more resource intensive and arduous than the many studies of polyUb binding to small tandem domain constructs. This is the first study I know of that looks in such quantitative detail at polyUb binding to a large, completely reconstituted complex.

Referee #3

There were no specific comments to address. We appreciate that the author finds the study of high technical quality.

Referee #4

We appreciate that the referee considers the question addressed to be ìintriguingî and is complimentary on the selection of tools we have brought to bear.

Mutational analysis of the STAM1 VHS domain supports the mode of association apparent in the crystal structure. ..This may in part be addressed through modeling the summation of the individual affinities as well as through analyzing the effects of mutations on ubiquitin-ESCRT-0 binding (as attempted indirectly in the in vivo analysis).

To address this question experimentally, we have analyzed binding to the Hrs VHS-FYVE-DUIM construct as compared to full ESCRT-0 and the isolated DUIM. Together with the STAM VHS-UIM this represents all of the binding domains of ESCRT-0 so provides a comprehensive account of the cooperative interactions contributed by each subunit to add to the analysis we already did for the full complex. It is satisfying that both of the multi-UBD constructs from each subunit so closely mirror the properties of the intact complex. The VHS-FYVE-DUIM construct behaves much as does

the DUIM alone, consistent with the weak binding of the Hrs VHS domain and a lack of any autoinhibitory interactions between the N-terminal domains of Hrs.

Extensive experiments on intact ESCRT-0 complex mutants are bordering on prohibitively time-consuming for issues raised above in response to ref. 2, and we do not believe would necessarily be incisive.

Additionally, the specificity of VHS-UIM combinations for K63 linked ubiquitin (as opposed to any tandem combination of ubiquitin binding domains represented within ESCRTs) should be addressed.

We also looked at the Vps27 tandem UIM, which was previously studied by Sims & Cohen, and obtained similar results to them, which are reported in the supplementary data. Also see above.

While the authors have presented compelling evidence for cooperative ubiquitin binding, a more detailed analysis of this association would provide more insight.
To explore the in vivo relevance of cooperative binding, Ren and Hurley examined yeast with various Vps27 or Hse1 ubiquitin binding mutants for defects in MVB sorting (using GFP-CPS). However, this analysis was not compelling. The authors utilize a qualitative assessment (limiting membrane versus lumenal distribution) to explore a process where ubiquitin recognition has been subtly altered. However, the extent to which affinity has been reduced in each context has not been addressed (as mentioned above) nor has a means to quantitatively assess the level of MVB sorting activity. Moreover, the presence of Class E compartments suggest a complete disruption in MVB sorting rather than a specific block in cargo recognition, as the authors are attempting to address. While it is possible that reducing ubiquitin binding by ESCRT-0 may compromise endosomal structure, it is difficult to conclude that the defects observed are specifically related to defects in cooperative recognition of K63-linked polyubiquitinated cargo. It is equally plausible that the combined defects have compromised additional aspects of ESCRT-0 function, and these possibilities (ESCRT-0 stability, ESCRT-0 recruitment, Rsp5 recruitement, etc.) must be addressed to reach the conclusion specifically related to cargo recognition.

Mutants blocking critical ESCRT functions lead to formation of the class E compartment. The precise molecular basis of class E compartment formation is not known. Class E compartments can be induced by mutations or gene deletion thought to be functioning in cargo binding, bud formation, bud scission, and the recycling of ESCRT components.

There is no reason to expect the Ub binding mutants of ESCRT-0 to perturb stability (all mutants are on the surface where no stability effects are expected, and none had effects on the stability of the recombinant proteins). The UIM mutants have been published previously and no loss of stability has been noted in past work. The VHS mutants were all characterized as recombinant domains, with no loss of stability noted.

Membrane recruitment is generally agreed to be mediated by the FYVE domain, which was not mutated in our study.

Rsp5 was shown by Piperís lab to bind to the C-terminus of Hse1, while we analyze Ub binding to the N-terminus. None of our mutants is found in the region involved in binding Rsp5.

Moreover, it is unclear if K63-linked polyubiquitination is required in yeast for MVB sorting as analysis by Lauwers et al. (2009) and Bilodeau et al. (2003) utilize different methods to reach distinct conclusions. In short, this in vivo analysis of the contribution of cooperative ubiquitin binding within ESCRT-0 is under-developed.

The Bilodeau paper described the redirection of Fth1 to the vacuolar lumen by fusion with various Ub constructs, all of which include the K63R mutations to block chain elongation through the Ub moiety. Unlike Cps1, Fth1 is not normally targeted to the lumen of the vacuole, consistent with a lack of reports that it is normally K63 polyubiquitinated. The Fth1 studies do show that in some instances, K63 Ub is not required for trafficking via ESCRTs. In our own work we have found that a monoUb-based synthetic cargo can be clustered in vitro by ESCRT-0. We carefully hedge our statements and carefully avoid stating that ESCRT-0 must bind K63 Ub to traffic cargo, and we are

careful to point out that aside from Gap1 and Cps1, it is not clear yet if any other cargoes require K63 polyubiquitination. What we do show is that ESCRT-0 is capable of binding K63 (and K48), which is a significant advance in addressing the plausibility of K63 recognition as a specificity determinant in ESCRT-mediated sorting of at least one cargo, Cps1.

2nd Editorial Decision 07 January 2010

Thank you for submitting your manuscript to the EMBO Journal. I asked the original referees #2 and 4 to review the revised manuscript and I have now received their comments. Both referees appreciate the added data. However there are still some issues that have to be resolved before acceptance here. Some of these issues can be resolved by appropriate text changes. The first issue concerns the date on di-Ub binding that was removed in the revised manuscript due to your findings on the longer Ub chains. Referee #2 strongly feels that this data should be re-incorporated into the manuscript and carefully discussed. I agree with the assessment of this referee and would ask you to include this data into the manuscript again and to discuss it. Referee #4 still feels that there is not sufficient data to support the conclusion that K63 binding activity is specific for MVB cargo recognition. This referee suggests either to add more data to address this point or to tone down this conclusion including in the title. I feel that this issue can be resolved by a more careful discussion and title that incorporate the concerns raised by this referee. Should you be able to resolve these last issues then we would consider a final revision.

When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review.

Looking forward to seeing the final version.

sincerely

Editor
The EMBO Journal

REFEREE REPORTS

Referee #2 (Remarks to the Author):

In the revised manuscript by Ren and Hurley, additional work was performed, mainly in the form of binding assays with K48-linked tetraUb chains, and this has led to major revision and reinterpretation of the data. The manuscript reads very well, and the figures are clear.

Strikingly however, the authors have excluded much binding data, which was part of the initial submission, in the revised manuscript, to incorporate one new finding, (ie that the ESCRT-0 complex lacks specificity, since K48 and K63 Ub4 chains bind with similar affinities). They have deleted all data regarding NC-linked linear chains and all data on diubiquitin binding.

This data was an integral part of the first version of this paper. The Ub2 binding data is a valuable addition since the differences between Ub2 and Ub4 binding is interesting, and may even be important (do we know how long Ub chains on endocytosed cargo become?). Dikic et al have recently proposed an important role for diUb in specific ubiquitin recognition (NRMCB, 2009). Ren and Hurley show clear differences in binding affinity between K48 Ub2 and K63 Ub2 (first version), but much smaller differences between K48 Ub4 and K63 Ub4 (revised version). This needs to be discussed rather than deleted.

Similarly, the NC-linked chain binding data (especially NC-Ub4) needs to be discussed. The authors earlier data seem to suggest that ESCRT-0 binds NC-linked Ub4 chains without cooperativity in contrast to K63 (and now K48) chains. Given the lack of specificity (K63,K48) on one hand, and similar structures of K63 and NC-chains on the other hand, the old data do maybe not fit the avidity

concept the authors propose? Having the NC-data in the figures would allow judging whether ESCRT-0 somehow selects against NC-linked chains. There is also an underlying biological question, ie whether NC-linked chains can substitute for K63-linked chains in endocytosis.

Therefore, now the authors provide clearer data to support their hypotheses regarding avidity within the ESCRT-0 complex mediated by its five UBDs, yet they exclude a large body of equally solid data that may be more difficult to explain, yet may trigger additional questions and make the study more interesting.

The novelty and strength of this manuscript lies in comprehensively analyzing the ubiquitin binding properties of the ESCRT-0 protein complex and its components. The authors have deleted most of this data now, yet for publication, this should be included and discussed.

Minor points:
1) The domain organization Fig 2B should include the same proteins discussed in Fig 2A and 3.
2) The clipping in Fig1C should be increased to show Trp26 in the background. This binding interface figure could be clearer.

Referee #4 (Remarks to the Author):

The revised manuscript from Ren and Hurley remains a solid body of work and has been improved in its revised form. However, a major assertion is that an ubiquitin binding activity within ESCRT-0 functions as a K63-linked polyubiquitin (cargo) receptor during MVB sorting. Support for this conclusion comes from the in vivo observation that the MVB cargo GFP-Cps1 is missorted in the relevant ESCRT-0 mutant context. This result is expected from previous studies documenting this phenotype both in class E vps mutants and MVB sorting mutants specifically defective for ubiquitin-dependent cargo recognition. However, class E vps mutants have been demonstrated to display a more dramatic phenotype, including an aberrant endosomal compartment referred to as the class E compartment. While the molecular basis of the class E compartment is not entirely clear, previous studies have been successful in separating cargo recognition and fundamental contributions to MVB sorting present in a subset of ESCRT UBDs. That is to say that cargo missorting has been observed in ESCRT UBD mutants that do not display the more severe class E phenotype. The significance is that this has allowed conclusions to be drawn pertaining to their role in cargo recognition (rather than, for instance, an interaction with other machinery which confers a more general defect in function of the pathway). The present studies are not performed at a level of resolution that allows any conclusion to made regarding the level at which this activity is relevant in vivo. The data presented reveals the presence of class E compartments in the context of mutant UBDs interpreted to be specifically defective for cargo recognition. As such, an ESCRT-0 ubiquitin binding activity capable of interacting with K63-linked polyubiquitin has been demonstrated to have a physiologically relevant role in MVB sorting. This is an interesting finding, even if it is less precise than the one the authors have reached. Whether this activity is relevant at the level of cargo recognition or for a fundamental aspect of ESCRT function remains to be determined. I can not support publication of the present work until the authors have either substantiated the conclusion that the K63-binging activity is specific for MVB cargo recognition with additional cell biological investigations or acknowledge that the site of action wherein this activity is relevant remains to be determined.

1) It is misleading to state that ESCRT UBD mutants confer a class E phenotype. The strength of structural biology is the ability to generate rationale mutants that are specifically defective for a given activity, as opposed to generating a secondary effect through structural perturbation. A number of studies have demonstrated that it is possible to make a distinction between ubiquitin-dependent cargo recognition and general ESCRT function. That does not mean the present avenue of investigation has to play out as such, but this has not been investigated. Conclusions regarding a role in cargo recognition are therefore unwarranted.

The authors justify the use of GFP-Cps1 as an in vivo reporter based on its use by others to assess the roles of UBDs in ESCRT-I and -II (e.g. the Shields et al. reference). This is true, however the fact that additional methods were utilized to substantiate the claims pertaining to cargo recognition by ESCRT-I and -II has been omitted. The present work does not employ any methods to support

the assertion that cargo recognition has been specifically impacted. Furthermore, the data would appear to contradict this interpretation. Again, it is not clear that this needs to be resolved in the present studies, but the data has been over-interpreted in present form.

2) It is not clear why the authors have referred to the UBDs within a number of ESCRT subunits as "mono-UBDs"? All would appear to be capable of binding to both mono- and poly-ubiquitin, and all display a higher avidity for polyubiquitin. The argument that these UBDs bind to mono-ubiquitin has been inferred from functional studies that suggest poly-ubiquitin is not required for targeting into the MVB pathway. A role of K63-linked poly-ubiquitin in MVB targeting has been in the literature for a number of years, without resolution. The present study may have identified a K63 effector, however as mentioned above the site of action remains unresolved as has an exclusive interaction with poly-ubiquitin. It seems premature to make a distinction between mono- and poly-UBDs at present.

2nd Revision - authors' response 07 January 2010

Referee #2

The diUb and NC-Ub data from the first version of the manuscript have been reincorporated into Table II and are discussed mainly as they are presented. Two new sentences regarding this were added near the beginning of the last paragraph of the discussion. In the interests of avoiding clutter in the already complex Fig. 4, the diUb and NC-Ub data are shown in an expanded Figure S2, where they join the complete set of data on the Vps27 tandem UIMs.

Minor

1) 13 protein sequences are shown in Fig. 2A, with eight of these schematicized in Fig. 2B. The reason the other five are not shown is that each of these five has essentially an identical domain structure to one of the eight shown in Fig. 2B. For example, STAM2_Hs has the same domain structure as STAM1_Hs, so the inclusion of STAM2_Hs would be redundant. A note has been made to this effect in the legend to Fig. 2B. With respect to Fig. 3, all but two of the proteins whose VHS domains are represented in this figure are also shown in Fig. 2B. Human GGA1 and GGA2 have domain structures essentially identical to GGA3, which is shown in Fig. 2B, and it is now noted in the legend that all of these domain structures are the same.

2) The figure has been amended as suggested to show Trp26 more clearly.

Referee #4

1. We believe that our data taken together with that in the literature, firmly establish that the VHS domains of ESCRT-0 are required not only for binding polyubiquitin but for sorting of the K63-polyubiquitinated cargo Cps1. We do feel that the requested alteration in wording to some extent shortchanges the significance of the accomplishment. In the interest of timely publication we have nevertheless made the requested changes to the wording in the title and discussion. The words "and sorting of" were deleted from the title, and the word "sorting" in the running title was changed to "binding". In the last paragraph of the discussion, the word "potentially" was added to the second sentence. The penultimate sentence in this paragraph was modified to remove mention of K63-ubiquitination and now reads "We have shown Ub recognition by VHS domains is important in sorting Cps1."

2. This point is well taken. "Mono UBD" was replaced simply by UBD in the first paragraph of the introduction, which is the only place this expression occurred.

