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The ubiquitin hydrolase Doa4 directly binds Snf7 to inhibit recruitment of ESCRT-III remodeling factors in *S. cerevisiae*

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MS TITLE: Doa4 interferes with ESCRT-III remodeling factors
to inhibit the membrane scission mechanism

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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In the manuscript by Odorizzi and colleagues, the authors build upon their previous finding that overexpression of Doa4 affects ESCRT-III polymerization dynamics. They had previously found that

overexpression of Doa4 increases Snf7 polymerization in yeast, the mechanism of which wasn't clear.

In the current manuscript, the authors propose a model in which Doa4 binds to the C-terminal MIM region of Snf7, which affects Vps2:Vps24 binding to Snf7, inducing higher amounts of Snf7 polymerization. Reduction of Snf7 binding to Vps2:24 had been previously associated with an increase in Snf7 polymerization, which is consistent with the model proposed. In addition to this insight, the authors find through in-vitro binding assays that the N-terminal region of Doa4 (including the residues 1-348) bind to Snf7's C-terminal MIM motif.
These data suggest that the C-terminal region of Snf7 and other ESCRT-III proteins may be involved in recruitment of ESCRT-III regulators. Overall, the regulatory factors that bind to the polymer through the sides of the filament may change filament dynamics, and in the case of Doa4, the authors propose that it may restrict ESCRT-III polymerization until all the cargo molecules are deubiquitinated via Doa4's deubiquitylation activity.

Comments for the author

The present study is interesting and will be informative to the field, however, the following issues need to be addressed before publication:

1. According to the model, Doa4 and Bro1 bind the same site on Snf7, while the in-vitro assays do not contain Bro1. We recognize that including Bro1 experiments is not feasible in the timeframe of this manuscript the authors should discuss how Bro1 association to the polymer would affect Snf7 polymerization and Vps2:24 binding.

2. The rate-zonal density gradients are important for the overall model of Doa4 affecting Snf7 polymerization in vivo. However, the data are not strong, especially considering the fact that the Doa4 overexpression data were published before by the authors. It is not appropriate to remove standard deviations from the quantification, and the quantification does not match the images of the gels. These data should be properly reanalyzed and presented.

3. To properly demonstrate the sorting defects induced by overexpression of Doa4 or the truncated version the authors could test sorting of an appropriate cargo (such as Mup1 or CPS).

4. It is not obvious that overexpression of the Doa4's N terminus changes the number of budding profiles from the images presented. Perhaps the authors can highlight the budding profiles in the tomograms by adjusting the contrast, or present additional images from their collected images where the budding events are clearly seen.

5. It is not obvious why addition of Vps4+ATP to Snf7/Vps24/Vps2 polymers in the presence of Doa4 should accelerate the dissociation of Vps2 from the polymers. In other words, why should Doa4 help accelerate dissociation of Vps2 in the presence of Vps4? Shouldn't the dissociation of Vps2 be already faster without the presence of Vps4? Also, these in-vitro polymerization data on model membranes can be strengthened by using the Snf7 MIM mutant in the assays, a reagent which the authors already have.

6. What is the effect of overexpressing the N terminal region of Doa4 (1-348) on the BiFc assay between Snf7 and Doa4?

Reviewer 2

Advance summary and potential significance to field

The authors report mapping of an interaction between Snf7 MIM1 and the N-terminal MIT-RHD domain of Doa4 in Fig. 1. They go on to study the role of this interaction in Snf7 polymerization, association of the Vps2 subunit required for membrane scission, and membrane budding (Fig. 2-5). These latter experiments rely on overexpression of the MIT-RHD fragment. The effects seem relatively small and are what would be expected if overexpression of MIT-RHD, which is larger than Snf7, occluded binding of other proteins to Snf7. The data in Fig. 2-5 serve to corroborate that

there probably is a physical interaction between Snf7 and the Doa4 MIT-RHD, but the leap in the title, abstract, and discussion to asserting that the interaction regulation membrane scission at normal expression levels in not justified. The study as presented has substantial flaws, but could potentially be acceptable if it were framed as of mapping the interaction between Snf7 MIM1 and Doa4 MIT-RHD (this could be significant to the community; perhaps it regulates cargo deubiquitination or some other biological function, even if it doesn't actually regulate scission) and the claims concerning regulation of membrane scission are dropped.

Comments for the author

Fig. 1D- Do the fusion proteins made for BiFC support function, or are the tags themselves inhibitory?

Fig. 1E- Why does bro1 have such a small effect and how is this reconciled with Luhtala et al 2004?

Fig. 2. The differences shown are quite small. SD values need to be shown. State statistical significance or lack thereof.

Fig. 3. The number of ILVs seems to be the same in all situations, which is hard to reconcile with the argument that MIT-RHD overexpression causes a scission defect. The numbers of budding profiles seen are so small that the differences seen are not necessarily statistically significant (a single asterisk is shown in Fig. 3E but the corresponding p-value is not stated).

Fig. 4. Preincubation with the Doa4 fragment causes only a small delay in Vps2 recruitment and simultaneous addition has no effect. The only strong effects are read visually are in panels E and F, and the only strong effect as quantitated is in H. It seems that the Doa4 fragment is interfering with the ability of Vps4 to access the substrate. It is not surprising that adding a large amount of any Snf7 binding protein added at a 4-fold molar excess would do this.

Fig. 5. Small effect over a very long time course with BiFC reagents that have not been shown to support function.

Reviewer 3

Advance summary and potential significance to field

In this manuscript, the authors addressed how Doa4 affect ESCRT-III assembly/dynamics and thus, inhibits membrane scission. The authors applied a battery of approaches, including protein pull-down, BiFC imaging, in vitro reconstitution, and EM tomography to address these questions. In my opinion, all the experiments were properly done and the data were accurately interpreted and quantified. The figures are well organized and the main text is written in a logical and coherent way. Thus, this manuscript is easy to read and comprehend. I believe this paper is suitable for publication in Journal of Cell Science in its current form.

Comments for the author

There is no minor comments.

First revision

Author response to reviewers' comments

Reviewer 1

1. According to the model, Doa4 and Bro1 bind the same site on Snf7, while the in-vitro assays do not contain Bro1. We recognize that including Bro1 experiments is not feasible in

the timeframe of this manuscript, the authors should discuss how Bro1 association to the polymer would affect Snf7 polymerization and Vps2:24 binding

We now address this issue in the discussion section (lines 346-352):

> "Like Doa4, Bro1 binds the MIM1 site in Snf7 and, as in the case of Doa4 overexpression, Bro1 overexpression inhibits Snf7 remodeling in yeast (Wemmer et al., 2011). Interestingly, Bro1 has been shown to nucleate polymerization of purified Snf7 on formvar grids (Tang et al., 2016). In contrast, we show that Doa4 has no effect on the rate at which Snf7 spontaneously polymerizes on membrane-coated coverslips. Future work using this in vitro system should reveal the extent to which Bro1 augments or interferes with Doa4-mediated regulation of Snf7 polymer association with its remodeling factors."

2. The rate-zonal density gradients are important for the overall model of Doa4 affecting Snf7 polymerization in vivo. However, the data are not strong, especially considering the fact that the Doa4 overexpression data were published before by the authors. It is not appropriate to remove standard deviations from the quantification, and the quantification does not match the images of the gels. These data should be properly reanalyzed and presented.

The rate-zonal density gradient data in Figure 2 was re-plotted to include the standard deviations as shaded margins to go along with the line graphs. Statistical significance was calculated for each fraction. Addition of the standard deviations helps to highlight the novel findings of this study in Figures 2C and 2E.

3. To properly demonstrate the sorting defects induced by overexpression of Doa4 or the truncated version, the authors could test sorting of an appropriate cargo (such as Mup1 or CPS).

As requested, the localization of GFP-Cps1 and the quantitative sorting of FLuc-Cps1 into the ILV pathway are now shown in Figs. 4A and 4B, respectively. These experiments show that overexpression of Doa4 and Doa4$^{1-348}$ caused a small but statistically significant defect in cargo sorting, consistent with the apparent reduction in ILV budding frequency observed under the same conditions.

4. It is not obvious that overexpression of the Doa4's N terminus changes the number of budding profiles from the images presented. Perhaps the authors can highlight the budding profiles in the tomograms by adjusting the contrast, or present additional images from their collected images where the budding events are clearly seen.

We have added an analysis of the ILV-to-budding profile ratio to highlight the effect on ILV biogenesis caused by overexpression of Doa4$^{1-348}$ (Fig. 3G) and we have selected a better representative of this effect for Figure 3D and Movie 4. Budding profiles are highlighted in green in the third panels of Figure 3A-D.

5. It is not obvious why addition of Vps4+ATP to Snf7/Vps24/Vps2 polymers in the presence of Doa4 should accelerate the dissociation of Vps2 from the polymers. In other words, why should Doa4 help accelerate dissociation of Vps2 in the presence of Vps4? Shouldn't the dissociation of Vps2 be already faster without the presence of Vps4? Also, these in-vitro polymerization data on model membranes can be strengthened by using the Snf7 MIM mutant in the assays, a reagent which the authors already have.

We now address the first question in the results section (lines 250-253):

> "[T]his latter result could be a secondary effect of the lower affinity Vps2:24 has for Snf7 in the presence of Doa4$^{1-348}$ (Fig. 5E, G), or it could signify that Vps2:24 copolymers are preferentially targeted by Vps4, as has been seen in the case of other ESCRT-III-associated factors (Pfitzner et al., 2019)."

We agree that using the Snf7^{MIM1} mutant in this assay would strengthen our argument; alas, multiple attempts to polymerize the purified mutant protein on membrane coated glass coverslips have been

unsuccessful (either due to technical limitations or an intrinsic property of this mutant). In light of this obstacle it is impossible to perform the suggested experiments.

6. What is the effect of overexpressing the N terminal region of Doa4 (1-348) on the BiFc assay between Snf7 and Doa4?

We now show that overexpressing untagged Doa4$^{1-348}$ has the same effect as overexpressing untagged full-length Doa4 with respect to BiFC between Snf7-VC and Doa4-VN (Fig. 1D,E). Additionally, we created a Doa4$^{1-348}$-VN/Snf7-VC BiFC strain and found that BiFC signal was observed at endosomal puncta (Fig. 1D), as we had seen for BiFC between Snf7-VC and full-length Doa4-VN (Fig. 1D).

Reviewer 2

The study as presented has substantial flaws, but could potentially be acceptable if it were framed as of mapping the interaction between Snf7 MIM1 and Doa4 MIT-RHD (this could be significant to the community; perhaps it regulates cargo deubiquitination or some other biological function, even if it doesn't actually regulate scission) and the claims concerning regulation of membrane scission are dropped.

We have changed the wording in the Title, Abstract, and Summary to focus on the direct binding between Doa4 and ESCRT-III. However, we believe we are justified in describing the data that show Doa4$^{1-348}$ overexpression inhibits ILV membrane scission (Figs. 3 and 4). The revisions addressing this effect are described in more detail in the reply to Reviewer 1 point 4 and in the reply to Reviewer 2 query about Fig. 3.

Fig. 1D- Do the fusion proteins made for BiFC support function, or are the tags themselves inhibitory?

We now address this issue with a supplementary figure (Fig. S1) and in the results section (lines 136-139):

> "C-terminal VN- or VC-tagging of ESCRT-III subunits renders them non-functional with respect to ILV cargo sorting (Fig. S1) but does not disrupt their subcellular localization on endosomal and vacuolar membranes, making them useful for testing direct protein interactions in vivo."

Fig. 1E- Why does bro1 have such a small effect and how is this reconciled with Luhtala et al 2004?

As the reviewer points out, we previously reported that Doa4-GFP localization to endosomes is diminished in the absence of Bro1 (Luhtala and Odorizzi, 2004), but we now show that BiFC fluorescence derived from Doa4-VN interaction with Snf7-VC persists in the absence of Bro1. We now address this issue in the results section (lines 155-158):

> "This observation is in agreement with residual Doa4 localization to endosomes seen in the absence of Bro1 (Richter et al., 2013) and is potentially enhanced in the context of BiFC analysis by VC-tagging the Snf7 C terminus, as C-terminal modifications are known to stabilize ESCRT-III subunits in their active conformations (Shim et al., 2007)."

Fig. 2. The differences shown are quite small. SD values need to be shown. State statistical significance or lack thereof.

Standard deviations have been added to Figs. 2B,C,E in the form of shaded margins to go along with the line graphs. Additionally, statistical significance was calculated for each fraction using a two-tailed Students *t*-test. Results were significantly different for fractions 7 and 8 in Fig. 2B, and both overexpression conditions (2μ *DOA4* and 2μ *doa4$^{1-348}$*) were significantly different from the empty-vector control in fractions 2, 8, and 9 in Fig. 2E. All other differences were not significant. This analysis was added to the legend of Fig. 2.

Fig. 3. The number of ILVs seems to be the same in all situations, which is hard to reconcile with the argument that MIT-RHD overexpression causes a scission defect. The numbers of

budding profiles seen are so small that the differences seen are not necessarily statistically significant (a single asterisk is shown in Fig. 3E but the corresponding p-value is not stated)

We now include analysis of the sum ratios of ILVs to budding profiles for all four strains (Fig. 3G). The data show that this ratio decreases upon overexpression of Doa4 or Doa4$^{1-348}$. Thus, ILVs are fewer when budding profiles are more frequently observed, which is consistent with a delay in ILV budding. The p value of the asterisk in Fig. 3E was added to the figure legend.

Fig. 4. Preincubation with the Doa4 fragment causes only a small delay in Vps2 recruitment and simultaneous addition has no effect. The only strong effects are read visually are in panels E and F, and the only strong effect as quantitated is in H. It seems that the Doa4 fragment is interfering with the ability of Vps4 to access the substrate. It is not surprising that adding a large amount of any Snf7 binding protein added at a 4-fold molar excess would do this.

The data demonstrating that Snf7 disassembly is unaffected by Doa4$^{1-348}$ (even at the high concentrations used in this experiment) contradict the reviewer's interpretation that Doa4$^{1-348}$ is interfering with Vps4 access to its substrate (Snf7). This issue is now addressed (lines 237-240):

> "Figures 5B and 5D show Doa4$^{1-348}$ had no effect on the rate at which Vps4 disassembled Snf7 polymers under these conditions, demonstrating that neither the catalytic inhibition of Vps4 nor steric hindrance of Vps4 access to Snf7 are responsible for Snf7 polymer accumulation in response to Doa4 overexpression in yeast (Johnson et al., 2017; Fig. 2)."

Fig. 5. Small effect over a very long time course with BiFC reagents that have not been shown to support function.

We now address the functionality of VN- and VC-tagged ESCRT-III subunits in the results section (lines 136-139):

> "C-terminal VN- or VC-tagging of ESCRT-III subunits renders them non-functional with respect to ILV cargo sorting (Fig. S1) but does not disrupt their subcellular localization on endosomal and vacuolar membranes, making them useful for testing direct protein interactions in vivo."

Also, we now address the duration of time required for BiFC fluorescence in the zygotic assay in the results section (lines 280-292):

> "The 300-min length of time required for Vps2-VN and Snf7-VC to achieve a steady-state level of interaction in the zygotic BiFC assay is much longer than the ~10 mins required for purified Vps2 and Snf7 proteins to reach binding equilibrium in vitro (Fig. 5). This difference reflects the fundamental contrast between the two techniques. The binding and fluorophore maturation kinetics of Venus when the VN fragment assembles with the VC fragment (Köker et al., 2018) are several orders of magnitude slower than the kinetics with which purified ESCRT-III subunits polymerize and depolymerize (Chiaruttini, et al., 2015). Thus, unlike the in vitro assays shown in Fig. 5, the zygotic BiFC assay does not have the temporal resolution to assess Vps2 association with Snf7 during a single round of ESCRT-III polymerization in vivo. Likely, we are observing BiFC puncta at endosomes that accumulate after multiple cycles of ESCRT-III polymerization, and the slower accumulation of BiFC seen upon DOA4 overexpression likely reflects either a smaller number of ESCRT-III polymerization events or a decrease in Vps2-Snf7 interaction at each event."

Reviewer 3

No issues to address. Thank you for the comments.

Second decision letter

MS ID#: JOCES/2019/241455

MS TITLE: Doa4 directly binds Snf7 to inhibit the recruitment of ESCRT-III remodeling factors

AUTHORS: Dalton Buysse, Anna-Katharina Pfitzner, Matt West, Aurelien Roux, and Greg Odorizzi
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.